Mechanistic Studies on the Bovine Liver Mitochondrial Dihydroorotate Dehydrogenase Using Kinetic Deuterium Isotope Effects†

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ABSTRACT: Dihydroorotates deuteriated at both C_5 and C_6 have been prepared and used to probe the mechanism of the bovine liver mitochondrial dihydroorotate dehydrogenase. Primary deuterium isotope effects on k_{cat} are observed with both (6*RS*)-[5(*S*)-^{2}H]- and (6*RS*)-[6-^{2}H]dihydroorotates (**3** and **6**, respectively); these effects are maximal at low pH. At pH 6.6, $^DV = 3.4$ for the C_5-deuteriated dihydroorotate (**3**), and $^DV = 2.3$ for the C_6-deuteriated compound (**6**). The isotope effects approach unity at pH 8.8. Analysis of the pH dependence of the isotope effects on k_{cat} reveals a shift in the rate-determining step of the enzyme mechanism as a function of pH. Dihydroorotate oxidation appears to require general base catalysis ($pK_B = 8.26$); this step is completely rate-determining at low pH and isotopically sensitive. Reduction of the cosubstrate, coenzyme Q_6, is rate-limiting at high pH and is isotopically insensitive; this step appears to require general acid catalysis ($pK_A = 8.42$). The results of double isotope substitution studies and analysis for substrate isotope exchange with solvent point toward a concerted mechanism for oxidation of dihydroorotate. This finding serves to distinguish further the mammalian dehydrogenase from its parasitic cognate, which catalyzes a stepwise oxidation reaction [Pascal, R., & Walsh, C. T. (1984) *Biochemistry 23*, 2745].

Over the past decade or so there has been considerable interest expressed in the chemical mechanisms of the dihydroorotate dehydrogenases (DHODases),[1] a diverse group of enzymes that catalyze the two-electron oxidation of dihydroorotate (DHO) to orotate (OA). It was orginally proposed that this dehydrogenation reaction in protozoans might be accomplished by a novel pterin-dependent hydroxylase (Gutteridge et al., 1979). This was a particularly intriguing suggestion in light of the fact that the DHODases from other eukaryotic organisms, including mammals, were thought to be simple metalloenzymes (Forman & Kennedy, 1977, 1978) or metalloflavoproteins (Miller & Curry, 1969; Miller, 1975). Consequently, some workers began to speculate that these implied mechanistic differences might be exploited for the development of a species-selective DHODase inactivator that would target pyrimidine biosynthesis in parasites while leaving the cognate process in mammals unaffected. Enthusiasm for this approach became understandably muted, however, when Pascal and colleagues (1983) demonstrated that the purified DHODases from *Crithidia fasciculata* and *Trypanosoma brucei* are not, in fact, pterin-linked enzymes but simple flavoprotein oxidases.

It remains, nonetheless, that there are significant differences between the parasite and mammalian DHODases with regard to molecular properties, kinetic behavior, and perhaps also chemical mechanism. Both the crithidial and trypanosomal enzymes are cytosolic flavoproteins that appear to deliver electrons directly to molecular oxygen (Pascal et al., 1983). The bovine enzyme, by contrast, is a mitochondrial dihydroorotate:ubiquinone oxidoreductase (Hines et al., 1986). This protein appears to be linked both structurally and functionally to membrane-associated electron-transport systems (Miller et al., 1968), as are a number of both prokaryotic and eukaryotic DHODases (Andrews et al., 1977; Miller & Adams, 1971; Chen & Jones, 1976; Gero & Sullivan, 1985; Miersch et al., 1986). We have recently shown that the bovine liver DHODase is a two-cofactor protein, containing both FMN and non-heme iron (Hines & Johnston, 1989a). These two redox-active components may, in fact, serve to link kinetically and structurally isolated sites on the protein for binding of DHO and coenzyme Q_6. In fact, the kinetic behavior of DHODase described in the accompanying paper (Hines & Johnston, 1989b) argues for a multisite ping-pong mechanism. And while the detailed formal kinetic mechanism of the protozoan DHODase is yet to be elucidated, the kinetic data reported (Pascal et al., 1983) appear to rule out a multisite ping-pong reaction for at least the crithidial enzyme.

There is good evidence from work on both the bovine (Keys & Johnston, 1985) and crithidial (Pascal & Walsh, 1984) DHODases to suggest that substrate oxidation involves proton abstraction from the C_5-*pro-S* position of DHO together with delivery of two electrons and a proton (perhaps as hydride) from C_6 of the substrate to flavin. Pascal and Walsh (1984) have argued—in part on the basis of kinetic isotope effects and in part on solvent-exchange reactions—that dehydrogenation may proceed by a stepwise process with intermediate formation of a kinetically significant C_5 carbanion. In this paper we report on studies designed also to identify the rate-determining steps in the reaction catalyzed by the purified bovine DHODase. Our results demonstrate a change in the rate-determining step as a function of pH and point toward a concerted,

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[1] Abbreviations: DHO, dihydroorotate; DHODase, dihydroorotate dehydrogenase; OA, orotate; KP_i, potassium phosphate; KPP_i, potassium pyrophosphate; Q_6, coenzyme Q_6 (ubiquinone).

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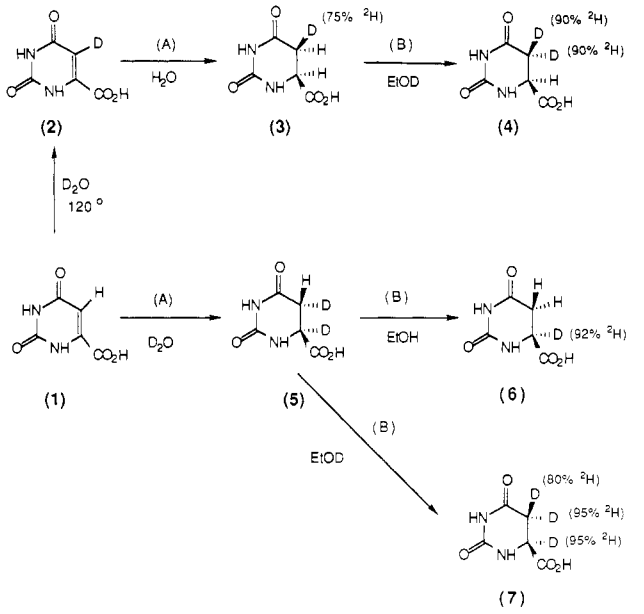


FIGURE 1: Synthetic routes for preparation of deuteriated dihydroorotates. The numbers in parentheses represent the percentage of deuterium incorporated into the designated position. (A) Rhodium-catalyzed reduction of orotic acid in the designated solvent. (B) Ethoxide-catalyzed exchange in the designated solvent. Details are given in the text.

rather than a stepwise, oxidation mechanism.

Experimental Procedures

Materials. (6*RS*)-Dihydroorotic acid was purchased from Sigma. Deuterium oxide (99.8 atom % ^2H), ethanol-*d* (99.5 atom % ^2H), and rhodium on alumina were purchased from Aldrich. All other reagents were of the best grade commercially available. Purified bovine dihydroorotate dehydrogenase was obtained from beef liver mitochondria as described previously (Hines et al., 1986; Hines & Johnston, 1989b).

Preparation of Deuteriated Compounds. Figure 1 summarizes the synthetic routes used to obtain the variously deuteriated dihydroorotates. These methods were designed to achieve ^2H incorporation approaching 100% into the positions indicated in the figure. [5-^2H]Orotic acid (**2**) was prepared according to the methods of Keys and Johnston (1985). Catalytic hydrogenation of orotic acid was accomplished following the procedure described by Pascal and Walsh (1984).

NMR Analysis of Deuterium Incorporation. Analysis of the extent of deuterium incorporation into the deuteriated DHOs was accomplished by using ^1H NMR. For orotic acid, the vinyl proton of thymine (δ 7.20 ppm) was integrated and compared to the signal intensity of the vinyl proton of deuteriated orotic acid (δ 5.99 ppm). For dihydroorotates, alanine was used as the internal standard, and the methine proton (δ 3.79 ppm) was used as an internal integration standard to determine the deuterium content at C_6 (δ 4.25 ppm), C_5-*pro-R* (δ 3.05 ppm), and C_6-*pro-S* (δ 2.85 ppm) of DHO. All NMR spectra were obtained by using a DS-1000 500-MHz instrument, equipped with a Nicolet 1180 computer. Chemical shift values (δ) are given in parts per million downfield from (trimethylsilyl)propionate, sodium salt, in D_2O.

[5-^2H]*Orotic Acid* (**2**). Orotic acid (**1**; 1.0 g, 6.41 mmol) was suspended in 150 mL of D_2O. The mixture, sealed in a glass bottle equipped with a magnetic stir bar, was heated for 12 days at 120 °C in an oil bath. After the solution had cooled to room temperature, the suspension was frozen and lyophilized. The residue was recrystallized twice from boiling water. The crystals were collected and dried in vacuo over P_2O_5: yield 0.64 g (64%); ^1H NMR (DMSO-d_6) δ 11.22 (s, 1 H, NH), 10.75 (s, 1 H, NH), 5.99 (s, 0.03 H, C_5 vinyl). NMR analysis revealed 97% ^2H incorporation at C_5.

(5*RS*,6*RS*)-[5-^2H]*Dihydroorotic Acid* (**3**). Powdered 5% rhodium on alumina (150 mg) was dissolved in 15 mL of chilled water and was placed into a hydrogenation flask. A solution of 150 mg of compound **2** (0.96 mmol) in 60 mL of water was slowly added. The system was then sealed and flushed with hydrogen gas. After 1 h of vigorous stirring under 1 atm of hydrogen, the atmosphere was changed by flushing with hydrogen gas, and stirring was continued for another 2 h. The suspension was filtered to remove the catalyst, and the resulting solution was frozen and lyophilized and then crystallized from boiling water. The product was dried in vacuo over P_2O_5: yield 100 mg (66%); ^1H NMR (D_2O) δ 4.25 (d, 1 H, C_6 H), 3.05 (d, 1 H, C_{5R} H), 2.85 (d, 0.25 H, C_{5S} H). NMR analysis revealed 75% ^2H incorporation at the C_{5S} position of the 6*S* isomer of DHO.

(6*RS*)-[5,5-$^2H_2$]*Dihydroorotic Acid* (**4**). Compound **3** (300 mg, 1.9 mmol) was dissolved in 125 mL of ethanol-*d*. Sodium metal (260 mg, 11 mmol) was added with vigorous stirring. When all the metal had dissolved, the reaction was refluxed for 50 h. Upon cooling, concentrated formic acid (11 mmol) was added to neutralize the solution, and the ethanol was removed. The solution was applied to a Dowex 50 column equilibrated and eluted in water. Fractions containing DHO were pooled, frozen, and lyophilized. The residue was recrystallized from boiling water, and the crystals were dried in vacuo over P_2O_5: yield 200 mg (67%); ^1H NMR (D_2O) δ 4.25 (s, 1 H, C_6 H), 3.05 (dd, 0.08 H, C_5 H), 2.85 (dd, 0.10 H, C_5 H). NMR analysis revealed 90% ^2H incorporation at both the C_{5S}- and C_{5R}-positions.

(5*RS*,6*RS*)-[5,6-$^2H_2$]*Dihydroorotic Acid* (**5**). Orotic acid (150 mg) was hydrogenated as in the synthesis of compound **3**, except that D_2O was used as solvent instead of H_2O. Isolation and recrystallization, as described for compound **3**, yielded 90 mg (59%) of **5**: ^1H NMR (D_2O) δ 2.85 (s, 0.8 H, C_5 H). NMR analysis revealed that the C_5-*pro-R* hydrogen and the C_6 hydrogen were greater than 95% ^2H but that the C_5-*pro-S* hydrogen was also 20% ^2H-enriched.

(6*RS*)-[6-^2H]*Dihydroorotic Acid* (**6**). Orotic acid was hydrogenated over 5% rhodium on alumina in D_2O as described for compound **5**. The isolated DHO (300 mg, 1.9 mmol) was thoroughly dried and dissolved in dry ethanol. The compound was refluxed in ethoxide, as in the synthesis of compound **4**, except that ethanol was used as the solvent: yield 230 mg (77%); ^1H NMR (D_2O) δ 4.25 (m, 0.08 H, C_6 H), 3.05 (d, 1 H, C_5 H), 2.85 (d, 1 H, C_5 H). NMR analysis revealed that the C_6-position is 92% ^2H and that both C_5 hydrogens are 100% ^1H.

(6*RS*)-[5,5,6-$^2H_3$]*Dihydroorotic Acid* (**7**). Compound **5** (300 mg, 1.9 mmol) was refluxed in ethanol-*d* as described for compound **4**. The compound was isolated by using the above procedures: yield 220 mg (73%). The NMR spectrum showed a single peak at 2.85 ppm, which, when compared to the internal standard, integrated to 0.2 H. This analysis revealed that the C_5-*pro-R* and C_6-positions are greater than 95% ^2H. The C_5-*pro-S*-position is 80% ^2H.

Enzyme Assays. Two buffer systems were used to monitor DHODase activity over the pH range from 6.6 to 8.8. From pH 6.6 to 8.0, assays were carried out in 100 mM KP_i buffer and 0.1% reduced Triton X-100. All buffers were brought to a constant ionic strength of μ = 0.3 by addition of KCl. Above pH 8, assays were carried out in 50 mM $NaPP_i$ buffer and

0.1% reduced Triton X-100, and the ionic strength of all buffers was adjusted to $\mu = 0.5$. Initial velocities were measured by monitoring orotic acid formation at 293 nm (Hines et al., 1986). Coenzyme Q_6 was 50 μM in all assays. Assays were carried out at 25 °C.

Determination of Substrate Concentration. The exact concentration of the 6*S* isomer of DHO in stock solutions used for steady-state kinetic assays was determined by an enzymatic method described by Keys and Johnston (1985). A 10.0-mL stock solution was prepared containing 100 μM DCIP, 70 μM Q_6, and 1000 units of DHODase in 0.1 M KP_i buffer, pH 7.8, with 0.1% reduced Triton X-100. Known concentrations of (6*S*)-DHO (1–10 nmol) were added to 1.0 mL of the enzyme mixture, and the net absorbance decrease was measured at 600 nm. In this way, a standard curve of ΔA_{600} versus [DHO] could be constructed for use in the determination of the concentration of (6*S*)-DHO in stock solutions containing both the 6*S* and 6*R* enantiomers of deuteriated DHO.

Kinetic Analysis. Individual kinetic parameters for all the substrates examined were determined by fitting the initial velocity data to eq 1. Deuterium isotope effects on initial

$$v = \frac{VA}{K_M + A} \tag{1}$$

velocities were obtained by varying the levels of deuteriated and nondeuteriated substrates. The initial velocities obtained were fitted to eq 2 (Schimerlik et al., 1977), where F is the

$$v = \frac{VA}{K_M(1 + FE_{V/K}) + A(1 + FE_V)} \tag{2}$$

fraction of deuteriated substrate and $E_{V/K}$ and E_V are the isotope effects minus 1 for the respective parameters. A nonlinear least-squares computer program was used for fitting the data to eq 2 (Yamaoka et al., 1981). Data for pH profiles showing a drop in k_{cat}/K_M as the pH was lowered were fitted to eq 3. When the values for k_{cat} decreased at both high and

$$k_{cat}/K_M^{app} = \frac{k_{cat}/K_M}{1 + [H^+]/K_B} \tag{3}$$

low pH, the data were fitted to eq 4. In eq 3 and 4, K_A and

$$k_{cat}^{app} = \frac{k_{cat}}{1 + [H^+]/K_B + K_A/[H^+]} \tag{4}$$

K_B represent the dissociation constants for groups on the enzyme.

NMR Analysis of Enzyme-Catalyzed Deuterium Incorporation into DHO. Experiments were carried out to determine whether solvent-deuterium exchange with DHO was catalyzed by DHODase. All exchange reactions were carried out in 100 mM KP_i deuterium oxide buffer (pD 7.8) containing 0.1% reduced Triton X-100. Five hundred units of DHODase, which had been dialyzed against deuteriated buffer, was added to a 5-mm NMR tube to initiate the reaction. Spectra (500 MHz) were obtained at timed intervals; the C_5 and C_6 proton resonances of DHO and the C_5 vinyl resonance of orotate were integrated against (trimethylsilyl)propionate (0.25 mM).

Since DHODase is characterized by a ping-pong mechanism (Hines & Johnston, 1989b), it is, in principle, possible to observe exchange reactions within the first half of the catalytic cycle in the absence of the second substrate (Q_6). Two such nonturnover experiments were conducted. In one experiment, 5 mM DHO was included in the buffer mixture, and the enzyme was incubated in this reaction mixture for 72 h. In a second experiment, 5 mM DHO and 1 mM OA were included in the mixture, and enzyme-catalyzed exchange was monitored for 72 h.

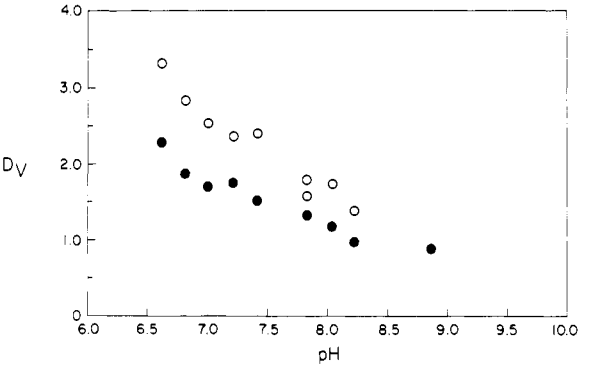


FIGURE 2: Kinetic isotope effects on V as a function of pH. Data are for **3** (○) and **6** (●) as substrates for DHODase.

Results

pH Profile of k_{cat}. Kinetic analysis of the DHODase reaction was carried out over the pH range from 6.6 to 8.8 using nondeuteriated DHO as substrate. The value for k_{cat} was observed to decrease at both low and high pH, with a maximum at pH 7.8–8.0. This bell-shaped profile of pH vs k_{cat} suggested the presence of two ionizable groups required for catalysis. Thus, the experimental data (not included) were fitted to eq 4, which generated two apparent molecular ionization constants: $pK_B = 7.31$ and $pK_A = 8.25$. Whether the two groups are involved in a single rate-limiting step or in two separate, partially rate-determining steps can be determined by studying the pH dependence of the deuterium kinetic isotope effects (vide infra).

Kinetic Isotope Effect on V. Preliminary studies using compounds **3** and **6** as substrates for DHODase indicated that only small isotope effects on V were observed at the pH maximum (pH 7.8) for the enzyme. This finding was not completely unexpected; the isotope effect on V is frequently small under maximal velocity conditions (Northrop, 1977). Since k_{cat} was found to decrease at both low and high pH, it seemed resonable to study the isotope effects with **3** and **6** over the pH range 6.6–8.8.

Figure 2 presents the kinetic isotope effects on V, as a function of pH, for the reactions of DHODase with both [5-^{2}H]- and [6-^{2}H]DHOs (**3** and **6**, respectively). The observed isotope effects with both compounds increased at low pH. At high pH, ^DV became unity despite the fact that V also decreases at high pH. Since ^DV increases only at low pH, the two ionizable groups observed in the k_{cat}/pH profile must be involved in separate partially rate-determining steps. The general base-catalyzed step is isotopically sensitive at low pH, and it follows, in turn, that the general acid group must promote a second step that is not sensitive to isotopic substitution on the substrate.

Scheme I describes a simple model for the DHODase reaction that attempts to account for the findings outlined above. In this model there are two rate-limiting steps that contribute to k_{cat}. At low pH, DHO oxidation must be rate-limiting, in accord with the large isotope effects observed below pH 7 (Figure 2). Therefore, k_2 must contribute to the expression for k_{cat}. At high pH, a second step, which is not isotopically sensitive, is rate-limiting. This step could be either product release, governed by k_3, or any step in quinone reduction, governed by k_4. The model of Scheme I also includes two molecular ionizations constants, K_A and K_B, which represent the ionization of two active-site residues that are involved in

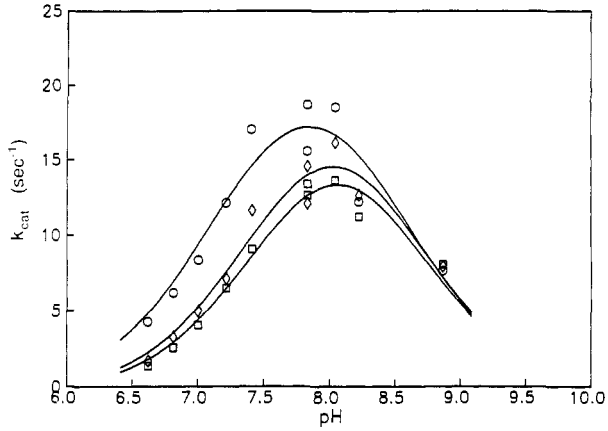
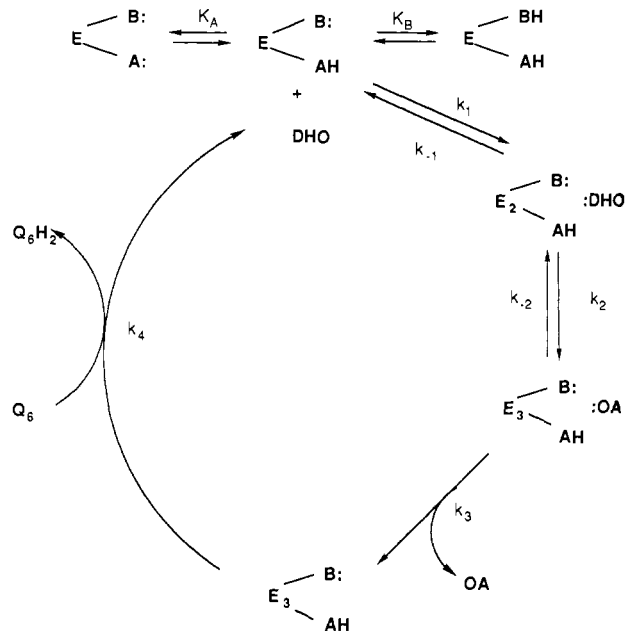


FIGURE 3: pH profile of k_{cat}. Data are for (6*RS*)-DHO (○), **3** (□), and **6** (◇) as substrates for bovine DHODase.

Scheme I



the two partially rate-limiting steps. K_B corresponds to the ionization of the group required for DHO oxidation. K_A represents the ionization of the residue that catalyzes the second rate-limiting step, which could be either product release (k_3) or quinone reduction (k_4). The rate equation for k_{cat} pertaining to the model reaction of Scheme I is given by eq 5.

$$k_{cat} = \frac{\dfrac{k_2}{1 + [H^+]/K_B}\dfrac{k_x}{1 + K_A/[H^+]}}{\dfrac{k_2}{1 + [H^+]/K_B} + \dfrac{k_x}{1 + K_A/[H^+]}} \quad (5)$$

The rate constant k_x in eq 5 represents either k_3 or k_4 or both rate constants, depending upon the assignment of the second rate-limiting step. The k_{cat} values determined for (6*RS*)-DHO, **3**, and **6** could all be fitted to this equation. The ionization constants K_A and K_B are assumed not to be affected by deuteriated substrates. Additionally, since the rate constant corresponding to the general acid-catalyzed step, k_x, is not affected by substrate isotopic substitution—as evidenced by the lack of any isotope effects at high pH (Figure 2)—this parameter is identical for all three substrates. Only k_2, the rate constant for the isotopically sensitive step, changes with different substrates.

Table I: Calculated Kinetic Parameters and Ionization Constants for the Two Rate-Limiting Steps in DHO Oxidation[a]

substrate	rate constant[b] k_2 (s^{-1})	k_x (s^{-1})
(6*RS*)-DHO	301.9 ± 49.5	27.7 ± 2.0
(6*RS*)-[5-^{2}H]DHO (**3**)	117.0 ± 11.1	27.9 ± 1.9
(6*RS*)-[6-^{2}H]DHO (**6**)	141.4 ± 18.0	29.0 ± 2.5
	pK_B = 8.26	pK_A = 8.42

[a] Parameters were obtained by fitting the data of Figure 3 to eq 5. See text for details. [b] Rate constants are not corrected for 100% deuterium incorporation.

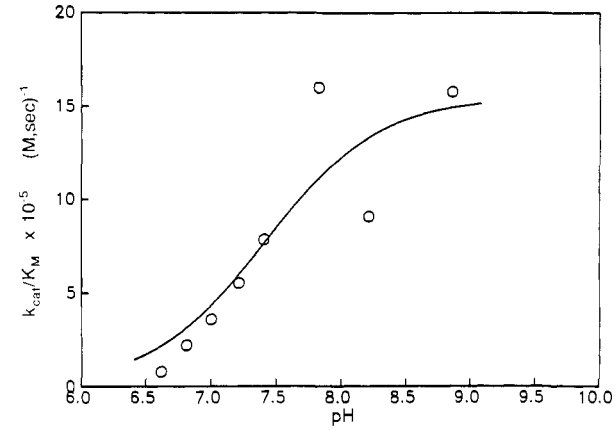


FIGURE 4: pH profile of k_{cat}/K_M. Data are for (6*RS*)-DHO as substrate for bovine DHODase.

Figure 3 presents the k_{cat} data for DHO and compounds **3** and **6** obtained over the pH range 6.6–8.8 together with the best-fit curves for all three compounds (as described by eq 5). The best-fit parameters determined for eq 5 with this model are listed in Table I. The two enzyme ionization constants that emerge from this analysis fall between pH 8.0 and 8.5; it is not clear, however, that these values represent the true molecular ionization constants inasmuch as both of these pK_as are quite similar and because $pK_B < pK_A$ (Tipton & Dixon, 1979).

As can be seen from Figure 3, the data sets fit the simple model of Scheme I quite well. Thus, the rate constants k_2 of Table I can be used to calculate the maximum isotope effect expected when DHO oxidation is completely rate-limiting. For compound **3**, the maximum ^DV = 3.40, and for compound **6**, the maximum ^DV = 2.32 (when normalized to 100% deuterium incorporation). The observed isotope effects at pH 6.6 are 3.3 and 2.3 for C_5- and C_6-deuteriated DHOs, respectively. This indicates that the step represented by k_2 in Scheme I is completely rate-limiting at pH 6.6. Identification of the second rate-limiting step, as defined by k_x, is possible through analysis of k_{cat}/K_M as a function of pH.

pH Profile of k_{cat}/K_M. Figure 4 gives the pH profile of k_{cat}/K_M for DHO; the experimental data were fitted to eq 3, which models k_{cat}/K_M as a function of a single ionization. Only one pK_a is apparent in the pH profile of Figure 4, which probably represents the general base-catalyzed step involved in dihydroorotate oxidation (pK_B = 7.41). As seen in the figure, the value for k_{cat}/K_M reaches a plateau at high pH. Since the expression for k_{cat}/K_M contains only those portions of the reaction mechanism up to and including the first irreversible step (which, in Scheme I, is product release), the pH dependence of subsequent steps will not be evident in the k_{cat}/K_M profile (Cleland, 1982). Insofar as pK_A is not observed in the pH profile of k_{cat}/K_M (Figure 4), this pH profile requires that the acid-catalyzed step, reflected in the pH profiles of k_{cat}

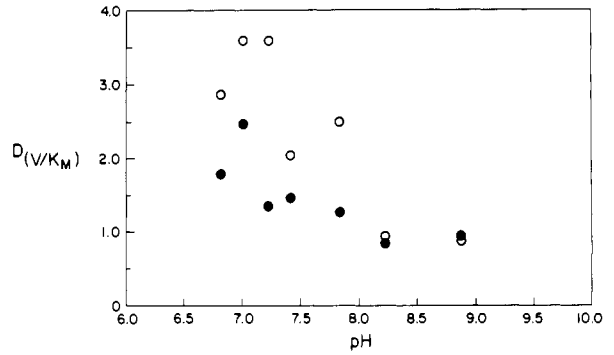


FIGURE 5: Kinetic isotope effects on V/K_M as a function of pH. Data are for **3** (○) and **6** (●) as substrates for bovine DHODase.

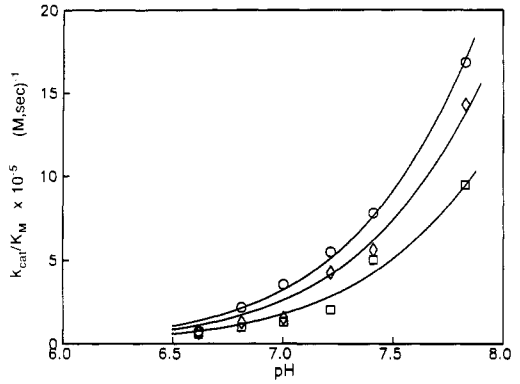


FIGURE 6: pH profile of k_{cat}/K_M. Data are for (6*RS*)-DHO (○), **3** (□), and **6** (◇) as substrates for bovine DHODase.

Table II: Calculated Values for k_{cat}/K_M and the Ionization Constant (pK_B) Determined for the Reactions of DHODase[a]

substrate	$10^{-5}k_{cat}/K_M$[b] (M^{-1} s^{-1})
(6*RS*)DHO	76.6 ± 1.5
(6*RS*)-[5-^{2}H]DHO (**3**)	42.6 ± 2.1
(6*RS*)-[6-^{2}H]DHO (**6**)	61.9 ± 2.5
	pK_B = 8.36

[a] Parameters were obtained by fitting the experimental data of Figure 6 to eq 3. See text for details. [b] Values are not corrected for 100% deuterium incorporation.

(Figure 3), must follow orotic acid release.

Isotope Effect on V/K. The isotope effects on V/K for compounds **3** and **6** are shown in Figure 5. Small isotope effects are seen with both compounds at high pH, and a large isotope effect is observed at low pH.

Since k_{cat}/K_M decreases at low pH for all three compounds (DHO, **3**, and **6**), the experimental data sets could be fitted to eq 3.[2] The best-fit curves for the data obtained with the three compounds are shown in Figure 6. The kinetic parameter k_{cat}/K_M for each substrate, calculated from eq 3, is listed in Table II.

Alternate Quinone Cosubstrates. We have reported previously that DHODase will reduce a variety of quinone cosubstrates (Hines et al., 1986). Consequently, we examined the kinetics of DHO oxidation, as a function of pH, using both Q_6 and menadione. Table III lists the kinetic parameters for these two quinone substrates at low pH (pH 6.6) and at the pH maximum for the enzyme (pH 7.8). The V_{max} for menadione at pH 7.8 is 50% that of Q_6 (Hines & Johnston, 1988b). At low pH, however, the V_{max} values for the two quinones are nearly identical (Table III).

Table III: Kinetic Parameters for Quinone Cosubstrates of DHODase

pH	substrate	K_M (μM)	V_{max} (μmol min^{-1} mg^{-1})
8.0	Q_6	14.2 ± 2.3	44.2 ± 2.2
	menadione	184.3 ± 28.1	18.3 ± 0.9
6.6	Q_6	2.8 ± 0.71	14.1 ± 0.6
	menadione	73.8 ± 11.3	12.6 ± 0.4

Table IV: Steady-State Kinetic Isotope Effects Observed with Dihydroorotate Dehydrogenase at pH 7.2

substrate	$^DV^a$	$^DV/K^a$
[5,5-$^2H_2$]DHO (**4**)	2.92 ± 0.16	2.95 ± 0.42
[6-^{2}H]DHO (**6**)	1.71 ± 0.06	1.31 ± 0.12
[5,5,6-$^3H_3$]DHO (**7**)	4.50 ± 0.3	3.90 ± 0.6
[5,5-^{2}H]DHO vs [5,6-$^2H_3$]DHO (**4** vs **7**)	1.32 ± 0.06	1.13 ± 0.14
[6-^{2}H]DHO vs [5,5,6-$^2H_3$]DHO (**6** vs **7**)	2.45 ± 0.11	2.80 ± 0.36

[a] All values are corrected for 100% deuterium incorporation.

Double Isotopic Substitution. It has recently become possible (Hermes et al., 1982; Belasco et al., 1983) to probe the concerted or stepwise nature of an enzyme-catalyzed reaction using substrates that have two isotopic substitutions. If a reaction contains two steps that are both isotopically sensitive, the observed isotope effect on one step will be dependent upon the corresponding isotopic substitution that affects the second step. This dependency can be observed in the isotope effects on V/K. If isotopic substitution at one position of the substrate decreases the observed isotope effect on V/K at a second position, then the two bond-breaking steps are distinct. If there is no change in the isotope effect or if the isotope effect increases (Hermes et al., 1984), then the two bond cleavages occur simultaneously.

In the reaction catalyzed by DHODase, there are two carbon–hydrogen bond cleavages, each of which we have shown to be isotopically sensitive. In order to determine whether the C_5–H and C_6–H bond scissions occur in a single step or in two sequential steps, we have applied the double-isotope methods to our study of the bovine enzyme. Table IV summarizes the results of these experiments. Since the isotope effects were small at pH 7.8, the studies were done at pH 7.2 to enhance the observed isotope effect. All isotope effects were corrected for 100% deuterium incorporation.

Rows 1–3 of Table IV include the isotope effects for each deuteriated compound as compared to all protio-DHO. Row 4 gives the isotope effect at C_6 when C_5 is deuteriated, which, using the nomenclature of Hermes et al. (1982), is $^{6D}V/K_{5D}$. Row 5 gives the isotope effect at C_5 when C_6 is deuteriated ($^{5D}V/K_{6D}$). Comparing the isotope effect at C_5, $^{5D}V/K_{6H}$ = 2.95 ± 0.42 and $^{5D}V/K_{6D}$ = 2.80 ± 0.36. Similarly, the isotope effects on V/K at C_6 were $^{6D}V/K_{5H}$ = 1.31 ± 0.12 and $^{6D}V/K_{5D}$ = 1.13 ± 0.14. In both cases, there is no significant decrease in the isotope effect upon isotopic substitution at the second position.

Analysis of Exchange with Solvent Deuterium. When an enzymatic reaction involves substrate proton abstraction by an active-site residue, it is often possible to observe deuterium wash-in into the position from which proton abstraction occurred. Figure 7 outlines two possible exchange reactions that might be catalyzed by DHODase. If DHODase promotes a stepwise oxidation initiated by proton abstraction at C_5, one might expect to see deuterium wash-in into the C_5-*pro-S*-

[2] When this modeling was done, it was necessary to exclude the data points at high pH due to buffer effects. When kinetic measurements for DHO were made in both phosphate and pyrophosphate buffers at the same pH, V_{max} was unaffected but K_M was buffer dependent. This resulted in buffer effects on k_{cat}/K_M as well, so only those values that were determined in phosphate buffer were used in the modeling.

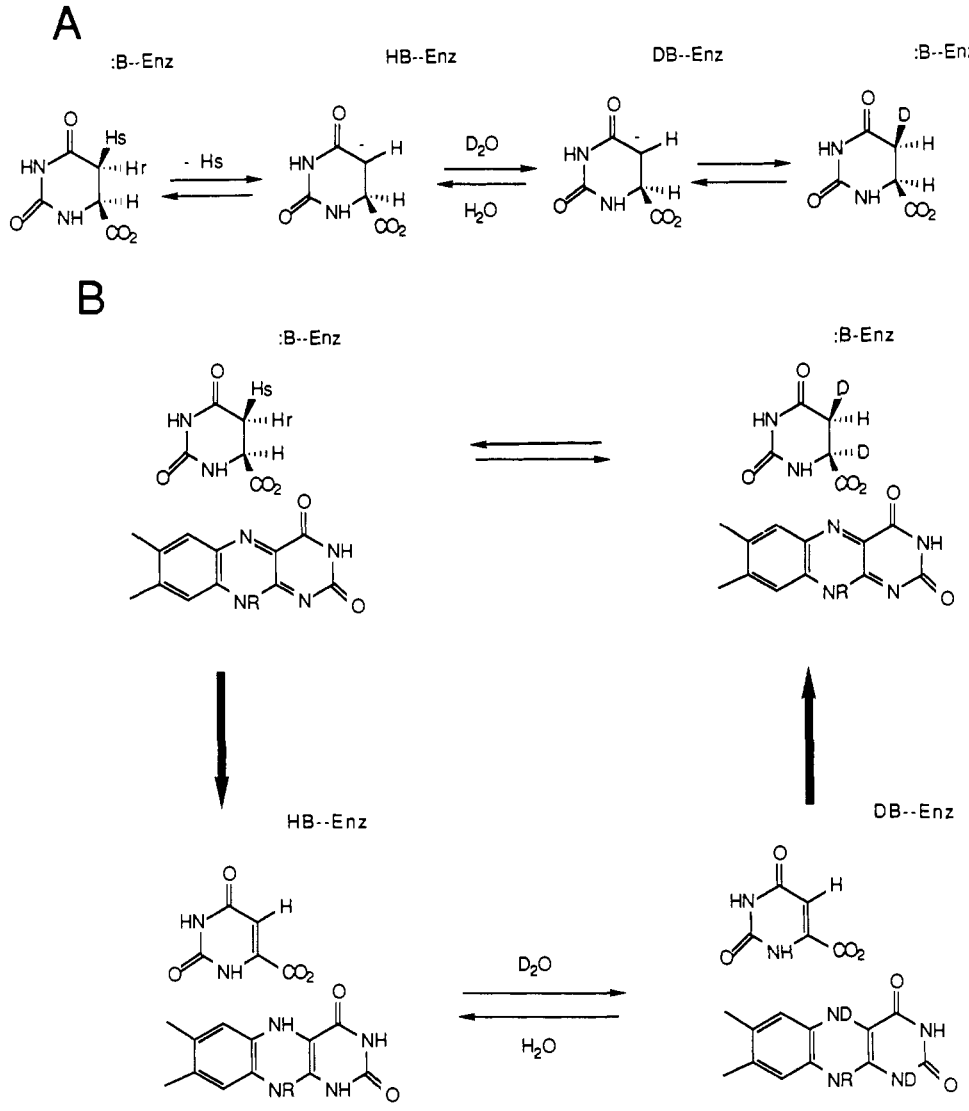


FIGURE 7: Hypothetical exchange reactions catalyzed by DHODase: (A) Exchange in the presence of DHO; (B) exchange with both DHO and OA.

positions when the enzyme is incubated with DHO (Figure 7A). If DHO and OA are present, exchange into both the C_5-*pro-S*- and the C_6-positions might be expected (Figure 7B). Since DHODase catalyzes a ping-pong reaction, deuterium incorporation into substrate DHO could occur in the absence of any quinone cosubstrate. When the enzyme was incubated with either DHO alone or with both DHO and OA for 72 h, no solvent deuterium wash-in could be detected under either condition.

Discussion

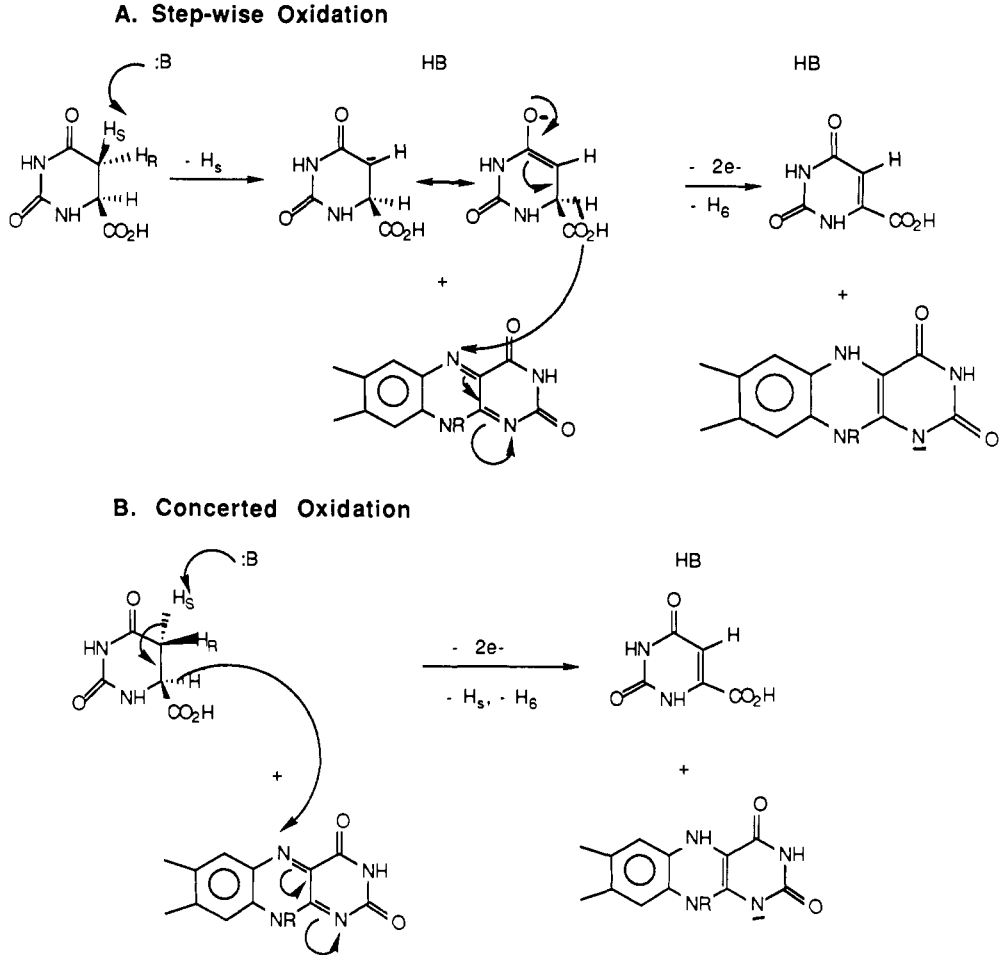
Rate-Limiting Steps in the Catalytic Mechanism. The pH profiles of the kinetic parameters and the deuterium isotope effects obtained in this study of the bovine mitochondrial DHODase-catalyzed reaction are consistent with a mechanism involving a change in rate-limiting steps as a function of pH. The bell-shaped pH profile of k_{cat} indicates the involvement of both a general acid-catalyzed and a general base-catalyzed step in the complete catalytic mechanism. At low pH, a general base-catalyzed step is rate-limiting and is sensitive to isotopic substitution at both the C_5- and C_6-positions of DHO (Figure 2). On the basis of the theory outlined by Cook and Cleland (1981), this is the expected result if the pH-dependent and isotopically sensitive steps are the same. Thus, at low pH, general base-catalyzed DHO oxidation is rate-limiting. At high pH, a separate acid-catalyzed step becomes rate-limiting and is not affected by isotopic substitution.

The pH profiles of k_{cat}/K_M (Figure 4 and 6) show a single enzyme ionization with $pK_a = 8.36$. This value corresponds closely to the pK_a determined for the k_{cat}/pH profile (Table I), a finding which indicates that substrate binding does not shift significantly the pK_a value of the group that promotes general base catalysis (Cleland, 1982; Tipton & Dixon, 1979). Since the isotope effects on k_{cat}/K_M are observed to increase at low pH, incorrect enzyme ionization would seem to prohibit catalysis but does not prevent DHO binding (Cook & Cleland, 1981; Northrop, 1982).

The second enzyme ionization observed in the k_{cat}/pH profile is not apparent in the pH profile of k_{cat}/K_M. This result demonstrates that the acid-catalyzed step must follow the first irreversible step in the catalytic mechanism. Since DHODase catalyzes a ping-pong reaction (Hines & Johnston, 1989b), the first irreversible step in the catalytic mechanism is orotate release (k_3). It follows, then, that a general acid is required for some step during the second half of the reaction involving quinone reduction. It is not, however, possible to determine from our studies whether this ionization is required simply for binding or is necessary for catalysis during quinone reduction.

Thus, all of the experimental data are consistent with a reaction mechanism where DHO oxidation is rate-limiting at

Scheme II: Mechanisms of Oxidation of Dihydroortate



low pH and Q_6 reduction is rate-limiting at high pH. The results with the alternate quinone cosubstrates are completely consistent with this model. At low pH, the V_{max} values obtained with two structurally distinct quinones were identical. At high pH, where quinone reduction becomes rate-limiting, the V_{max} values were different for Q_6 and menadione.

Chemical Mechanism of DHO Oxidation. Mechanistic studies on flavoprotein dehydrogenases suggest that these proteins oxidize their substrates by proton abstraction and hydride delivery to flavin (Tober et al., 1970; Ghisla et al., 1984; Bruice, 1980). Studies in our laboratory indicate that both C_5 protons of DHO are considerably more acidic than is the C_6 proton (Keys & Johnston, 1985). In addition, resonance stabilization of any carbanionic intermediate, resulting from proton abstraction on DHO, is only possible if the carbanion is located at C_5. It seems reasonable, therefore, to expect that enzymatic oxidation of DHO would be initiated by C_5 proton abstraction, as has also been suggested by Pascal and Walsh (1984).

It is not clear, however, whether substrate oxidation catalyzed by the bovine DHODase is a stepwise or a concerted process. Each of these possible mechanisms for DHO oxidation is outlined in Scheme II. In a stepwise reaction (Scheme IIA), initial proton abstraction from C_5 generates a kinetically significant and stabilized carbanionic intermediate. The second step entails hydride delivery from C_6 of DHO to flavin, forming orotate and reduce flavin. An alternate chemical mechanism for DHO oxidation would be a concerted reaction wherein both C_5 proton abstraction and C_6 hydride removal occur simultaneously. This possibility is outlined in Scheme IIB. Several lines of evidence from the present work indicate that DHO oxidation promoted by the bovine DHODase is concerted.

When the isotope effects at C_5 and C_6 were studied individually as a function of pH, both isotope effects increased at low pH. This indicates that neither hydrogen abstraction is cleanly rate-limiting. Therefore, the reaction is either concerted or, alternatively, stepwise with both hydrogen abstractions being partially rate-limiting.

A concerted mechanism can often be distinguished from an equal, stepwise reaction by the use of multiple isotope effects (Hermes et al., 1982). We found that the kinetic isitope effects at C_5 and C_6 change little, if any, upon isotopic substitution at the second possible. This result is consistent with a concerted reaction. However, since the errors in the isotope effects determined where rather large, it is not possible to completely rule out a stepwise reaction by this analysis. In addition, Belasco et al. (1983) have pointed out that, under certain conditions, a stepwise reaction can also show isotope effects that do not change upon double isotopic substitution.

The solvent deuterium exchange experiments carried out in this work, however, also indicate a concerted reaction. If both hydrogens are abstracted in a single step, there is no opportunity to obtain solvent exchange, as there is no kinetically significant carbanionic intermediate that can be reprotonated by using a solvent deuteron. Even with prolonged incubation of the enzyme, we found that solvent exchange could not be detected under both nonturnover or catalytic conditions (data not included).

While these results are consistent with a concerted reaction, it is also possible that the active site is sequestered from solvent and, therefore, would not show any exchange even in a stepwise

mechanism. This type of solvent shielding of a carbanionic intermediate has been demonstrated for the flavoprotein D-amino-acid oxidase (Walsh et al., 1973). Thus, it is not possible to assert conclusively from the exchange studies alone that DHODase catalyzes a concerted oxidation of DHO. Nonetheless, the results of deuterium isotope studies and NMR exchange reactions, taken together, point toward a concerted oxidation. We note also that Ghisla and his co-workers (Pohl et al., 1986) have concluded, on the basis of a similar study of doubly substituted isotope effects, that the acyl-CoA dehydrogenase reaction proceeds by a concerted mechanism. Acyl-CoA dehydrogenase and the bovine DHODase appear to be chemically similar. They are both, for example, flavoprotein oxidoreductases.

Mechanistic studies similar to those reported here have been carried out with the DHODase from *C. fasciculata*. Pascal and Walsh (1984) studied multiple isotope effects and solvent-exchange reactions with the parasitic enzyme, and their results contrast sharply with those reported here for the mammalian enzyme. The isotope effects observed in the study of the crithidial enzyme with [5-^{2}H]DHO were $^{5D}V/K_{6H}$ = 2.92 and $^{5D}V/K_{6D}$ = 2.04, where C_6 was either hydrogen or deuterium, respectively. The isotope effects observed with [6-^{2}H]DHO were $^{6D}V/K_{5H}$ = 2.99 and $^{6D}V/K_{5D}$ = 2.09, where C_5 was either hydrogen or deuterium, respectively. In both cases, deuterium substitution decreased the kinetic isotope effects significantly. When exchange studies were carried out in the presence of both DHO and OA, both the C_5-*pro-S* hydrogen and the C_6 hydrogen of DHO exchanged with solvent. In addition, the exchange of the C-*pro-S* hydrogen was twice as fast as the C_6 hydrogen exchange. All of these results suggest that the parasitic enzyme catalyzes a stepwise oxidation of DHO.

We note in conclusion that the apparent catalytic difference between the crithidial and bovine enzymes—that is, stepwise oxidation in the former and concerted dehydrogenation in the latter—leaves open the possibility for design of an inhibitor of pyrimidine biosynthesis that might have selectivity against the parasite DHODases only. One can imagine, as has been suggested previously (Pascal & Walsh, 1984), the functionalization of DHO at C_5 with a group that would unravel catalytically to an electrophilic Michael system consequent of C_5 carbanion formation.

We have, in fact, begun to explore synthetic approaches to a (5*R*)-halomethyl dihydroorotate, with the expectation that carbanion-assisted HX elimination would uncover and suicidally active 1,3-enone of DHO. There is some support for this speculation in the finding that 5-ethynylorotate seems to function as a mechanism-based inactivator of the *Clostridium oroticum* orotate reductase (Bhatt et al., 1981). In this case, one suspects that inactivation proceeds by nucleophilic addition to a conjugated allene, formed by intermediate propargylic rearrangement of an anionic acetylene (Walsh, 1982). A concerted mechanism for oxidation of DHO, by contrast, would conceivably insulate the mammalian protein from inactivation by either of these types of chemistries. Whether these theories will bear up to examination awaits the successful synthesis of the appropriately derivatized dihydroorotate.

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Registry No. **1** (dihydro derivative), 6202-10-4; **2**, 90109-17-4; **3**, 90241-95-5; **4**, 90109-21-0; **5**, 90242-05-0; **6**, 90109-19-6; **7**, 90109-20-9; DHODase, 9029-03-2; Q_6, 1065-31-2; D_2, 7782-39-0; menadione, 58-27-5.

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