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The Effects of pH and Inhibitors upon the Catalytic Activity of the Dihydroorotase of Multienzymatic Protein *pyr1-3* from Mouse Ehrlich Ascites Carcinoma*

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We have studied factors affecting the catalytic activity of dihydroorotase (EC 3.5.2.3), purified as part of a multienzymatic protein which contains carbamyl phosphate synthetase, aspartate transcarbamylase, and dihydroorotase (ME *pyr1-3*) and which initiates *de novo* pyrimidine biosynthesis in mouse Ehrlich ascites carcinoma. The apparent K_m value for *N*-carbamyl-L-aspartate increases by 2 orders of magnitude as the pH increases from 7.0 to 8.3, consistent with equilibration of dihydroorotase (*E*) between four states of protonation ($E \rightleftarrows EH \rightleftarrows EH_2 \rightleftarrows EH_3$), where EH_3 is the only catalytically active form of dihydroorotase for the biosynthetic reaction, having a K_m for *N*-carbamyl-L-aspartate of 30 μM. The apparent K_m for L-dihydroorotate shows a converse dependence upon pH, remaining relatively constant at alkaline pH and increasing progressively as the pH is decreased below 7.0. These data are consistent with the above model if *E* and *E*H are catalytically active for the degradative reaction, both having K_m values of 4.4 μM for L-5,6-dihydroorotate.

The D isomers of carbamylaspartate and dihydroorotate are also substrates for dihydroorotase. At pH 7.33, the apparent K_m values for *N*-carbamyl-L-aspartate and *N*-carbamyl-D-aspartate are 247 and 204 μM, respectively, but the V_{max} for *N*-carbamyl-D-aspartate is only 1.7% of that obtained with N-carbamyl-L-aspartate. Orotate and a series of 5-substituted derivatives are competitive inhibitors of dihydroorotase. At pH 7.27, the apparent K_i for orotate using *N*-carbamyl-L-aspartate as substrate is 170 μM and with L-5,6-dihydroorotate as substrate, the apparent K_i value is 9.6 μM, suggesting that the enzyme exists in different forms in the presence of each substrate. Dihydroorotase is inhibited in a time-dependent manner by 50 mM L-cysteine and the presence of *N*-carbamyl-L-aspartate or L-5,6-dihydroorotate protects against this ultimately complete inactivation. 2-Mercaptoacetate, 2-mercaptoethylamine, 3-mercaptopropionate, and L-2,3-diaminopropionate have a similar although less potent inhibitory effect.

To account for the data obtained, we propose a model for the equilibria existing between various protonated forms of dihydroorotase which is consistent with the pH dependencies of the apparent K_m values observed and the V_{max} values observed previously (Christopherson, R. I., and Jones, M. E. (1979) *J. Biol. Chem.* 254, 12506-12512). In addition, a catalytic mechanism is presented for the interconversion of *N*-carbamyl-L-aspartate and L-5,6-dihydroorotate.

Dihydroorotase (EC 3.5.2.3) catalyzes the third reaction in the *de novo* biosynthesis of pyrimidines, the cyclization of *N*-carbamyl-L-aspartate (L-CA)[1] to form L-5,6-dihydroorotate (L-DHO):

$$\text{L-CA}^{2-} + H^+ \rightleftarrows \text{L-DHO}^- + H_2O \quad (1)$$

In mammals, dihydroorotase is part of a multienzymatic protein, ME *pyr1-3* (Shoaf and Jones, 1973), which also contains the enzymatic activities catalyzing the first two steps of the pathway, carbamyl phosphate synthetase (EC 2.7.2.9) and aspartate transcarbamylase (EC 2.1.3.2). The substrate kinetics and regulation of the first two enzymes of the pathway in mammals have been studied (Tatibana and Shigesada, 1972; Mori *et al.*, 1975; Shoaf and Jones, 1973) and the general catalytic mechanisms for these two enzymes have been established from detailed studies with *Escherichia coli* of the glutamine-dependent carbamyl phosphate synthetase (Powers *et al.*, 1977; Powers and Meister, 1978; Raushel *et al.*, 1978) and aspartate transcarbamylase (Collins and Stark, 1969, 1971). By contrast, little is known about dihydroorotase from mammalian or bacterial sources, although this enzyme has been purified to apparent homogeneity from *E. coli* (Sander and Heeb, 1971) and *Clostridium oroticum* (Taylor *et al.*, 1976) and characterized to some extent.

There are conflicting reports in the literature of the effectiveness of various compounds as inhibitors of mammalian dihydroorotase. Bresnick and Hitchings (1961) found that dihydroorotase from mouse Ehrlich ascites carcinoma is inhibited by orotate and 5-fluoro orotate and Bresnick and Blatchford (1964) found that L-cysteine and 2-mercaptoacetate also inhibited the enzyme. In addition, it was claimed from these studies that a number of other pyrimidine compounds and analogs, thiol and disulfide-cleaving compounds, and $S_2O_4^{2-}$, CN^-, SO_3^{2-}, Ag^+, and Hg^{2+} ions were also inhibitory. Kennedy (1974) confirmed the inhibition of dihydroor-

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[1] The abbreviations used are: L-CA, *N*-carbamyl-L-aspartate; L-DHO, L-5,6-dihydroorotate; ME *pyr1-3*, the multienzymatic protein containing carbamyl phosphate synthetase, aspartate transcarbamylase, and dihydroorotase (this protein has been called Complex A (Christopherson and Jones, 1979) or CAD (Padgett *et al.*, 1979), but we have adopted the more systematic abbreviation ME *pyr1-3* to indicate that this is a multienzymatic protein, catalyzing the first three reactions of pyrimidine biosynthesis, which is encoded by a single mRNA (Padgett *et al.*, 1979)); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

otase from rat liver by orotate and by Ag^+ and Hg^{2+} ions but found no inhibition by many of the pyrimidine compounds and analogs, L-cysteine, 2-mercaptoacetate, and other thiol and disulfide-cleaving compounds, $S_2O_4^{2-}$, CN^-, and SO_3^{2-}, which were tested in the earlier studies.

Bresnick and Blatchford (1964) found that the apparent K_m for *N*-carbamyl-DL-aspartate varied with pH and a minimal value of 1.2 mM was obtained at pH 6.5. Kennedy (1974) found that the K_m for *N*-carbamyl-L-aspartate of a racemic mixture of *N*-carbamyl-DL-aspartate varied with enzyme concentration at pH 6.5, the minimal value being 120 μM, and he found that the K_m for dihydroorotate of unspecified stereochemistry was approximately 40 μM at pH 9.0. A major difficulty in measuring subtle variations in dihydroorotase activity as a function of pH, substrate, or inhibitor concentrations has been the lack of a sensitive and reproducible assay procedure. Recently, we developed a very sensitive radioassay for dihydroorotase using as substrate L-[^{14}C]CA synthesized chemically from potassium [^{14}C]cyanate and L-aspartate (Christopherson *et al.*, 1978). This assay has enabled us to study in detail factors affecting the catalytic activity of dihydroorotase.

In a previous publication (Christopherson and Jones, 1979), we determined the pH-independent equilibrium constant of Reaction 1 and the maximal rates of the forward and reverse reactions as a function of pH. The experimental data obtained were consistent with a model of catalysis where cyclization of *N*-carbamyl-L-aspartate or ring cleavage of L-5,6-dihydroorotate are dependent upon the acidic and basic forms, respectively, of a catalytic residue of the enzyme·substrate complex with a pK_a of 7.1. The presence of *N*-carbamyl-L-aspartate or L-5,6-dihydroorotate protects dihydroorotase against inactivation by diethylpyrocarbonate, suggesting that at least 1 histidine residue is involved in catalysis.

In this paper, we extend these studies and find that the apparent K_m for *N*-carbamyl-L-aspartate exhibits a remarkable dependence upon pH, increasing from 100 μM at pH 7.0 to 10 mM at pH 8.3. Using as substrate L-[^{14}C]DHO synthesized and purified on a microscale, we find that the apparent K_m for L-5,6-dihydroorotate shows a converse but less pronounced pH dependence. The D isomers of carbamylaspartate and dihydroorotate are less effective substrates for dihydroorotase and orotate and a series of 5-substituted orotate derivatives are competitive inhibitors of the enzyme. The apparent K_i values for orotate measured in the presence of *N*-carbamyl-L-aspartate are considerably higher than those measured with L-5,6-dihydroorotate as substrate. We find that L-cysteine does inactivate dihydroorotase and that this ultimately complete inactivation is prevented by the presence of *N*-carbamyl-L-aspartate or L-5,6-dihydroorotate.

EXPERIMENTAL PROCEDURES

DL-Dithiothreitol, glutathione, L-cysteine (free base), 2-mercaptoacetate (thioglycolic acid, sodium salt), 3-mercaptopropionic acid, and 2-mercaptoethylamine HCl were supplied by Sigma Chemical Co.; 2-mercaptoethanol was from Fisher Scientific Co.; and L-2,3-diaminopropionic acid HCl was from Chemical Dynamics Corp. 1,10-Phenanthroline was obtained from Eastman Organic Chemicals, disodium ethylenediaminetetraacetate was from Fisher Scientific Co., *p*-nitrobenzenesulfonamide was from Eastman Kodak Co., diethyldithiocarbamic acid and dipicolinic acid were from Sigma. Fumaric acid (disodium salt) was purchased from Calbiochem, maleic and malonic acids were purchased from Sigma, and succinic acid was from J. T. Baker Chemical Co. *N*-Carbamyl-β-alanine, *N*-carbamyl-L-α-alanine, and *N*-carbamyl-L-glutamic acid were supplied by Sigma and *N*-acetyl-L-aspartic acid was from Cyclo Chemical Co. Orotic acid (monosodium salt) and the 5-methyl, 5-bromo, and 5-iodo derivatives of orotic acid were obtained from Sigma; 5-amino orotic acid was from Heterocyclic Chemical Corp.; and 5-fluoro orotic acid was from P-L Biochemicals Inc. Dihydrouracil, dihydrothymine, azauracil, orotidine 5′-phosphate (OMP), UMP, UTP, and 5-phosphoribosyl 1-pyrophosphate were purchased from Sigma; orotidine was from Calbiochem; and barbituric acid was from Eastman Organic Chemicals. All other compounds used in these studies were of the highest grade available.

Buffers used in the pH dependence studies were: potassium Mes, pH range 5.2 to 6.8; potassium Hepes, pH range 6.4 to 8.0; and Tris-HCl, pH range 7.6 to 9.2. The buffers have been described in detail previously (Christopherson and Jones, 1979).

Preparation of Unlabeled Carbamylaspartate and Dihydroorotate—L-5,6-Dihydroorotate and L-aspartic acid were supplied by Sigma and D-aspartic acid was from Calbiochem. The optical rotation of L-5,6-dihydroorotate was measured with a Perkin-Elmer 241 polarimeter, $[\alpha]_D^{23} = +31.5°$ compared with a value of $[\alpha]_D^{25} = +33.2°$ obtained by Miller *et al.* (1953). *N*-Carbamyl-L-aspartate and *N*-carbamyl-D-aspartate are not commercially available and so were synthesized from potassium cyanate and the appropriate enantiomer of aspartate (Christopherson *et al.*, 1978). *N*-Carbamyl-D-aspartate showed similar properties to *N*-carbamyl-L-aspartate during purification. Lyophilization of fractions from the DEAE-cellulose column yielded an oily residue from which *N*-carbamyl-D-aspartate was precipitated by addition of ice cold anhydrous ethanol:acetone (1:1, v/v) (Christopherson *et al.*, 1978). The optical rotations of the *N*-carbamyl-L-aspartate and *N*-carbamyl-D-asparate synthesized were $[\alpha]_D^{23} = +39.6°$ for the L isomer and $[\alpha]_D^{23} = -39.9°$ for the D isomer. Measurements were made at pH 7.0 with solutions equivalent to 8.87% of the free acid of *N*-carbamyl-L-aspartate and 9.93% of the free acid of *N*-carbamyl-D-aspartate. The optical rotation obtained for *N*-carbamyl-L-aspartate of +39.6° is considerably higher than $[\alpha]_D^{25} = +24.1°$ reported by Reichard and Lagerkvist (1953) for a 3% solution of *N*-carbamyl-L-aspartate prepared as the barium salt, suggesting some racemization of their preparation which may have occurred during lyophilization of pooled fractions containing HCl. The concentrations of stock solutions of the *N*-carbamyl-L-aspartate and *N*-carbamyl-D-aspartate were determined colorimetrically by the method of Prescott and Jones (1969) using as a standard *N*-carbamyl-DL-aspartate (Sigma) dried over P_2O_5.

Preparation of L-[^{14}C]CA and D-[^{14}C]CA—L-[^{14}C]CA (52.0 Ci/mol) and D-[^{14}C]CA (52.0 Ci/mol) were synthesized from potassium [^{14}C]cyanate (52.0 Ci/mol, New England Nuclear) and the same solutions of L- and D-aspartate used for the syntheses of unlabeled *N*-carbamyl-L-aspartate and *N*-carbamyl-D-aspartate described above. Because the preparation of ^{14}C-labeled *N*-carbamyl-L-aspartate and *N*-carbamyl-D-aspartate was identical in every respect with the preparation of unlabeled *N*-carbamyl-L-aspartate and *N*-carbamyl-D-aspartate, except for the amount of material processed and the use of potassium [^{14}C]cyanate, it is assumed that the stereochemistry of the L-[^{14}C]CA and D-[^{14}C]CA so prepared is identical to that of the unlabeled compounds. The radiochemical purities of these two enantiomers, as determined by chromatography on polyethyleneimine-cellulose (Christopherson *et al.*, 1978), were: L-[^{14}C]CA, 99.7%; D-[^{14}C]CA, 99.5%. The concentrations of stock solutions of L-[^{14}C]CA and D-[^{14}C]CA were determined by measuring the content of ^{14}C of a number of different dilutions of each solution spotted on polyethyleneimine-cellulose. The concentrations were calculated from the slope of a line fitted by the method of least squares to a plot of volume of [^{14}C]CA *versus* cpm using a counting efficiency of 78.3% and the specific activity of the [^{14}C]cyanate of 52.0 Ci/mol.

Preparation of L-[^{14}C]DHO—L-[^{14}C]DHO of high purity and specific radioactivity was synthesized enzymatically from L-[^{14}C]CA using dihydroorotase partially purified from mouse Ehrlich ascites carcinoma (Christopherson and Jones, 1979). L-[^{14}C]DHO was separated from residual L-[^{14}C]CA by elution with a LiCl gradient from a DEAE-cellulose column (Christopherson *et al.*, 1978) and LiCl and any other impurities were subsequently removed by two crystallizations of the L-[^{14}C]DHO. Lieberman and Kornberg (1953) synthesized approximately 340 μmol of L-[^{14}C]DHO of low specific radioactivity (2 mCi/mol) by enzymatic reduction of [^{14}C]orotate; the final product was dissolved in hot water and recrystallized in the cold. Because we proposed to synthesize smaller amounts of L-[^{14}C]DHO of much higher specific radioactivity, the crystallization conditions of Lieberman and Kornberg (1953) were optimized for satisfactory use on a microscale. Preliminary testing indicated that in the presence of 340 mM LiCl at 60°C, the saturating concentration for L-5,6-dihydroorotate, converted to the free acid by 2 molar equivalents of HCl, was 106 mM. To test the crystallization of L-5,6-dihydroorotate in the presence of LiCl as it would be eluted from a DEAE-cellulose column (Christopherson *et al.*, 1978), a mixture of 70 μmol of L-5,6-dihydroorotate and 202 μmol of LiCl was dissolved at 60°C in 590 μl of water,

acidified with 140 μmol of HCl, and cooled to 0°C. After standing for at least 30 min at 0°C, the precipitated L-5,6-dihydroorotate was collected by centrifugation at 10,000 × *g* for 20 min. The supernatant was removed and the L-5,6-dihydroorotate was redissolved in a minimal volume of water at 60°C (to give approximately 106 mM L-5,6-dihydroorotate) and HCl was added to a concentration of 10 mM. The clear solution was cooled to 0°C and the recrystallized L-5,6-dihydroorotate (free acid) was recovered as described above. After two crystallizations, 85% of the L-5,6-dihydroorotate was recovered with only 0.25% of the LiCl. Chloride ion concentration was determined by titration with standardized 10 mM $Hg(NO_3)_2$ (Sigma) in the presence of diphenylcarbazone-bromphenol blue indicator (Furman, 1968). L-5,6-Dihydroorotate was assayed by its absorbance at 230 nm in 133 mM sodium phosphate (pH 5.5) using a molar extinction coefficient of 1.17×10^3 liters·mol^{-1} cm^{-1} (Sander *et al.*, 1965).

For the enzymatic synthesis of L-[^{14}C]DHO from L-[^{14}C]CA, the reaction mixture contained 67 mM potassium Mes (pH 6.29), 1.85 mCi of L-[^{14}C]CA (52.0 Ci/mol), and dihydroorotase (maximal activity, 40.6 nmol/min at pH 6.29) (Christopherson and Jones, 1979) in a total volume of 1.74 ml. After incubation for 19 h at 37°C, the entire mixture was loaded onto a DEAE-cellulose column (18.9 × 0.7 cm) equilibrated with water which was eluted with a 40-ml gradient of 0 to 300 mM LiCl (pH 5.5). Fractions of 0.405 ml were collected, L-[^{14}C]DHO eluted between 18.6 and 22.3 ml, and L-[^{14}C]CA eluted between 24.7 and 30.4 ml (Christopherson *et al.*, 1978). Appropriate fractions were pooled to yield 6.8 μmol L-[^{14}C]DHO and 20.5 μmol of L-[^{14}C]CA. To increase the volume of the subsequent recrystallization solution to a workable size, 9.3 μmol of unlabeled L-5,6-dihydroorotate was added to the pooled L-[^{14}C]DHO and both pools were lyophilized. The resultant oily residue of L-[^{14}C]CA was further purified as described by Christopherson *et al.* (1978). The L-[^{14}C]DHO was purified by the recrystallization procedure described above with minor modifications to facilitate crystallization of a smaller amount of material (16.1 μmol compared with 70.0 μmol). The yellow oily residue of L-[^{14}C]DHO and LiCl was redissolved in a minimal volume of water at 60°C and 40.3 μmol of HCl was added to give a final volume of 150 μl. The solution was stored overnight at −10°C and clear crystals of L-[^{14}C]DHO were apparent the next morning. The crystals were collected by centrifugation at 10,000 × *g* for 20 min and redissolved in 150 μl of 10 mM HCl. In contrast to the first crystallization, L-[^{14}C]-DHO precipitated readily upon cooling to 0°C, presumably because most of the LiCl from the DEAE-cellulose column eluate was removed in the supernatant of the first crystallization. The recrystallized L-[^{14}C]DHO was dissolved in water and the pH was adjusted to 7.0 with KOH to give a final volume of 450 μl. The concentration of the final product was determined from the absorbance at 230 nm of appropriate dilutions (Sander *et al.*, 1965) and the content of ^{14}C/μl was determined as described above for L-[^{14}C]CA. The synthesis and purification of L-[^{14}C]DHO yielded 7.2 μmol of final product with a specific radioactivity of 22.0 Ci/mol and a radiochemical purity of 99.9% as determined by chromatography on polyethyleneimine-cellulose (Christopherson *et al.*, 1978). The final yield of L-[^{14}C]DHO was 45% of the total ^{14}C-labeled L-5,6-dihydroorotate originally synthesized. A further crop of L-[^{14}C]DHO of lower specific radioactivity could be crystallized from the pooled supernatants of the two crystallizations by addition of more carrier L-5,6-dihydroorotate.

Racemic DL-[^{14}C]DHO (2.76 Ci/mol) was obtained from New England Nuclear and 50% of this preparation could be oxidized to [^{14}C]orotate by mouse dihydroorotate dihydrogenase.[2] Quantitation of ^{14}C-labeled compounds in aqueous samples or on polyethyleneimine-cellulose by scintillation counting was as previously described (Christopherson *et al.*, 1978).

Assay of Dihydroorotase—Dihydroorotase used in these studies was partially purified as part of the multienzymatic protein ME *pyr1-3* from mouse Ehrlich ascites carcinoma (Christopherson and Jones, 1979). The preparation had a maximal specific activity in the biosynthetic direction at pH 7.4 of 89.9 pmol/min/μg and a protein concentration of 7.24 mg/ml and showed a linear dependence of dihydroorotase activity upon volume of the preparation assayed within the range of dilutions used in these experiments. ME *pyr1-3* was prepared in a solution of 30% (v/v) glycerol, 2.0 mM dithiothreitol, 100-μM L-glutamine, and 10 mM potassium Hepes (pH 7.5) (Christopherson and Jones, 1979) and appropriate dilutions of dihydroorotase were always prepared with this solution. Dihydroorotase activity was measured by a modification of the method of Christopherson *et al.* (1978).

For determination of apparent K_m values as a function of pH and apparent K_i values for orotate derivatives, assay mixtures contained in a total volume of 25 μl: appropriate concentrations of L-[^{14}C]CA (52.0 Ci/mol) or L-[^{14}C]DHO (22.0 Ci/mol) and an inhibitor where required and 50 mM buffer with a pK_a value appropriate for the pH required (see above).[3] The reaction was initiated by addition of 5 μl of dihydroorotase diluted to a suitable activity to obtain a constant initial reaction velocity over the three sampling times used for each assay. At appropriate times, 5-μl samples were transferred onto polyethyleneimine-cellulose chromatograms (Brinkmann Instruments, Inc.) for isolation of L-[^{14}C]CA and L-[^{14}C]DHO. The thin layer chromatograms were developed by ascending chromatography with 0.34 M LiCl (pH 5.5) in a closed chromatography tank. Where relatively high K_m values were to be determined (at alkaline pH for *N*-carbamyl-L-aspartate and at acidic pH for L-5,6-dihydroorotate), the ^{14}C-labeled substrate was diluted to a lower specific radioactivity with unlabeled *N*-carbamyl-L-aspartate or L-5,6-dihydroorotate. The equilibrium ratio R = [DHO]/[CA] is extremely dependent upon pH (Christopherson and Jones, 1979) and we determined experimentally that at a given pH, the initial reaction rate is constant only up to 30% of formation of the final equilibrium concentration of product. Consequently, the dilution of dihydroorotase and the sampling times to be used for an experiment were of critical importance and were calculated from the equilibrium constant for Reaction 1 (K_{eq} = 1.51 × 10^6 liters·mol^{-1}) and the pH activity profiles for dihydroorotase (Christopherson and Jones, 1979) using the mathematical relationships derived under "Appendix 1."[4]

To screen for potential inhibitors of dihydroorotase, assay mixtures contained, in a total volume of 25 μl: 250 μM L-[^{14}C]CA (1.07 Ci/mol) or 10 μM L-[^{14}C]DHO (22.0 Ci/mol), 50 mM potassium Hepes (pH 7.4), and a 5.0 mM concentration of the compound to be tested when L-[^{14}C]CA was substrate and 200 μM when L-[^{14}C]DHO was substrate. The reaction was initiated by addition of 5 μl of dihydroorotase (maximal biosynthetic activity, 22.0 pmol/min at pH 7.4) and samples were taken and processed as described above. For every experiment, duplicate control incubations lacking inhibitor were run enabling calculation of percentage of inhibition of the compounds tested. Effective inhibitors of dihydroorotase (orotate and 5-substituted derivatives, L-cysteine, and related compounds) were subsequently studied in more detail.

For studies of the inactivation of dihydroorotase by L-cysteine and related compounds, preincubation mixtures contained in a total volume of 25 μl: 50 mM potassium Hepes (pH 7.27) or 50 mM Tris-HCl (pH 8.40), a 50 mM concentration of the inactivator, and L-[^{14}C]CA (1.07 Ci/mol) or L-[^{14}C]DHO (2.00 Ci/mol) when substrate protection was studied. The experiment was started by addition of 5 μl of dihydroorotase and 5-μl samples of the preincubation mixture were removed at appropriate times for determination of dihydroorotase activity (Christopherson and Jones, 1979). When inactivation of dihydroorotase was studied at pH 7.27, assays were done in the biosynthetic direction at pH 6.6; when protection by L-[^{14}C]DHO against inactivation by L-cysteine was studied at pH 7.27, assays were done in the degradative direction at pH 8.0. When inactivation of dihydroorotase at pH 8.4 was studied, assays were done in the degradative direction at pH 8.4. Solutions of all inactivators containing a thiol group were made up just before use in degassed water.

RESULTS

The dependence of the apparent K_m for *N*-carbamyl-L-aspartate upon pH is shown in Fig. 1. The apparent K_m has a minimal value at acidic pH of 30 μM and increases from 100 μM at pH 7.0 to 10.0 mM at pH 8.3. These K_m values (Fig. 1) are in contrast to those obtained by Bresnick and Blatchford (1964), who found that the apparent K_m for *N*-carbamyl-DL-

[2] J.-J. Chen, personal communication.

[3] Apparent K_m values obtained for dihydroorotase were similar at the same pH using potassium Mes or potassium Hepes and potassium Hepes or Tris-HCl.

[4] Derivation of equations for determining the maximal permitted conversion of substrate to product for measurement of initial reaction velocities, Figs. 3a, 3b, 4, 5a, 5b, 6a, and 6b, and determination of functional relationships describing the pH dependencies of apparent K_m and V_{max} values appear in a miniprint section. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document No. 79M-2240, cite author(s), and include a check or money order for $3.60 per set of photocopies.

aspartate varied in a somewhat unsystematic manner with pH with a minimal value of 1.2 mM at pH 6.5. Kennedy (1974) obtained a minimal apparent K_m value for *N*-carbamyl-L-aspartate of 120 μM at pH 6.5 by extrapolating the values obtained to zero enzyme concentration. Commercially available *N*-carbamyl-DL-aspartate was used in Kennedy's study and the concentrations of *N*-carbamyl-DL-aspartate used were halved to give an apparent K_m for *N*-carbamyl-L-aspartate.

The remarkable increase of 2 orders of magnitude in the apparent K_m for *N*-carbamyl-L-aspartate between pH 7.0 and 8.3 (Fig. 1) suggests that an acidic form of free dihydroorotase, binding one or more H^+ ions, is the catalytically active species for the biosynthetic reaction. To test this hypothesis, models were constructed where dihydroorotase takes up 1, 2, or 3 H^+ ions to attain a catalytically active form which binds *N*-carbamyl-L-aspartate ("Appendix 2"). With the assumption that the true K_m for *N*-carbamyl-L-aspartate is equivalent to the dissociation constant of the catalytically active enzyme·substrate complex, equations were derived from these three models which give the functional dependence of the apparent K_m upon pH ("Appendix 2"). Using these equations and substituting various values for the acid dissociation constant(s) (K_a values) and a value of 30 μM for the true K_m for *N*-carbamyl-L-aspartate, theoretical curves were generated for the 1-, 2-, and 3-proton models. The theoretical curve for each model which best fits the experimental data is shown in Fig. 1. The curve for the 1-proton model, generated from a single pK_a value of 5.7, does not fit the experimental data. The curve for the 2-proton model, generated from two pK_a values of 6.8, fits the experimental data up to pH 7.4 but cannot account for the remarkable increase in the apparent K_m at more alkaline pH values. The curve for the 3-proton model fits the experimental data (*solid line* of Fig. 1) and was generated from the equation:

$$(K_m^{CA})_H = K_m^{CA} \frac{([H^+]^3 + K_{a3}[H^+]^2 + K_{a2}K_{a3}[H^+] + K_{a1}K_{a2}K_{a3})}{[H^+]^2([H^+] + K_{a4})} \quad (2)$$

where $(K_m^{CA})_H$ is the apparent K_m for *N*-carbamyl-L-aspartate at a given pH, K_m^{CA} is the true K_m for *N*-carbamyl-L-aspartate of the acidic, catalytically active form of dihydroorotase, K_{a1}, K_{a2}, and K_{a3} are the acid dissociation constants for the various protonated forms of dihydroorotase (pK_{a1} = 7.6, pK_{a2} = 6.8, pK_{a3} = 6.8), and K_{a4} is the acid dissociation constant for the enzyme·substrate complex (pK_{a4} = 7.1) (Christopherson and Jones, 1979). The full derivation of Equation 2 appears under "Appendix 2c."

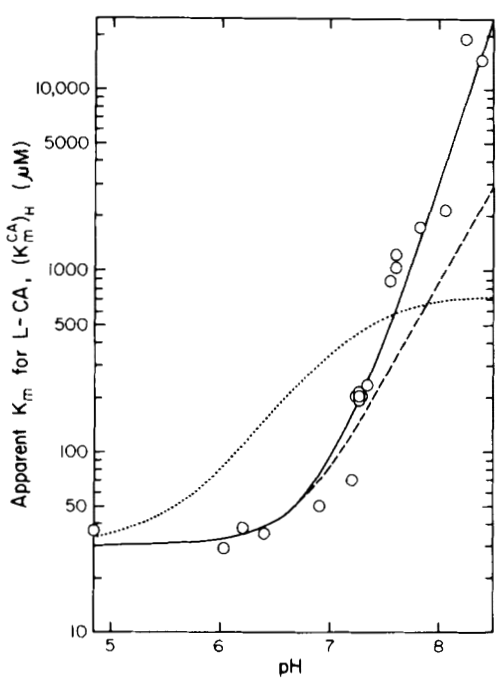

FIG. 1. **The effect of pH on the apparent K_m for L-CA $(K_m^{CA})_H$.** Assay mixtures contained 0.20 μg of dihydroorotase in a total volume of 25 μl. Each value of $(K_m^{CA})_H$ was determined from at least six different concentrations of L-[^{14}C]CA. Details of the assay are described under "Experimental Procedures." Lines of best fit through the experimental points were generated from a 1-proton model (····) ("Appendix 2a"), a 2-proton model (– – –) ("Appendix 2b"), and a 3-proton model (——) ("Appendix 2c") using appropriate equations from the appendices listed above.

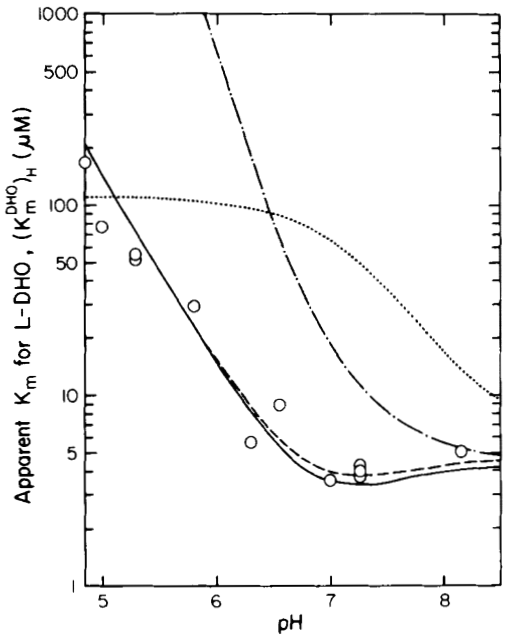

FIG. 2. **The effect of pH on the apparent K_m for L-DHO $(K_m^{DHO})_H$.** Procedures used were the same as described in the legend to Fig. 1. Lines of best fit through the experimental points were generated from a 1-proton model (····) ("Appendix 2a"), a 2-proton model (– – –) ("Appendix 2b"), a 3-proton model (–·–·–) ("Appendix 2c"), and a 3-proton model where L-DHO binds to *E* and *E*H (——) ("Appendix 2d") using appropriate equations from the appendices listed above.

The pH dependence of the apparent K_m for L-5,6-dihydroorotate is shown in Fig. 2. The apparent K_m has a minimal value of 3.8 μM at pH 7.3 and increases progressively to 140 μM as the pH decreases to 5.0. The values obtained for the apparent K_m at alkaline pH (Fig. 2) are 10-fold lower than the value reported by Kennedy (1974) of approximately 40 μM at 9.0 for dihydroorotate of unspecified stereochemistry.

The increase of the apparent K_m for L-5,6-dihydroorotate by 1 order of magnitude as the pH decreases from 7.0 to 5.6 implicates a basic form of dihydroorotase as the catalytically active form for the degradative reaction. The weaker dependence of the apparent K_m for L-5,6-dihydroorotate upon pH (Fig. 2) when compared with that of the apparent K_m for *N*-carbamyl-L-aspartate (Fig. 1) suggests that less than 3 H^+ ions may be involved in the equilibria between the putative catalytically active and inactive forms of dihydroorotase. In a similar analysis to that for the apparent K_m for *N*-carbamyl-L-aspartate, theoretical models were constructed where a single active form of dihydroorotase takes up 1, 2, or 3 H^+ ions giving rise to inactive forms of the enzyme ("Appendix 2"). Using equations derived from these three models, theoretical

curves were generated using various values for the acid dissociation constants of the protonated forms of dihydroorotase and the true K_m value of the catalytically active form of dihydroorotase for L-5,6-dihydroorotate (an optimal value of 4.4 μM was used). The theoretical curve for each model which best fits the experimental data is presented in Fig. 2. The best curve for the 1-proton model, generated from a single pK_a value of 8.5, does not fit the experimental data and, as expected, the curve for the 3-proton model gives an apparent K_m for L-5,6-dihydroorotate which is too strongly dependent upon pH (Fig. 2). The curve for the 2-proton model fits the experimental data of Fig. 2 and was generated from the equation:

$$(K_m^{DHO})_H = K_m^{DHO} \frac{K_{a4}([H^+]^2 + K_{a3}[H^+] + K_{a2}K_{a3})}{K_{a2}K_{a3}([H^+] + K_{a4})} \quad (3)$$

where $(K_m^{DHO})_H$ is the apparent K_m for L-5,6-dihydroorotate at a given pH, K_m^{DHO} is the true K_m for L-5,6-dihydroorotate of the basic catalytically active form of dihydroorotase, K_{a2} and K_{a3} are the acid dissociation constants for the various protonated forms of dihydroorotase ($pK_{a2} = 6.8$, $pK_{a3} = 6.8$, as for Equation 2 describing $(K_m^{CA})_H$), and K_{a4} is the acid dissociation constant of the enzyme·substrate complex ($pK_{a4} = 7.1$) (Christopherson and Jones, 1979). Equation 3 is derived under "Appendix 2b."

The involvement of four forms of dihydroorotase (3-proton model), of which the most acidic form binds *N*-carbamyl-L-aspartate ("Appendix 2c"), and the involvement of only three forms of dihydroorotase (2-proton model), the most basic of which binds L-5,6-dihydroorotate ("Appendix 2b"), is inconsistent since dihydroorotase should have the same number of ionizing groups in the presence of either substrate. A 3-proton model, in which dihydroorotase exists in four forms similar to those present for *N*-carbamyl-L-aspartate and in which L-5,6-dihydroorotate binds equally well to the two most basic forms of the enzyme, also accounts for the pH dependence of the apparent K_m for L-5,6-dihydroorotate (*solid line* of Fig. 2). The equation for this model is derived under "Appendix 2d":

$$(K_m^{DHO})_H = K_m^{DHO} \frac{K_{a4}([H^+]^3 + K_{a3}[H^+]^2 + K_{a2}K_{a3}[H^+] + K_{a1}K_{a2}K_{a3})}{K_{a2}K_{a3}([H^+] + K_{a1})([H^+] + K_{a4})} \quad (4)$$

The terms of Equation 4 have been defined in the descriptions of Equations 2 and 3. To generate the theoretical curve of Fig. 2, a value of 4.4 μM was assigned to K_m^{DHO} and the values of K_{a1}, K_{a2}, K_{a3}, and K_{a4} used were identical to those substituted into Equation 2 to generate the theoretical curve of Fig. 1.

Fig. 3a shows that *N*-carbamyl-D-aspartate is also a substrate for dihydroorotase. D-[^{14}C]CA was prepared as described under "Experimental Procedures" under conditions identical to those used for preparation on a larger scale of unlabeled *N*-carbamyl-D-aspartate. It is assumed, therefore, that the D-[^{14}C]CA was of the same high degree of stereochemical purity as the unlabeled *N*-carbamyl-D-aspartate ($[\alpha]_D^{23} = -39.9°$ compared with $[\alpha]_D^{23} = +39.6°$ for *N*-carbamyl-L-aspartate). After prolonged incubation at pH 6.0, 46% of the D-[^{14}C]CA, was converted by excess dihydroorotase to D-[^{14}C]DHO. The equilibrium value of 60% conversion of D-[^{14}C]CA to D-[^{14}C]DHO (Christopherson and Jones, 1979) was not reached because of the relatively low rate of catalysis and, ultimately, inactivation of dihydroorotase. The apparent K_m values for *N*-carbamyl-L-aspartate and *N*-carbamyl-D-aspartate are similar, 247 and 204 μM, respectively, at pH 7.33, but the V_{max} for *N*-carbamyl-D-aspartate is only 1.7% of that obtained with *N*-carbamyl-L-aspartate (Fig. 3b). The deviation of the Lineweaver-Burk plot for *N*-carbamyl-D-aspartate from linearity at high concentrations of *N*-carbamyl-D-aspartate (2 and 5 mM, Fig. 3b) was a reproducible effect which was not observed for *N*-carbamyl-L-aspartate.

D-5,6-Dihydroorotate can also be used as a substrate by dihydroorotase (Fig. 4). Because D-[^{14}C]DHO was not available, a racemic mixture of DL-[^{14}C]DHO was incubated with an excess of dihydroorotase at pH 9.2. The resultant time course for synthesis of DL-[^{14}C]CA from DL-[^{14}C]DHO (Fig. 4) is biphasic; half of the racemic DL-[^{14}C]DHO was consumed at a rapid rate and the remainder reacted at a slower rate, suggesting that L-[^{14}C]DHO is a more effective substrate than D-[^{14}C]DHO. At the end of the experiment, 93% of the DL-[^{14}C]DHO was converted to DL-[^{14}C]CA, compared with 99.9% conversion predicted at equilibrium, a value not reached presumably because of the low rate of catalysis with D-5,6-dihydroorotate as substrate. Kinetic studies using DL-[^{14}C]DHO indicated an apparent K_m for the racemic substrate of 25 μM at pH 7.33 compared with an apparent K_m for L-[^{14}C]DHO of 3.8 μM (Fig. 2), but a value of high precision could not be obtained because of the low specific radioactivity of the DL-[^{14}C]DHO (2.76 Ci/mol). The maximal reaction velocities obtained with saturating concentrations of DL-[^{14}C]DHO (Christopherson and Jones, 1979) and L-[^{14}C]DHO from pH 6.4 to 9.2 were similar.

Kennedy (1974) reported a K_i for orotate of 250 μM at pH 6.5 for dihyroorotase from rat liver using *N*-carbamyl-DL-aspartate as substrate. We have studied the dependence of dihydroorotase activity upon the concentrations of L-[^{14}C]CA or L-[^{14}C]DHO at pH 7.33 using four concentrations of orotate. Fig. 5a shows Lineweaver-Burk plots of the kinetic data obtained using L-[^{14}C]CA as substrate. The resultant straight lines for various orotate concentrations intersect on the $1/v$ axis indicating simple competitive inhibition by orotate. A plot of the slopes of these lines (Fig. 5a) *versus* orotate concentration (Segel, 1975) gave a straight line with a negative intercept of 170 μM on the orotate abscissa. Thus, using *N*-carbamyl-L-aspartate as substrate for dihydroorotase, orotate is a competitive inhibitor with an apparent K_i value of 170 μM.

An analogous experiment was done using L-[^{14}C]DHO as substrate and a pattern of lines intersecting on the $1/v$ axis was again obtained (Fig. 5b). However, a plot of the slopes of these lines *versus* orotate concentration gave an apparent K_i value for orotate of 9.6 μM, 18-fold lower than the value determined using L-[^{14}C]CA as substrate. The intersecting Lineweaver-Burk plots obtained (Figs. 5a and 5b) are consistent with simple competitive inhibition involving binding of orotate at the active site of dihydroorotase to the exclusion of substrate. The lower apparent K_i value for orotate found in the presence of L-[^{14}C]DHO suggests that dihydroorotase using this substrate has a higher affinity for orotate.

Bresnick and Hitchings (1961) found that 5-fluoro orotate inhibits dihydroorotase and Finney and O'Sullivan (1979) reported that the 5-bromo and 5-methyl derivatives of orotate are also effective inhibitors. We have carried out a systematic study of the inhibition of dihydroorotase by 5-substituted derivatives of orotate (Table I). The tabulated apparent K_i values obtained using L-[^{14}C]CA or L-[^{14}C]DHO as substrate show that the 5-fluoro, 5-amino, and 5-methyl derivatives of orotate are more effective inhibitors of dihydroorotase than unsubstituted orotate. 5-Bromo orotate is a more effective inhibitor than orotate when *N*-carbamyl-L-aspartate is substrate but is less effective than orotate when L-5,6-dihydroorotate is substrate, while 5-iodo orotate is a less effective inhibitor than orotate using either substrate (Table I). A considerably lower apparent K_i value was obtained for each orotate derivative using L-5,6-dihydroorotate as substrate, as

TABLE I

Inhibition of the biosynthetic and degradative activities of dihydroorotase by 5-substituted orotate derivatives

Assay mixtures contained 50 mM potassium Hepes, pH 7.27, purified dihydroorotase (0.14 μg of protein), 400 μM L-[^{14}C]CA (52.0 Ci/mol) or 10 μM L-[^{14}C]DHO (22.0 Ci/mol), and six different concentrations of each orotate (OA) derivative. K_i values were calculated from Dixon plots of the experimental data (Segel, 1975) using a value for V_{max} determined for each experiment from at least six substrate concentrations. The assay of dihydroorotase is described in detail under "Experimental Procedures."

Substrate or OA derivative	Diameter of substituent in position 5[a]	Apparent K_m or K_i for biosynthetic reaction	Apparent K_m or K_i for degradative reaction
	Å	μM	
L-CA	3.8[b]	205	
L-DHO	3.5/4.0[c]		3.9
OA	2.0/3.4[d]	170[e]	9.6[e]
F-OA	2.7	24	6.0
NH_2-OA	2.9	28	6.0
CH_3-OA	3.5	36	6.6
Br-OA	3.8	46	20
I-OA	4.1	312	54

[a] The diameter of the substituent in position 5 of the pyrimidine ring (between the carbonyl group in position 4 and the exocyclic carboxyl group in position 6) is the distance perpendicular to the plane of the ring occupied by this chemical group. Molecular models of the pyrimidines above were constructed to scale from Ealing CPK space-filling atoms and the distances were measured using calibrated calipers.

[b] Maximal diameter of the β-methylene group of L-CA.

[c] The pyrimidine ring of L-DHO is in the "half-chair" conformation, thus the perpendicular distance between the axial and equatorial hydrogen atoms in position 5 is 3.5 Å while the total perpendicular distance occupied by the ring and the axial hydrogen atom is 4.0 Å.

[d] The diameter of the hydrogen atom in position 5 of OA is 2.0 Å; the perpendicular distance through the pyrimidine ring of all orotate derivatives is 3.4 Å.

[e] K_i values obtained from Figs. 5*a* and 5*b*, K_i values obtained from Dixon plots, were 107 μM for the biosynthetic reaction and 14 μM for the degradative reaction.

was found for orotate (Figs. 5a and 5b). Arranging the 5-substituted orotate derivatives in order of ascending apparent K_i values, there is a corresponding increase in the size of the 5 substituent (Table I). The distance in Angstroms perpendicular to the plane of the pyrimidine ring occupied by the substituent in position 5 on the ring was measured from scale molecular models constructed from space-filling atoms. Table I indicates that the apparent K_i values for orotate derivatives begin to increase when the size of the 5 substituent exceeds 3.5 Å, suggesting some steric hindrance to binding with bulky substituents.

In a previous paper (Christopherson and Jones, 1979), we postulated that mammalian dihydroorotase might contain zinc at the active site as dihydroorotase from *C. oroticum* probably does (Taylor *et al.*, 1976). To obtain further evidence for this postulate, partially purified mouse dihydroorotase was dialyzed under an atmosphere of nitrogen against a solution of 30% glycerol, 1.0 mM dithiothreitol, 100 μM L-glutamine, and 10 mM potassium Hepes (pH 7.5) in ultrapure water containing 1 mM 1,10-phenanthroline, 10 mM diethyldithiocarbamate, or 100 mM L-cysteine. These metal chelators are the most effective in removing zinc from horse liver alcohol dehydrogenase (Drum *et al.*, 1969). Dihydroorotase activity rapidly decreased to less than 1% in the presence of L-cysteine while 55% of the activity (relative to a control) remained after dialysis for 84 h against 1,10-phenanthroline or diethyldithiocarbamate.[5]

[5] R. I. Christopherson, E. E. Floyd, and M. E. Jones, unpublished experiment.

TABLE II

Inactivation of dihydroorotase by L-cysteine and some structural analogs

Dihydroorotase was preincubated at pH 7.27 or pH 8.40 with a 50 mM concentration of inactivator at 37°C. Samples of the preincubation mixture were removed for assay of dihydroorotase at 0.5, 3.5, 6.5, and 9.5 min for L-cysteine and at 0.5, 15, 30, and 45 min for the other inactivators and control preincubations lacking inactivator. Pseudo-first order rate constants for inactivation of dihydroorotase were calculated from the slope of a line fitted by the method of least squares to a plot of the logarithm of dihydroorotase activity *versus* time. Conditions for preincubation and assay of dihydroorotase are described under "Experimental Procedures."

Inactivator	pK_a value(s)	Pseudo-first order rate constant for inactivation of dihydroorotase	
		pH 7.27	pH 8.40
		$\times 10^3$ min^{-1}	
No addition		2.66	1.51
L-cysteine	8.58, 10.35 8.66, 10.28[a]	418	1244
2-Mercaptoacetate	10.56[b]	50.1	35.3
2-Mercaptoethylamine	8.35, 10.81[c]	25.3	86.1
3-Mercaptopropionate	10.84[d]	6.98	5.64
L-2,3-Diaminopropionate		6.70	11.1

[a] pK_a values are the microscopic ionization constants starting with the neutral zwitterion of L-cysteine. The upper two values are for dissociation of a proton from the thiol group followed by dissociation of a proton from the ammonium group. The lower two values are for dissociation of these two protons in the reverse order (Wrathall *et al.*, 1964).

[b] pK_a of the thiol group (Wrathall *et al.*, 1964).

[c] Li and Manning, 1955.

[d] pK_a of the thiol group (Irving *et al.*, 1964).

Biosynthetic dihydroorotase activity is almost totally inhibited by incubation with 50 mM L-cysteine for 9.5 min (Fig. 6a). Increasing concentrations of added L-[^{14}C]CA progressively protect dihydroorotase against this inactivation; 5 mM L-[^{14}C]CA gives total protection (Fig. 6a). Increasing concentrations of L-[^{14}C]DHO also progressively protect dihydroorotase against inactivation by 50 mM L-cysteine; 5 mM L-[^{14}C]DHO gives almost total protection (Fig. 6b). The data of Figs. 6a and 6b provide strong evidence for a direct interaction of L-cysteine with the active site of dihydroorotase.

Our finding that L-cysteine inhibits mouse dihydroorotase is in contradiction to the finding of Kennedy (1974) but confirms the earlier report by Bresnick and Blatchford (1964). However, the latter workers also found that 2-mercaptoacetate (thioglycollate), glutathione, 2-mercaptoethanol, sulfite, and cyanide inhibited dihydroorotase. Kennedy (1974) could not confirm these results and we found that of all the compounds cited above, only 2-mercaptoacetate (50 mM) was inhibitory, although less inhibitory than L-cysteine. To study further the mechanism of inhibition of dihydroorotase by these thiol compounds, we tested structural analogs of L-cysteine as inhibitors. The data of Table II show that in addition to L-cysteine and 2-mercaptoacetate, 2-mercaptoethylamine (L-cysteine lacking the α-carboxyl group), 3-mercaptopropionate (L-cysteine lacking the α-amino group), and L-2,3-diaminopropionate (the thiol group of L-cysteine replaced with an amino group) also inhibit dihydroorotase. Plots of the logarithm of dihydroorotase activity *versus* time from data similar to that of Figs. 6a and 6b gave straight lines for the decay of activity consistent with first order kinetics with respect to the concentration of active dihydroorotase. When the pH of the incubation medium was increased from pH 7.27 to 8.40, the pseudo-first order rate constant for inactivation of dihydroorotase by L-cysteine increased 3.0-fold, the constant for 2-mercaptoethylamine increased 3.4-fold, and the constant

for L-2,3-diaminopropionate increased 1.7-fold. In contrast, the constants for 2-mercaptoacetate and 3-mercaptopropionate decreased slightly (Table II). A likely explanation of these effects is that the basic forms of L-cysteine, 2-mercaptoethylamine, and L-2,3-diaminopropionate are involved in inactivation of dihydroorotase, whereas the state of ionization of 2-mercaptoacetate and 3-mercaptopropionate changes little between pH 7.27 and 8.4.

A wide variety of compounds were tested as potential inhibitors of dihydroorotase using assay conditions described under "Experimental Procedures." Ethylenediaminetetraacetate, imidazole, *p*-nitrobenzenesulfonamide, and dipicolinate, effective inhibitors of certain other zinc metalloenzymes (Kannan *et al.*, 1977; Pradham and Sander, 1973; Pocker and Fong, 1979) had no effect upon dihydroorotase activity. The following structural analogs of *N*-carbamyl-L-aspartate had no effect upon dihydroorotase: *N*-carbamyl-β-alanine, *N*-carbamyl-L-α-alanine, *N*-carbamyl-L-glutamate, *N*-acetyl-L-aspartate, fumarate, maleate, malonate, and succinate. The following pyrimidine compounds and analogs were not inhibitory: orotidine, orotidine 5'-phosphate (OMP), UMP, UTP, 5-phosphoribosyl 1-pyrophosphate, azauracil, barbiturate, dihydrouracil, and dihydrothymine. Thus, dihydroorotase is highly specific for its natural substrates; the only effective inhibitors of the enzyme found after extensive screening were orotate and its 5-substituted derivatives (Table I), L-cysteine and certain structural analogs (Table II), and diethylpyrocarbonate (Christopherson and Jones, 1979).

DISCUSSION

There is a complex interplay of pH effects for the enzymatic cyclization of *N*-carbamyl-L-aspartate to form L-5,6-dihydroorotate. The equilibrium between *N*-carbamyl-L-aspartate and L-5,6-dihydroorotate (Reaction 1) is pH-dependent (Christopherson and Jones, 1979); the biosynthetic reaction consumes an H^+ ion and the degradative reaction requires an OH^- ion. Consequently, one would expect the biosynthetic reaction to be catalyzed by an acidic form of dihydroorotase and the degradative reaction to be catalyzed by a basic form of the enzyme. Consistent with this proposal, the V_{max} values for the biosynthetic and degradative reactions show a complementary dependence upon pH (Fig. 4 of Christopherson and Jones, 1979) as do the apparent K_m values for *N*-carbamyl-L-aspartate and L-5,6-dihydroorotate (Figs. 1 and 2). By fitting theoretical curves generated from equations derived under "Appendix 2" to the experimental data for the pH dependencies of the apparent K_m values for *N*-carbamyl-L-aspartate and L-5,6-dihydroorotate, we have devised a minimal model which describes the equilibria existing between the various protonated forms of dihydroorotase (Fig. 7).

The various species of dihydroorotase depicted in Fig. 7 are aligned horizontally as free enzyme or enzyme·substrate complexes at each stage of catalysis and vertically according to the various states of protonation of the enzyme (except for the nonproductive enzyme·substrate complex EH'·DHO). *Diagonal arrows* indicate donation of a proton by dihydroorotase to convert carbamylaspartate to dihydroorotate and the net abstraction of a proton by dihydroorotase to convert dihydroorotate to carbamylaspartate. The pH dependence of the apparent K_m for *N*-carbamyl-L-aspartate (Fig. 1) is consistent with equilibration of dihydroorotase (E) between four states of protonation (E, EH, EH_2, EH_3), where EH_3 is the only catalytically active form of dihydroorotase for the biosynthetic reaction (Fig. 7) having a K_m value for *N*-carbamyl-L-aspartate of 30 μM. The functional dependence of the apparent K_m for *N*-carbamyl-L-aspartate $(K_m^{CA})_H$ upon pH is given by Equation 2. The enzyme·substrate complex (EH_3·CA) can lose a proton with an acid dissociation constant K_{a4} (Fig. 7) to give a nonproductive complex (EH_2·CA), which accounts for the biosynthetic pH profile for V_{max} (this dissociation has already been established, see Equation 7 and Fig. 4 of Christopherson and Jones, 1979, and "Appendix 2c"). The pH dependence of the apparent K_m for L-5,6-dihydroorotate (Fig. 2) is consistent with this model if E and EH are catalytically active for the degradative reaction, both having K_m values of 4.4 μM for L-5,6-dihydroorotate. The dependence of the apparent K_m for L-5,6-dihydroorotate $(K_m^{DHO})_H$ upon pH is given by Equation 4. The two active enzyme·substrate complexes (E·DHO and EH·DHO) may both gain a proton with an acid dissociation constant K_{a4} (Fig. 7) to form nonproductive complexes (EH'·DHO and EH_2·DHO), which explains the degradative pH profile for V_{max} (see Equation 8 and Fig. 4 of Christopherson and Jones, 1979, and "Appendix 2d"). The 3-proton model was preferred over an alternative 2-proton model ("Appendix 2b," Equation 3), which is also consistent with the experimental data because the former is consistent with the 3-proton model for $(K_m^{CA})_H$.

The multiple states of protonation of dihydroorotase involve only four distinct ionizing groups and the vertical align-

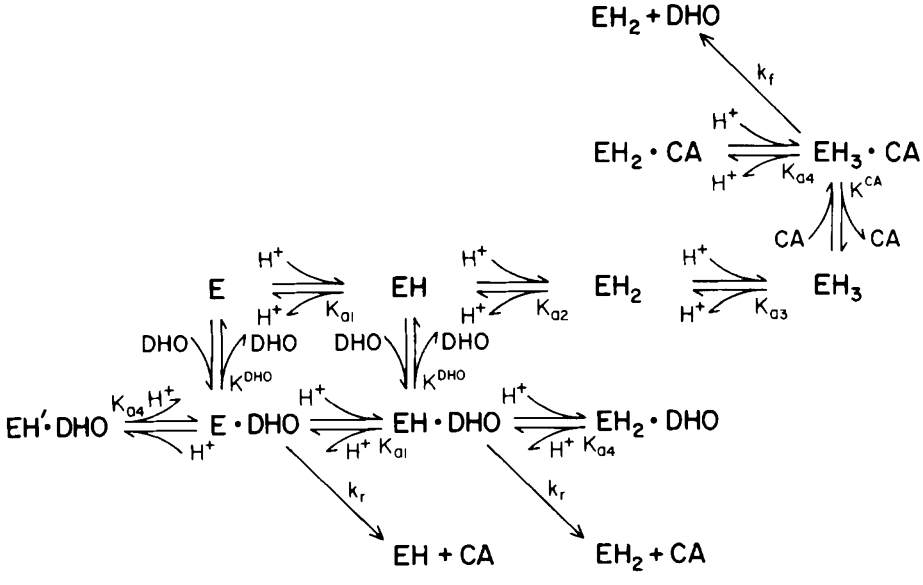

FIG. 7. **A minimal model describing equilibration of dihydroorotase between states of protonation significant for catalysis.** K_{a1}, K_{a2}, K_{a3}, and K_{a4} are acid dissociation constants, K^{CA} and K^{DHO} are the dissociation constants for the catalytically active enzyme·substrate complexes, k_f and k_r are the rate constants for synthesis of L-DHO and L-CA, respectively, and EH'·DHO is a catalytically inactive species differing from EH·DHO. For derivation of Equation 2 describing the dependence of the apparent K_m for L-CA upon pH ("Appendix 2c") and Equation 4 describing the pH dependence of the apparent K_m for L-DHO ("Appendix 2d"), the dissociation constants K^{CA} and K^{DHO} of this model were assumed to be equal to K_m^{CA} and K_m^{DHO}, respectively.

ment of enzyme species with the same number of protons (Fig. 7) does not necessarily indicate protonation of the same enzyme residues. The acid dissociation constants determined from curve-fitting procedures: $pK_{a1} = 7.6$, $pK_{a2} = 6.8$, $pK_{a3} = 6.8$ (Figs. 1 and 2), and $pK_{a4} = 7.1$ (see Fig. 4 of Christopherson and Jones, 1979), must be considered as approximate values but the model presented (Fig. 7) is well substantiated by the experimental data. The identity of the 3 or 4 ionizing residues involved in the equilibria between various states of protonation of dihydroorotase is unknown but there is evidence for at least 1 histidine residue at the active site (Christopherson and Jones, 1979) and the possibility of an ionizing water molecule bound to zinc at the active site (see below). The proportions of the various protonated species of free dihydroorotase may be calculated from their pK_a values using the mathematical expressions for each species of "Appendix 2c." At pH 7.0: *E*, 11%; *E*H, 44%; EH_2, 28%; EH_3, 17%. At pH 7.4: *E*, 33%; *E*H, 51%; EH_2, 13%; EH_3, 3%. Thus, in the physiological range of pH 7.0 to 7.4, 55 to 84% of dihydroorotase is in a form to catalyze ring cleavage of L-5,6-dihydroorotate and 17 to 3% will catalyze the biosynthetic ring closure of *N*-carbamyl-L-aspartate. Such a high proportion of dihydroorotase which is inactive for pyrimidine biosynthesis except in the presence of high concentrations of *N*-carbamyl-L-aspartate (Fig. 7) may indicate some form of regulation of biosynthetic dihydroorotase activity when it is functioning in concert with carbamyl phosphate synthetase and aspartate transcarbamylase of ME *pyr1-3*. We are investigating this possibility.

The structural similarity between orotate and L-5,6-dihydroorotate suggests that orotate may bind to the same forms of dihydroorotase as L-5,6-dihydroorotate does (*E*, *E*H). The apparent K_i values obtained for orotate as a competitive inhibitor (170 and 9.6 μM at pH 7.27 with *N*-carbamyl-L-aspartate and L-5,6-dihydroorotate, respectively, as substrates) are certain to be pH-dependent. However, the derived functional dependencies of the apparent K_i values upon pH should be identical using either substrate unless the dissociation constants for the enzyme·orotate complex(es) are higher in the presence of *N*-carbamyl-L-aspartate than with L-5,6-dihydroorotate or the equilibria between protonated species of free dihydroorotase are different in the presence of either substrate. If binding of L-5,6-dihydroorotate increased the affinity for orotate at the second or third active site of dihydroorotase on a molecule of trimeric ME *pyr1-3* (Mori and Tatibana, 1975; Coleman *et al.*, 1977: native ME *pyr1-3* exists as a mixture of trimers and hexamers), cooperativity should be observed in the substrate-velocity curves for *N*-carbamyl-L-aspartate or L-5,6-dihydroorotate, or both. An alternative explanation is that dihydroorotase exists in only three protonated forms (Equation 3 and "Appendix 2b") in the presence of L-5,6-dihydroorotate, the most basic of which binds L-5,6-dihydroorotate or orotate with high affinity, but again cooperativity should be observed in the substrate-velocity curves. Hill plots of kinetic data down to L-5,6-dihydroorotate concentrations of 0.5 μM (pH 7.0) and down to *N*-carbamyl-L-aspartate concentrations of 3 μM (pH 6.3) showed no such cooperativity. It is possible that positive cooperativity with respect to L-5,6-dihydroorotate exists at concentrations below 0.5 μM, the limit of our assay procedure using L-[^{14}C]DHO of specific radioactivity 22.0 Ci/mol.

At pH 7.33, the apparent K_m values for *N*-carbamyl-L-aspartate and *N*-carbamyl-D-aspartate are 247 and 204 μM, respectively (Fig. 3b) but the V_{max} for *N*-carbamyl-D-aspartate is only 1.7% of the value obtained with the L isomer. The data described above indicate that binding of carbamylaspartate to dihydroorotase is not affected by the disposition of the α-carboxyl group, although there is disruption of the stereochemistry for catalysis. By contrast, the apparent K_m for L-5,6-dihydroorotate is 3.8 μM (Fig. 2) and the apparent K_m for DL-5,6-dihydroorotate is approximately 25 μM; V_{max} values are similar with either substrate. The biphasic curve for conversion of DL-5,6-dihydroorotate to *N*-carbamyl-DL-aspartate (Fig. 4) indicates that the V_{max} is maintained until L-5,6-dihydroorotate is depleted. Thus, in contrast to *N*-carbamyl-D-aspartate and *N*-carbamyl-L-aspartate, D-5,6-dihydroorotate binds less effectively to dihydroorotase than L-5,6-dihydroorotate. The nitrogen and carbon atoms at positions 1, 2, 3, and 4 of the pyrimidine ring of dihydroorotate form a conjugated system of partial double bonds and are coplanar, while the saturated carbon atoms at positions 5 and 6 are disposed towards opposite sides of the plane of the ring to give a "half-chair" conformation (Brown, 1970). The constraint of the pyrimidine ring forces the bulky carboxyl group into the axial position on two different half-chair conformations taken up by L-5,6-dihydroorotate and D-5,6-dihydroorotate, while the hydrogen atom of position 6 is equatorial to the ring for both enantiomers. D-5,6-Dihydroorotate may not bind as effectively as L-5,6-dihydroorotate because the difference in the position of the carboxyl groups between L-5,6-dihydroorotate and D-5,6-dihydroorotate would be far greater than for *N*-carbamyl-D-aspartate and *N*-carbamyl-L-aspartate where the α-carboxyl groups have more rotational freedom. Thus, the "D orientation" of the carboxyl group of D-5,6-dihydroorotate disrupts binding and probably catalysis as well.

The hierarchy of the apparent K_i values observed for orotate derivatives is the same as the order of increasing size of the 5 substituent, except for unsubstituted orotate (Table I). The changes observed in the apparent K_i values are not consistent with possible electronic effects of the substituents on the pyrimidine ring. The halogen substituents would be electron-withdrawing while the amino and methyl substituents would be electron-donating. The observation that 5-fluoro, 5-amino, and 5-methyl orotate have a higher apparent affinity for dihydroorotase than orotate (Table I) may be due to occupation by these 5 substituents of a space at the active site normally filled by the equatorial and axial protons of carbon atom 4 of L-5,6-dihydroorotate in the half-chair conformation but not adequately filled by the small hydrogen atom of planar, unsubstituted orotate. Using L-5,6-dihydroorotate as substrate, there is a 3-fold increase in the apparent K_i going from 5-methyl orotate to 5-bromo orotate as the diameter of the 5-bromo substituent (3.8 Å) exceeds the distance perpendicular to the pyrimidine ring occupied by the equatorial and axial protons in position 5 of L-5,6-dihydroorotate (3.5 Å, Table I). The steric hindrance to binding of 5-bromo orotate is even more pronounced for the larger 5-iodo substituent (Table I). Using *N*-carbamyl-L-aspartate as substrate, there is a progressive increase in the apparent K_i values for 5-fluoro, 5-amino, and 5-methyl orotate, the apparent K_i for 5-bromo orotate is only 1.3-fold greater than the K_i for 5-methyl orotate, and then there is a 6.8-fold increase in K_i between 5-bromo and 5-iodo orotate (Table I). Thus, the transition to higher K_i occurs between the 5-methyl and 5-bromo substituents with L-5,6-dihydroorotate as substrate and between the 5-bromo and 5-iodo substituents with *N*-carbamyl-L-aspartate as substrate. The data of Table I suggest a more closed conformation for the active site with L-5,6-dihydroorotate as substrate, which may be correlated with the possible tighter binding of the orotate derivatives indicated by the considerably lower apparent K_i values obtained using L-5,6-dihydroorotate as substrate.

The rapid inactivation of dihydroorotase by L-cysteine and the slower effect of 2-mercaptoacetate (Table II) are very similar to their effects on bovine carboxypeptidase A (Coombs

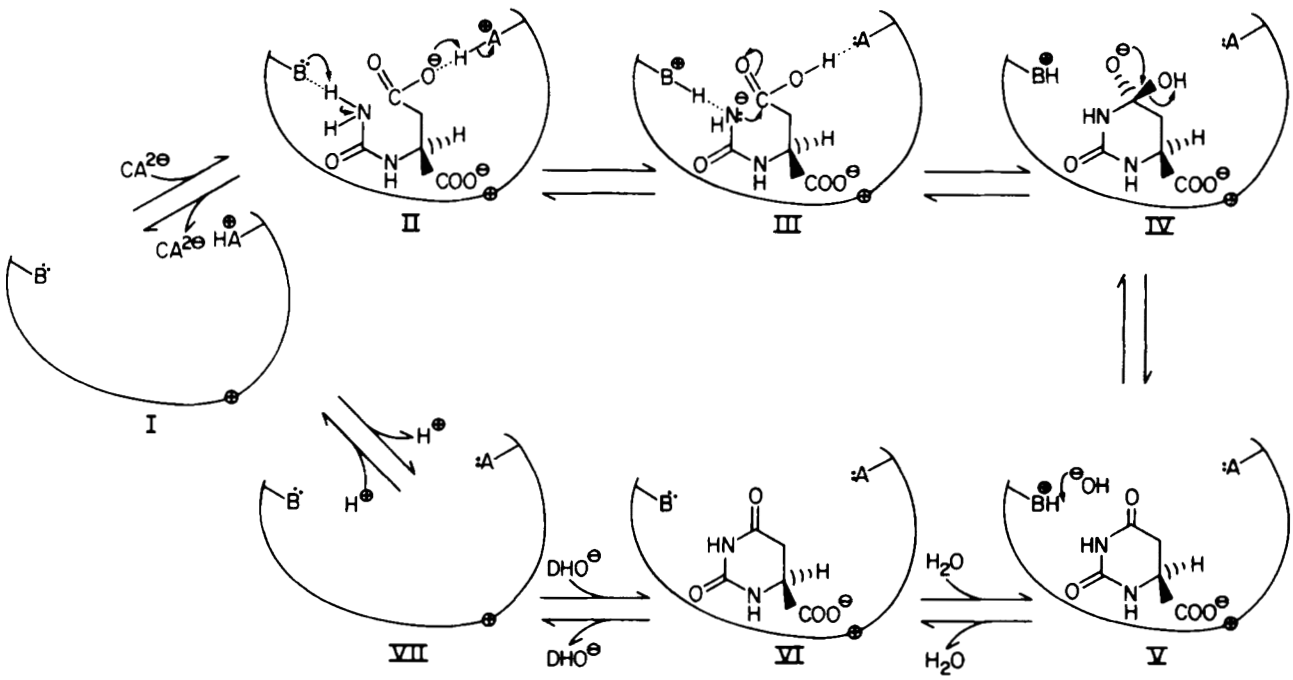

FIG. 8. **Proposed mechanism for the cyclization of L-CA to L-DHO catalyzed by mouse dihydroorotase.**

et al., 1962) where catalytic activity decreases in a time-dependent manner as zinc is removed from the active site. In aqueous solution, L-cysteine has two microscopic ionization constants for the thiol group (pK_a values of 8.58 and 10.28) and two for the amino group (pK_a values of 8.66 and 10.35) (Table II) and the two microspecies produced by loss of a proton from the zwitterion $(COO^-C(H)\ (NH_3^+)CH_2S^-$ and $COO^-C(H)\ (NH_2)CH_2SH)$ are present in approximately equal proportions (Wrathall *et al.*, 1964). Li and Manning (1955) found that the first stability constant for formation of the L-cysteine·Zn(II) and 2-mercaptoethylamine·Zn(II) complexes were $10^{9.86}$ and $10^{9.90}$, respectively, indicating complexes of equal stability. Their studies showed that L-cysteine forms a bidentate complex with Zn(II) through the basic forms of the thiol group ($-S^-$) and the amino group ($-NH_2$). The stability constant for formation of the 2-mercaptoacetate·Zn(II) complex was $10^{7.44}$, indicating weaker coordination of Zn(II) through the thiolate and carboxylate groups. The increase in the pseudo-first order rate constant for inactivation of dihydroorotase by L-cysteine, 2-mercaptoethylamine, and L-2,3-diaminopropionate as the pH is increased from 7.27 to 8.40 may be attributed to formation of the basic forms of their thiol or amino groups, or both (Table II). No increase was observed in the constant for 2-mercaptoacetate or 3-mercaptopropionate because of the high pK_a values of their thiol groups, 10.56 and 10.84, respectively (Table II). L-Cysteine may inhibit dihydroorotase more rapidly than 2-mercaptoethylamine because of the presence of a greater proportion of the ionized thiolate group on one of the microspecies of L-cysteine with a pK_a of 8.58 (Wrathall *et al.*, 1964), compared with the pK_a of the thiol group of 2-mercaptoethylamine of 10.81 (Li and Manning, 1955). 2-Mercaptoethanol with a pK_a of 9.72 for the thiol group (Irving *et al.*, 1964) did not inhibit dihydroorotase, presumably because the basic form of the hydroxyl group which might coordinate a metal atom does not exist to a significant extent in the pH range used in these studies.

The thiol group is the only functional group common to all the inactivators, except L-2,3-diaminopropionate. If the thiolate ion reacted as a nucleophile with a residue at the active site of dihydroorotase, one would expect 2-mercaptoethanol to inactivate dihydroorotase. The data presented in Table II implicate at least two basic groups of each inhibitor in the inactivation of dihydroorotase and the simplest explanation is coordination of a metal atom bound at the active site. Inhibition of dihydroorotase by L-2,3-diaminopropionate (Table II) shows that the inhibition by L-cysteine and other thiol-containing analogs is not due to cleavage of a disulfide bond as postulated by Bresnick and Blatchford (1964).

From the data presented here and in a previous paper (Christopherson and Jones, 1979) and from studies on the mechanism of alkaline hydrolysis of dihydropyrimidines (Sander, 1969), we propose a model for the catalytic mechanism of dihydroorotase (Fig. 8). The fact that carbamyl-β-alanine (an analog of *N*-carbamyl-L-aspartate lacking the α-carboxylate group) and dihydrouracil (an analog of L-5,6-dihydroorotate lacking the carboxylate group in the corresponding position 6) are not competitive inhibitors of the biosynthetic and degradative reactions demonstrates that this carboxylate group is essential to the binding of either substrate. Therefore, a positive charge has been placed on our representation of the active site of dihydroorotase in close proximity to the negative charge of the carboxylate group of the bound substrate (Fig. 8, *II* and *VI*). Similarly, carbamyl-L-α-alanine (an analog of *N*-carbamyl-L-aspartate lacking the β-carboxylate group) is not a competitive inhibitor of dihydroorotase, indicating that the β-carboxylate group of *N*-carbamyl-L-aspartate is also essential to binding. To account for the pH dependence of the apparent K_m for *N*-carbamyl-L-aspartate (Fig. 1), we propose that binding of L-CA^{2-} (pK_{a1} = 2.8, pK_{a2} = 4.3; Christopherson and Jones, 1979) is dependent upon an electrostatic interaction between the β-carboxylate group of L-CA^{2-} and the acidic, cationic form of a residue (AH^+) at the active site of dihydroorotase (Fig. 8*II*). Thus, the binding of *N*-carbamyl-L-aspartate may be attributed to two electrostatic interactions and must bring the terminal nitrogen atom of the conjugated, inflexible ureido group and the carbon atom of the β-carboxylate group into close proximity. The initial step of catalysis would be abstraction of a proton from the terminal nitrogen of the ureido group of *N*-carbamyl-L-aspartate and

donation of a proton to the β-carboxylate group of *N*-carbamyl-L-aspartate by AH^+ (Fig. 8*II*). Ring closure of *N*-carbamyl-L-aspartate would occur by nucleophilic attack by the resultant anionic nitrogen on the carbon atom of the β-carboxylate group of *N*-carbamyl-L-aspartate (Fig. 8*III*), forming a transient tetrahedral intermediate (Fig. 8*IV*). This transition state collapses, eliminating OH^- to form L-5,6-dihydroorotate (Fig. 8*V*). The form of the active site of dihydroorotase required for binding and cyclization of *N*-carbamyl-L-aspartate (Fig. 8*I*) is equivalent to EH_3 of Fig. 7. The involvement of 1 proton on the catalytic residue AH^+ is shown in Fig. 8 and the role of the other 2 protons is unknown. The catalytic mechanism (Fig. 8) is based upon the general base-catalyzed mechanism proposed by Sander (1969) (Equation 6b of that paper) for the hydrolysis of dihydropyrimidines in dilute alkali. Ring cleavage of L-5,6-dihydroorotate would proceed from Fig. 8*VI* in the reverse direction, the state of protonation of dihydroorotase being equivalent to *E* and *E*H of Fig. 7. The catalytic residue A (Fig. 8*VI*) would act as a general base generating an OH^- ion from water which attacks the carbonyl group at position 4 of L-5,6-dihydroorotate to form the transient tetrahedral intermediate (Fig. 8*IV*) which collapses to form *N*-carbamyl-L-aspartate.

If catalysis by dihydroorotase proceeds according to the mechanism of Fig. 8, then the zinc atom which we propose at the active site might coordinate a titrateable water molecule to form a catalytic residue equivalent to AH^+ (Fig. 8*II*). Such a catalytic group has been found at the active site of bovine carboxypeptidase A (Makinen *et al.*, 1979), which catalyzes cleavage of a peptide bond in perhaps an analogous manner to cleavage of the peptide-like bond of L-5,6-dihydroorotate by dihydroorotase. Dihydroorotase from *C. oroticum* contains 2 g atoms of zinc/subunit, probably at the active site (Taylor *et al.*, 1976). Walsh (1979) postulated that zinc bound at the active site of this enzyme stabilizes the transition state of the reaction (see Fig. 8*IV*). This stabilization could occur by formation of an inner sphere coordination complex through the 2 oxygen atoms at position 4 of the pyrimidine ring of the transition state (Fig. 8*IV*). Such a role for zinc in catalysis by mammalian dihydroorotase could still be consistent with the observed pH dependencies of K_m and V_{max}. The acidic form of a catalytic residue necessary for binding *N*-carbamyl-L-aspartate would be a water molecule coordinated to Zn(II) bound at the active site. The β-carboxylate group of *N*-carbamyl-L-aspartate would displace the water to form an inner sphere coordination complex with the Zn(II). Ring cleavage of L-5,6-dihydroorotate would be initiated by nucleophilic attack at carbon atom 4 of L-5,6-dihydroorotate by OH^- formerly coordinated to Zn(II). Although the pH activity profiles for the biosynthetic and degradative reactions of dihydroorotase from *C. oroticum* (Figs. 2 and 3 of Taylor *et al.*, 1976) are quite similar to those for mouse dihydroorotase (Fig. 4 of Christopherson and Jones, 1979), it would be premature to assume that the catalytic mechanisms are analogous without analysis of homogeneous ME *pyr1-3*, or the dihydroorotase domain of this multienzymatic protein, for zinc content.

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Supplementary Material to

The Effects of pH and Inhibitors Upon the Catalytic Activity of the Dihydroorotase of Multienzymatic Protein pyr1-3 from Mouse Ehrlich Ascites Carcinoma

Richard I. Christopherson and Mary Ellen Jones

Additional Figures Cited in the Text

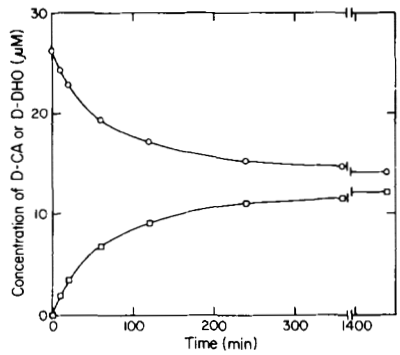


Fig. 3a. Conversion of D-CA to D-DHO by dihydroorotase. The assay mixture contained in a total volume of 100 μl: 50 mM KMes (pH 6.01), 25 μM D-[^{14}C]CA (52.0 Ci/mol) and 10 μg of dihydroorotase. Samples of 5 μl were taken at the indicated times and D-[^{14}C]CA (O) was separated from D-[^{14}C]DHO (□) as described under "Experimental Procedures".

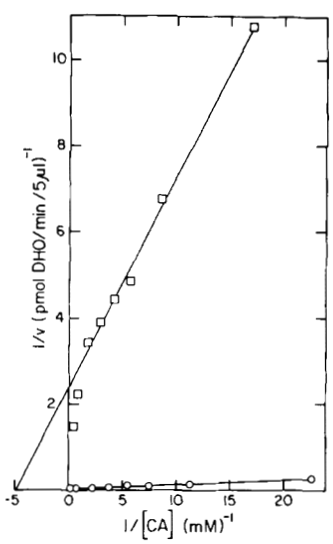


Fig. 3b. Lineweaver-Burk plots of the dependence of dihydroorotase activity upon the concentrations of L-CA and D-CA. Assay mixtures contained in a total volume of 25 μl: 50 mM KHepes (pH 7.33), the indicated concentrations of L-[^{14}C]CA (20.0 Ci/mol) or D-[^{14}C]CA (33.4 Ci/mol) and 0.24 μg of dihydroorotase. Details of the assay are described under "Experimental Procedures". Substrate used: O , L-[^{14}C]CA; □ , D-[^{14}C]CA.

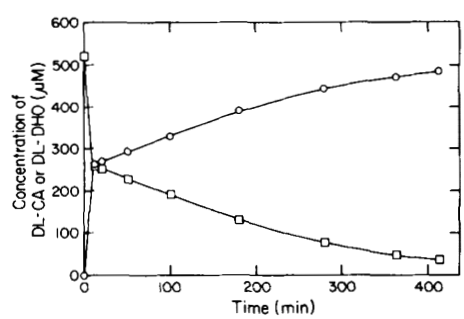


Fig. 4. Conversion of DL-DHO to DL-CA by dihydroorotase. The assay mixture contained in a total volume of 100 μl: 50 mM Tris-HCl (pH 9.2), 500 μM DL-[^{14}C]DHO (2.75 Ci/mol) and 40 μg of dihydroorotase. Other procedures were identical to those of Fig. 3a. DL-[^{14}C]CA, O ; DL-[^{14}C]DHO, □

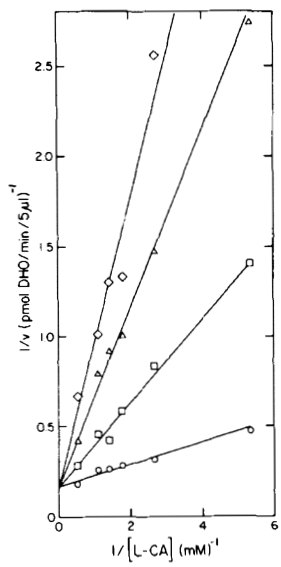


Fig. 5a. Lineweaver-Burk plots of dihydroorotase activity using L-CA as substrate and various concentrations of orotate. Assay mixtures contained in a total volume of 25 μl: 50 mM KHepes (pH 7.27), the indicated concentrations of L-[^{14}C]CA (26.0 Ci/mol), orotate (O , no orotate; □ , 250 μM; △ , 750 μM; ◇ , 1250 μM) and 0.28 μg of dihydroorotase. Details of the assay are described under "Experimental Procedures".

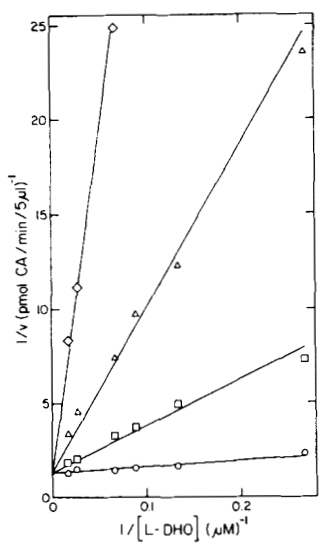
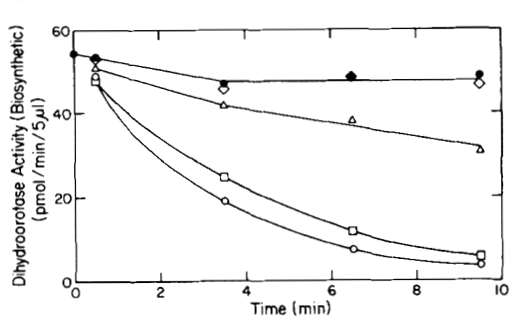
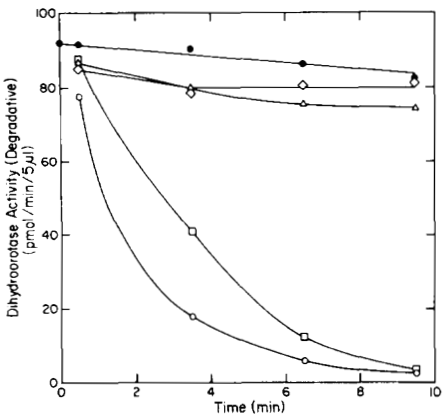


Fig. 5b. Lineweaver-Burk plots of dihydroorotase activity using L-DHO as substrate and various concentrations of orotate. Assay mixtures contained in a total volume of 25 μl: 50 mM KHepes (pH 7.27), the indicated concentrations of L-[^{14}C]DHO (22.0 Ci/mol), orotate (O , no orotate; □ , 50 μM; △ , 200 μM; ◇ , 750 μM) and 0.089 μg of dihydroorotase. Other experimental details were as for Fig. 5a.

Fig 6. Inactivation of dihydroorotase by L-cysteine. Partially purified ME pyr1-3 (18 μg) was incubated at 37°C in the presence of 50 mM L-cysteine and 50 mM KHepes (pH 7.27) in a total volume of 25 μl and dihydroorotase activity was assayed at the indicated times as described in "Experimental Procedures". Protection experiments were done with L-[^{14}C]CA or L-[^{14}C]DHO included in the incubation medium at the same specific radioactivities as those subsequently used in the biosynthetic (pH 6.6) or degradative (pH 8.4) assays, respectively.



a) Protection by L-[^{14}C]CA (1.07 Ci/mol) of dihydroorotase activity. Control vessel incubated in the absence of L-cysteine and L-[^{14}C]CA; ● . Experimental vessels incubated with 50 mM L-cysteine: O , no L-[^{14}C]CA; □ , 0.05 mM L-[^{14}C]CA; △ , 0.50 mM L-[^{14}C]CA; ◇ , 5.0 mM L-[^{14}C]CA.



b) Protection by L-[^{14}C]DHO (2.00 Ci/mol) of dihydroorotase activity. Control vessel incubated in the absence of L-cysteine and L-[^{14}C]DHO, ● . Experimental vessels incubated with 50 mM L-cysteine; ○ , no L-[^{14}C]DHO; □ , 0.05 mM L-[^{14}C]DHO; △ , 0.50 mM L-[^{14}C]DHO; ◇ , 5.0 mM L-[^{14}C]DHO.

Appendix 1. Calculation of Permitted Fractional Conversion of Substrate to Product for Measurement of Initial Velocities.

The equilibrium between L-CA and L-DHO includes an H^+ ion (reaction 1) and consequently, the equilibrium ratio R=DHO/CA is extremely dependent upon pH (Fig. 3b of Christopherson and Jones, 1979). We determined experimentally that at a given pH, the initial reaction rate is constant only up to 30% formation of the final equilibrium concentration of product. We derive relationships here which have enabled calculation of the permitted fractional conversion of substrate to product for measurement of true initial velocities at any pH value.

From reaction 1:

$$K_{eq} = \frac{[DHO^-]}{[CA^{2-}][H^+]} = \frac{d\ DHO}{c\ CA[H^+]} = \frac{R\ d}{c\ 10^{-pH}} = 1.51 \times 10^6\ 1.mol^{-1} \qquad (5)$$

where: CA and DHO are the total amounts of all ionic species of L-CA and L-DHO present at a given pH; c and d are the molar fractions of CA^{2-} and DHO^-, respectively.

$$c = \frac{10^{pH-4.32}}{1 + 10^{pH-4.32}} \qquad d = \frac{10^{pH-3.10}}{1 + 10^{pH-3.10}}$$

and R is the experimentally determined ratio of DHO/CA. These relationships are derived in more detail in a previous paper (equations 1 to 6 of Christopherson and Jones, 1979).

For assay of biosynthetic dihydroorotase activity, the fractional conversion of L-CA to L-DHO will be:

$$F = \frac{DHO}{DHO + CA}$$

$$F\ DHO + F\ CA = DHO$$

$$F\frac{DHO}{CA} + F = \frac{DHO}{CA}$$

Substituting R = DHO/CA:

$$F\ R + F = R$$

$$F = \frac{R}{R+1} \qquad (6)$$

Rearranging equation 5:

$$R = \frac{c\ 10^{-pH} K_{eq}}{d} \qquad (7)$$

For 30% formation of the final equilibrium concentration of product (L-DHO) substituting equation 7 into equation 6:

$$0.3\ F = \frac{0.3\ c\ 10^{-pH} K_{eq}/d}{\frac{c\ 10^{-pH} K_{eq}}{d} + 1} \qquad (8)$$

Because of the low pK_a values for L-CA (2.83 and 4.32) and L-DHO (3.10) (Christopherson and Jones, 1979), above pH 6.0 c and d are approximately equal to unity and equation 8 simplifies to:

$$0.3\ F = \frac{0.3\ 10^{-pH} K_{eq}}{10^{-pH} K_{eq} + 1} \qquad (9)$$

For a particular assay of biosynthetic dihydroorotase activity above pH 6.0 containing initially X pmol of L-[^{14}C]CA, the maximal pmol of L-[^{14}C]DHO (Y) which can be formed at a constant initial reaction velocity is:

$$Y = 0.3\ F\ X = \frac{0.3\ 10^{-pH} K_{eq}\ X}{10^{-pH} K_{eq} + 1} \qquad (10)$$

For assay of degradative dihydroorotase activity, the fractional conversion of L-DHO to L-CA will be:

$$G = \frac{CA}{DHO + CA}$$

$$G\ DHO + G\ CA = CA$$

$$G\frac{DHO}{CA} + G = 1$$

Substituting R = DHO/CA

$$G\ R + G = 1$$

$$G = \frac{1}{R+1} \qquad (11)$$

For 30% formation of the final equilibrium concentration of product (L-CA), substituting the relationship for R from equation 7:

$$0.3\ G = \frac{0.3}{\frac{c\ 10^{-pH} K_{eq}}{d} + 1} \qquad (12)$$

Above pH 6.0 equation 12 simplifies to:

$$0.3\ G = \frac{0.3}{10^{-pH} K_{eq} + 1} \qquad (13)$$

For a particular assay of degradative dihydroorotase activity above pH 6.0 containing initially Y pmol of L-[^{14}C]DHO, the maximal pmol of L-[^{14}C]CA (X) which can be formed at a constant initial reaction velocity is:

$$X = 0.3\ G\ Y = \frac{0.3\ Y}{10^{-pH} K_{eq} + 1} \qquad (14)$$

Knowing the pmol of L-[^{14}C]DHO or L-[^{14}C]CA to be synthesized at a particular pH from using equations 10 and 14, respectively, the amount of dihydroorotase to be added to the assay and the sampling times could be calculated from the specific activity of the enzyme and the pH-activity profiles for the biosynthetic and degradative reactions (Fig. 4 of Christopherson and Jones, 1979).

Appendix 2. Derivation of Rate Equations for Catalysis by Dihydroorotase Existing in Multiple States of Protonation.

The complementary pH-dependencies of the apparent K_m values for L-CA and L-DHO (Figs. 1 and 2) and the V_{max} values for the biosynthetic and degradative reactions (Fig. 4 of Christopherson and Jones, 1979) suggest that the biosynthetic reaction is catalyzed by an acidic form of dihydroorotase and the degradative reaction is catalyzed by a basic form of the enzyme. In the following sections, rate equations will be derived for the biosynthetic and degradative reactions of the form:

$$\frac{v}{V_{max}} = \frac{[S]}{K_m^S\ (\text{slope factor}) + [S]\ (\text{intercept factor})} \quad \text{(Segel, 1975)} \qquad (15)$$

where S is L-CA or L-DHO and v is the reaction velocity at that concentration of S. A Lineweaver-Burk plot of the kinetic data obtained at a particular pH will have an intercept on the 1/v ordinate of $1/(V_{max})_H$ and an intercept on the negative 1/S abscissa of $1/(K_m^S)_H$. The relationships between these apparent constants, $(V_{max})_H$ and $(K_m^S)_H$, and the true constants for the catalytically active forms of dihydroorotase are:

$$(V_{max})_H = \frac{V_{max}}{(\text{intercept factor})} \qquad (16)$$

$$(K_m^S)_H = \frac{K_m^S\ (\text{slope factor})}{(\text{intercept factor})} \qquad (17)$$

From rate equations of the form of equation 15, functional relationships are determined for the dependence of $(V_{max})_H$ and $(K_m^S)_H$ upon H^+ concentration using equations 16 and 17, respectively. Theoretical curves have been generated from these functional relationships using various acidity constants for dissociation of protons from dihydroorotase ($pK_{a1} \leq pK_{a2} \leq pK_{a3}$) and the lines of best fit for each model are presented in Figs. 1 and 2 of this paper and Fig. 4 of Christopherson and Jones (1979). The derivation of equations 2 and 4 which fit the experimental data of Fig. 1 for $(K_m^{CA})_H$ and Fig. 2 for $(K_m^{DHO})_H$, will be given in detail. It is assumed for these models that the true K_m for L-CA or L-DHO is equivalent to the dissociation constant (K_S) of the catalytically active enzyme-substrate complex and k_f and k_r and the rate constants for catalysis of the biosynthetic and degradative reactions, respectively.

In all models, the active enzyme-substrate complex for the biosynthetic reaction loses a proton with an acid dissociation constant K_{a4} to become inactive giving the pH-dependence for the maximal velocity (using equation 16).

$$(V_{max})_H = \frac{V_{max}\ [H^+]}{[H^+] + K_{a4}}$$

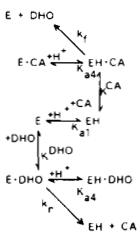
(equation 7 of Christopherson and Jones, 1979).

The active enzyme-substrate complex(es) for the degradative reaction gain a proton with the same acidity constant, K_{a4}, to become inactive giving the pH-dependence for the maximal velocity (using equation 16).

$$(V_{max})_H = \frac{V_{max} K_{a4}}{[H^+] + K_{a4}}$$

(equation 8 of Christopherson and Jones, 1979).

2a. One Proton Model



For the biosynthetic reaction:

$$\frac{v}{(V_{max})_H} = \frac{[CA]}{K_m^{CA}\frac{([H^+] + K_{a1})}{[H^+]} + [CA]\frac{([H^+] + K_{a4})}{[H^+]}}$$

From equation 17:

$$(K_m^{CA})_H = K_m^{CA}\frac{([H^+] + K_{a1})}{([H^+] + K_{a4})}$$

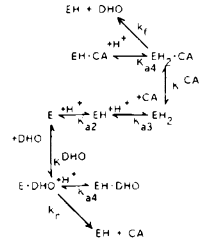
For the degradative reaction:

$$\frac{v}{(V_{max})_H} = \frac{[DHO]}{K_m^{DHO}\frac{([H^+] + K_{a1})}{K_{a1}} + [DHO]\frac{([H^+] + K_{a4})}{K_{a4}}}$$

From equation 17:

$$(K_m^{DHO})_H = K_m^{DHO}\frac{K_{a4}([H^+] + K_{a1})}{K_{a1}([H^+] + K_{a4})}$$

2b. Two Proton Model



For the biosynthetic reaction:

$$\frac{v}{(V_{max})_H} = \frac{[CA]}{K_m^{CA}\,\dfrac{([H^+]^2 + K_{a3}[H^+] + K_{a2}K_{a3})}{[H^+]^2} + [CA]\,\dfrac{([H^+] + K_{a4})}{[H^+]}}$$

From equation 17:

$$(K_m^{CA})_H = K_m^{CA}\,\frac{([H^+]^2 + K_{a3}[H^+] + K_{a2}K_{a3})}{[H^+]\,([H^+] + K_{a4})}$$

For the degradative reaction:

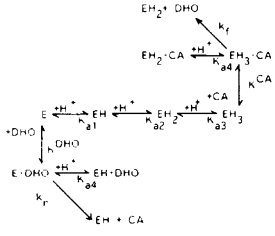
$$\frac{v}{(V_{max})_H} = \frac{[DHO]}{K_m^{DHO}\,\dfrac{([H^+]^2 + K_{a3}[H^+] + K_{a2}K_{a3})}{K_{a2}K_{a3}} + [DHO]\,\dfrac{([H^+] + K_{a4})}{K_{a4}}}$$

From equation 17:

$$(K_m^{DHO})_H = K_m^{DHO}\,\frac{K_{a4}\,([H^+]^2 + K_{a3}[H^+] + K_{a2}K_{a3})}{K_{a2}K_{a3}\,([H^+] + K_{a4})} \qquad (3)$$

This is equation 3 from "Results".

2c. Three Proton Model



For the biosynthetic reaction:

$$v = k_f\,[EH_3{\cdot}CA] \qquad (18)$$

$$[E]_t = [E] + [EH] + [EH_2] + [EH_3] + [EH_3{\cdot}CA] + [EH_2{\cdot}CA] \qquad (19)$$

$$(V_{max})_H = k_f\,[E]_t \qquad (20)$$

where v is the rate of synthesis of L-DHO and $[E]_t$ is the total concentration of all species of dihydroorotase. Dividing equation 18 by equation 19 and substituting expressions containing only $[E]$ for each of the terms of $[E]_t$:

$$\frac{v}{[E]_t} = \frac{k_f[E][H^+]^3[CA]/K_{a1}K_{a2}K_{a3}K_m^{CA}}{[E] + \dfrac{[E][H^+]}{K_{a1}} + \dfrac{[E][H^+]^2}{K_{a1}K_{a2}} + \dfrac{[E][H^+]^3}{K_{a1}K_{a2}K_{a3}} + \dfrac{[E][H^+]^3[CA]}{K_{a1}K_{a2}K_{a3}K_m^{CA}} + \dfrac{K_{a4}[E][H^+]^2[CA]}{K_{a1}K_{a2}K_{a3}K_m^{CA}}}$$

Rearranging and substituting equation 20:

$$\frac{v}{(V_{max})_H} = \frac{[CA]}{\dfrac{K_{a1}K_{a2}K_{a3}K_m^{CA}}{[H^+]^3} + \dfrac{K_{a2}K_{a3}K_m^{CA}}{[H^+]^2} + \dfrac{K_{a3}K_m^{CA}}{[H^+]} + K_m^{CA} + [CA] + \dfrac{K_{a4}[CA]}{[H^+]}}$$

$$= \frac{[CA]}{K_m^{CA}\,\dfrac{([H^+]^3 + K_{a3}[H^+]^2 + K_{a2}K_{a3}[H^+] + K_{a1}K_{a2}K_{a3})}{[H^+]^3} + [CA]\,\dfrac{([H^+] + K_{a4})}{[H^+]}}$$

From equation 17:

$$(K_m^{CA})_H = K_m^{CA}\,\frac{([H^+]^3 + K_{a3}[H^+]^2 + K_{a2}K_{a3}[H^+] + K_{a1}K_{a2}K_{a3})}{[H^+]^2\,([H^+] + K_{a4})} \qquad (21)$$

This is equation 2 from "Results".

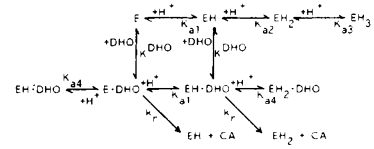
For the degradative reaction:

$$\frac{v}{(V_{max})_H} = \frac{[DHO]}{K_m^{DHO}\,\dfrac{([H^+]^3 + K_{a3}[H^+]^2 + K_{a2}K_{a3}[H^+] + K_{a1}K_{a2}K_{a3})}{K_{a1}K_{a2}K_{a3}} + [DHO]\,\dfrac{([H^+] + K_{a4})}{K_{a4}}}$$

From equation 17:

$$(K_m^{DHO})_H = K_m^{DHO}\,\frac{K_{a4}\,([H^+]^3 + K_{a3}[H^+]^2 + K_{a2}K_{a3}[H^+] + K_{a1}K_{a2}K_{a3})}{K_{a1}K_{a2}K_{a3}\,([H^+] + K_{a4})}$$

2d. Three Proton Model Where E and EH are Catalytically Active for the Degradative Reaction.



$$v = k_r\,([E{\cdot}DHO] + [EH{\cdot}DHO]) \qquad (21)$$

$$[E]_t = [E] + [EH] + [EH_2] + [EH_3] + [EH{\cdot}DHO] + [E{\cdot}DHO] + [EH{\cdot}DHO] + [EH_2{\cdot}DHO] \qquad (22)$$

$$(V_{max})_H = k_r[E]_t \qquad (23)$$

where v is the rate of synthesis of L-CA and $[E]_t$ is the total concentration of all species of dihydroorotase. Dividing equation 21 by equation 22 and substituting expressions containing only $[E]$ for each of the terms of $[E]_t$:

$$\frac{v}{[E]_t} = \frac{k_r\,[([E][DHO]/K_m^{DHO}) + ([E][H^+][DHO]/K_{a1}K_m^{DHO})]}{[E] + \dfrac{[E][H^+]}{K_{a1}} + \dfrac{[E][H^+]^2}{K_{a1}K_{a2}} + \dfrac{[E][H^+]^3}{K_{a1}K_{a2}K_{a3}} + \dfrac{[E][DHO]}{K_m^{DHO}} + \dfrac{[E][H^+][DHO]}{K_{a1}K_m^{DHO}} + \dfrac{[E][H^+]^2[DHO]}{K_{a1}K_m^{DHO}K_{a4}} + \dfrac{[E][H^+]^3[DHO]}{K_m^{DHO}K_{a1}K_{a2}K_{a3}}}$$

Rearranging and substituting equation 23:

$$\frac{v}{(V_{max})_H} = \frac{[DHO]}{\dfrac{1}{([H^+]+K_{a1})}\left(K_m^{DHO}K_{a1} + K_m^{DHO}[H^+] + K_m^{DHO}\dfrac{[H^+]^2}{K_{a2}} + K_m^{DHO}\dfrac{[H^+]^3}{K_{a2}K_{a3}}\right) + K_{a1}[DHO] + [H^+][DHO] + \dfrac{[H^+]^2[DHO]}{K_{a4}} + K_{a1}\dfrac{[H^+][DHO]}{K_{a4}}}$$

$$= \frac{[DHO]}{K_m^{DHO}\,\dfrac{([H^+]^3 + K_{a3}[H^+]^2 + K_{a2}K_{a3}[H^+] + K_{a1}K_{a2}K_{a3})}{K_{a2}K_{a3}\,([H^+] + K_{a1})} + [DHO]\,\dfrac{([H^+]^2 + K_{a1}[H^+] + K_{a4}[H^+] + K_{a1}K_{a4})}{K_{a4}\,([H^+] + K_{a1})}}$$

The coefficient of $[DHO]$ in the denominator (intercept factor, equation 15) may be factorized and simplified:

$$\frac{v}{(V_{max})_H} = \frac{[DHO]}{K_m^{DHO}\,\dfrac{([H^+]^3 + K_{a3}[H^+]^2 + K_{a2}K_{a3}[H^+] + K_{a1}K_{a2}K_{a3})}{K_{a2}K_{a3}\,([H^+] + K_{a1})} + [DHO]\,\dfrac{([H^+] + K_{a1})}{K_{a4}}}$$

From equation 17:

$$(K_m^{DHO})_H = K_m^{DHO}\,\frac{K_{a4}\,([H^+]^3 + K_{a3}[H^+]^2 + K_{a2}K_{a3}[H^+] + K_{a1}K_{a2}K_{a3})}{K_{a2}K_{a3}\,([H^+] + K_{a1})\,([H^+] + K_{a4})} \qquad (4)$$

This is equation 4 from "Results".