Mechanistic Studies with Deuterated Dihydroorotates on the Dihydroorotate Oxidase from *Crithidia fasciculata*†

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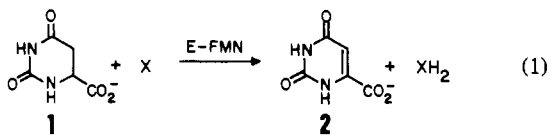
ABSTRACT: Deuterium-labeled dihydroorotates bearing one, two, or three deuteriums at the pair of C^4 and C^5 positions have been synthesized in high isotopic and chiral purity and characterized by NMR and mass spectroscopy. These substrates have been used with the FMN-containing biosynthetic dihydroorotate oxidase from *Crithidia fasciculata* [Pascal, R., Trang, N., Cerami, A., & Walsh, C. (1983) *Biochemistry 22*, 171] to probe stereochemistry and mechanism. At pH 6.0 the (4*RS*)-[5,5-$^2H_2$]dihydroorotate shows a V_{max} isotope effect (^DV) of 2.83; since the (4*S*,5*R*)-[5-^2H]dihydroorotate shows a ^DV of no more than 1.1, a secondary effect, the overall stereochemistry of desaturation is anti as previously reported for the degradative orotate reductase from *Clostridium oroticum*. The (4*RS*)-[4-^2H]dihydroorotate shows a ^DV of 2.97, indicating removal of the C^4-H is also partially rate limiting at pH 6.0. When trideuterio (4*RS*)-[4,5,5-$^2H_3$]dihydroorotate was tested, a ^DV of 8.0, a value close to the product of the separate isotope effects at the 4- and 5*S*-positions, was observed. At this pH then, both C–H cleavage steps are partly rate limiting in catalysis. Under anaerobic conditions without an electron acceptor the enzyme catalyzes the preferential exchange of the 5*S* hydrogen with solvent protons. The aggregate isotope effects on V_{max} (^DV) and on V_{max}/K_m [$^D(V/K)$] are analyzed and suggest a stepwise rather than a concerted mechanism for this biosynthetic desaturation in pyrimidine biosynthesis.

In the metabolism of such parasitic protozoa as trypanosomes and plasmodia, de novo biosynthesis is the major route for provision of pyrimidines, whereas purines are gathered by salvage pathways only (Wang, 1981). In pyrimidine ring construction, the dehydrogenation of dihydroorotate to orotate is the sole redox step. It had been suggested that *Crithidia* and trypanosomes might effect this conversion by a novel pterin-dependent hydroxylase (Gutteridge et al., 1979), but we have recently purified the dihydroorotate oxidizing enzymes from *Crithidia fasciculata* and from *Trypanosoma brucei* to homogeneity and established that both are simple flavoprotein oxidases (Pascal et al., 1983) (eq 1, X = O_2).



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As a prelude to the design of specific inhibitors or inactivators of the parasitic dihydroorotate dehydrogenases/oxidases, we have undertaken a mechanistic study of the desaturation process. Knowledge of the order and nature of C^4–H and C^5–H cleavages and the stereochemical preference for recognition of the two diastereotopic C^5 hydrogens will condition inhibitor design. In fact, despite the metabolic centrality of the dihydroorotate to orotate conversion almost nothing has been reported on the nature of the biosynthetic dehydrogenases. This is put in perspective by the fact that the *Clostridium oroticum* dehydrogenase, purified and studied by Vennesland (Graves & Vennesland, 1957; Friedman & Vennesland, 1960) and Handler and colleagues (Aleman & Handler, 1967), is degradative in physiological function. Elicited by growth on orotate as carbon source, that enzyme is an Fe/S-, FMN-, and FAD-containing orotate reductase, with orotate reduction driven by coupled NADH oxidation. In contrast the protozoan biosynthetic enzymes do not contain iron and cannot transfer electrons to NAD. The biosynthetic electron acceptor is either O_2 or a membrane respiratory chain component (Pascal et al., 1983).

In this paper we report the synthesis and characterization of all possible stably deuterated forms of dihydroorotate and their use in mechanistic analysis of the dihydroorotate dehydrogenase from *Crithidia fasciculata*. This enzyme and substrate pair may offer advantages for the study of enzymic desaturation α to a carbonyl group because of the availability of the specifically deuterated substrate forms and the structural simplicity of this flavoprotein biosynthetic desaturase.

Materials and Methods

General. Unless otherwise stated, all solvents and inorganic chemicals were of analytical reagent grade. Cysteine hydrochloride, 2-mercaptoethanol, and NADH were purchased from Sigma Chemical Co. Deuterium oxide (99.7 atom % D) and methanol-*d* (99 atom % D) were purchased from Merck Sharp & Dohme. Deuterated buffers were prepared by dissolving appropriate quantities of buffer salts in D_2O, evaporating the solutions to dryness, redissolving the residue in D_2O, and making slight adjustments in pD with solutions of DCl or NaOD.

Melting points were recorded on a Mel-Temp apparatus and are uncorrected. Ultraviolet–visible spectroscopy was performed on a Perkin-Elmer 554 spectrophotometer. Proton nuclear magnetic resonance (NMR) spectra were recorded on D_2O solutions by using Bruker WM-250 and WM-270 Fourier transform spectrometers operating at 250 and 270 MHz, respectively. Chemical shifts are reported in parts per million (δ) relative to an internal standard of acetone at δ 2.04. Mass spectra were obtained on a Varian MAT 44 spectrometer using an electron beam energy of 70 eV.

Enzymes. Dihydroorotate oxidase was purified from *Crithidia fasciculata* as described previously (Pascal et al., 1983). The enzyme (M_r 60 000) contains one molecule of flavin mononucleotide as its sole redox cofactor. Enzyme of greater than 90% purity as judged by SDS–polyacrylamide gel elec-

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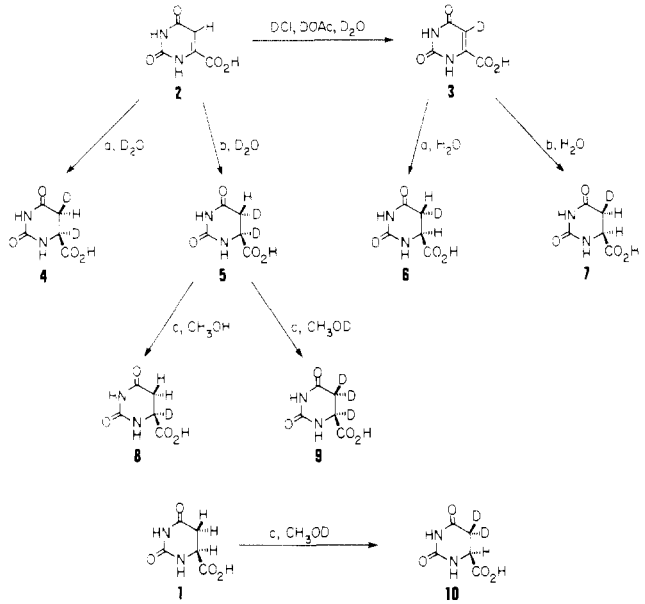


FIGURE 1: Synthesis of deuterium-labeled dihydroorotates. Reagents: a, *Clostridium oroticum* dihydroorotate dehydrogenase, NADH; b, H_2 (1 atm), 5% rhodium on alumina; c, sodium methoxide.

trophoresis and with a ratio of absorbance A_{276}/A_{454} = 5.7–5.9 was employed for all experiments.

Dihydroorotate dehydrogenase from *Clostridium oroticum* was purchased from Sigma Chemical Co.

Pyrimidines. Orotic acid hydrate, (4*S*)-dihydroorotic acid [(4*S*)-DHO],[1] and (4*R*)-dihydroorotic acid were purchased from Sigma. Prior to use in enzyme assays, (4*S*)-DHO was recrystallized from hot water to give material melting at 270–272 °C dec [lit. mp 269–271 °C dec (Liebermann & Kornberg, 1953)]. (4*RS*)-Dihydroorotic acid (mp 270–272 °C) was prepared by catalytic hydrogenation of orotic acid over 5% rhodium on alumina as described below. Deuterium-labeled pyrimidines were synthesized as described below and as outlined in Figure 1. The proton NMR spectrum of dihydroorotic acid is illustrated in Figure 2, spectrum 1: NMR δ 2.72 (dd, 1 H, J = 17.2, 5.3 Hz, 5-*pro*-*S*-H), 2.90 (dd, 1 H, J = 17.2, 7.1 Hz, 5-*pro*-*R*-H), and 4.21 (dd, 1 H, J = 7.1, 5.3 Hz, 4-H).

[5-^2H]*Orotic Acid* (**3**). Acetyl chloride (1.5 mL) and D_2O (3 mL) were mixed with cooling in a screw-capped tube until homogeneous. Orotic acid hydrate (21 mg, 0.12 mmol) was added, and the mixture was heated to 110 °C for 16 h. After cooling to <100 °C, the mixture was filtered rapidly through a preheated fritted glass funnel. Deuterated orotic acid crystallized from the filtrate upon cooling. After two recrystallizations from hot water (protium oxide) to remove exchangeable deuterons on nitrogen and oxygen, the yield of **3** was 5 mg: mp 345 °C dec [lit. mp for unlabeled orotic acid, 345 °C dec (Johnson & Schroeder, 1931), 343–345 °C dec (Nyc & Mitchell, 1947)]; mass spectrum, m/z 157 (M^+, 22), 114 (M – HNCO, 7), 69 (M – HNCO – CO_2H, 100). Analysis of the mass spectrum indicated that 91% of the molecules contained one deuterium.

(4*S*,5*S*)-[4,5-$^2H_2$]*Dihydroorotic Acid* (**4**). Orotic acid hydrate (22 mg, 0.13 mmol), cysteine hydrochloride (30 mg), and *Clostridium oroticum* dihydroorotate dehydrogenase (~1.5 IU) were dissolved in deuterated sodium phosphate (pD 6.6, 200 mM, 25 mL). After 10 min, NADH (115 mg, 0.15 mmol) was added, and the solution was incubated at 25 °C for 2 h. The reaction mixture was applied directly to a column of Dowex 1-X8 (formate form, 200–400 mesh, 18 mL), and it was eluted with a solution of 0.07 M sodium formate adjusted to pH 3.2 with formic acid. Thirty 12.2-mL fractions were collected. Fractions 8–15 were pooled and passed through a column of Dowex 50-X8 (H^+ form, 200–400 mesh, 18 mL) to remove the sodium ions. The solvent was evaporated, and the residue was recrystallized from water to yield compound **4** (12 mg); mp 270–271 °C dec; NMR (Figure 2, spectrum 2) δ 2.88 (s); mass spectrum, m/z 160 (M^+, 1), 115 (M – CO_2H, 96). The NMR and mass spectral data indicated that the 5-*pro*-*S*-H was 95% deuterated and the 4-H was 97% deuterated.

(4*RS*,5*SR*)-[4,5-$^2H_2$]*Dihydroorotic Acid* (**5**). Orotic acid hydrate (36 mg, 0.21 mmol) was dissolved in D_2O (25 mL). Powdered 5% rhodium on alumina (40 mg) was added, and the mixture was stirred overnight under 1 atm of hydrogen. The catalyst was filtered away, and the solvent was evaporated. The residue was dissolved in 500 mL of water, and the solvent was evaporated again (removing exchangeable deuterons). The residue was recrystallized from water to yield **5** (18 mg); mp 269–270 °C dec; NMR (Figure 2, spectrum 3), δ 2.69 (s); mass spectrum, m/z 160 (M^+, 2), 115 (M – CO_2H, 100). The NMR and mass spectral data taken together indicated that the 4-H was 97% deuterated, that the 5-*pro*-*R*-H was 89% deuterated, but that the 5-*pro*-*S*-H also contained 26% deuterium (the *pro*-*R* and *pro*-*S* designations are relative to the 4*S* isomer).

(4*S*,5*R*)-[5-^2H]*Dihydroorotic Acid* (**6**). [5-^{2}H]Orotic acid (30 mg, 0.19 mmol) was reduced by *Clostridium oroticum* dihydroorotate dehydrogenase as described for compound **4**, except that *protic* 200 mM sodium phosphate, pH 6.5, was employed as the buffer. Isolation and recrystallization as before yielded **6** (21 mg); mp 271–272 °C dec; NMR δ 2.70 (d, 1 H, J = 5.3 Hz) and 4.20 (d, 1 H, J = 5.3 Hz), superimposed on a 20% background of the spectrum of unlabeled dihydroorotate; mass spectrum, m/z 159 (M^+, 1), 114 (M – CO_2H, 72). The NMR and mass spectral data indicated that the 5-*pro*-*R*-H was 83% deuterated.

(4*RS*,5*RS*)-[5-^2H]*Dihydroorotic Acid* (**7**). [5-^{2}H]Orotic acid (52 mg, 0.33 mmol) was hydrogenated as in the synthesis of compound **5**, except that a protic solvent was employed. Isolation and recrystallization as before yielded compound **7** (35 mg); mp 270–271 °C dec; NMR δ 2.88 (d, 1 H, J = 7.1 Hz and 4.20 (d, 1 H, J = 7.1 Hz), superimposed on the spectrum of unlabeled dihydroorotate of equal intensity; mass spectrum, m/z 159 (M^+, 2), 114 (M– CO_2H, 67). The NMR and mass spectral data indicated that the 5-*pro*-*S*-H (of the 4*S* isomer) was 53% deuterated.

(4*RS*)-[4-^2H]*Dihydroorotic Acid* (**8**). Orotic acid hydrate was hydrogenated over 5% rhodium on alumina in D_2O as described in the synthesis of compound **5**. The isolated cis-deuterated dihydroorotate was thoroughly dried, and a solution of 0.2 g of sodium in methanol (50 mL) was added. The resulting mixture was sonicated in a bath type sonicator for 2 h. The solution was acidified with formic acid, diluted with water (50 mL), and passed through a column of Dowen 50-X8 (H^+) to remove the sodium. The solvent was evaporated, and the residue was recrystallized from water to give **8** (52 mg); mp 269–270 °C dec; NMR (Figure 2, spectrum 5) δ 2.72 (d, 1 H, J = 17.3 Hz) and 2.90 (d, 1 H, J = 17.3 Hz); mass spectrum, m/z 159 (M^+, 7), 114 (M – CO_2H, 100). The NMR spectrum (Figure 2) clearly indicates that the 4-H is

[1] Abbreviations: DHO, dihydroorotate; ^Dk, deuterium isotope effect on an elementary step; ^DV, deuterium isotope effect on V_{max}; $^D(V/K)$, a deuterium isotope effect on V/K.

greater than 98% deuterated, with no deuterium at other positions.

(*4RS*)-[*4,5,5*-$^2H_3$]*Dihydroorotic Acid* (**9**). Orotic acid hydrate (45 mg, 0.26 mmol) was hydrogenated over 5% rhodium on alumina in D_2O as described for the synthesis of compound **5**. The product was thoroughly dried under vaacuum, and a solution of 0.1 g of sodium in methanol-*d* (25 mL) was added. The mixture was sonicated for 2 h. The resulting solution was acidified with formic acid, diluted with water (25 mL), and passed through a column of Dowex 50-X8 (H^+). The solvent was evaporated, water was added, and it was evaporated again (to remove exchangeable deuterons). The residue was recrystallized from water to give **9** (22 mg); mp 271–273 °C dec; NMR, no signals observed; mass spectrum, *m/z* 161 (M^+, 8), 116 (M – CO_2H, 100). From the NMR and mass spectral data we conclude that the 4-H and both 5-H's are greater than 98% deuterated.

(*4RS*)-[*5,5*-$^2H_2$]*Dihydroorotic Acid* (**10**). Unlabeled (4*RS*)-dihydroorotic acid (45 mg, 0.29 mmol) was added to a solution of 0.1 g of sodium in methanol-*d* (25 mL). The mixture was sonicated for 2 h. The resulting solution was acidified with formic acid, diluted with water (25 mL), and passed through a column of Dowex 50-X8 (H^+). The solvent was evaporated, water was added, and it was evaporated. The residue was recrystallized from water to give **10** (37 mg); mp 269–270 °C dec; NMR (Figure 2, spectrum 4) δ 4.20 (s); mass spectrum, *m/z* 159 (M^+, 6), 114 (M – CO_2H, 100). The NMR and mass spectral data indicate that both 5-H's are greater than 98% deuterated.

All samples of labeled and unlabeled dihydroorotic acid used in enzymatic experiments showed a single band on TLC (MN 300 Cellulose, Analtech) with an R_f 0.43 (2-propanol–ammonium hydroxide–water, 7:1:2 v/v). The 250-MHz proton NMR spectra of all samples showed no peaks not attributable to dihydroorotic acid or the NMR solvents.

Determination of Substrate Concentrations. The concentration of the 4*S* isomer of dihydroorotate in stock solutions used for steady-state kinetic experiments was determined by complete oxidation of an aliquot by dihydroorotate oxidase at 30 °C in air-saturated 100 mM sodium pyrophosphate buffer, pH 9.0. The increase in absorbance at 278 nm due to the formation of orotate (ϵ_{pH9} = 6900 M^{-1} cm^{-1}) permitted the estimation of the original (4*S*)-dihydroorotate concentration to within 1% (the 4*R* isomer does not react with the enzyme). The precision and accuracy of the method was verified with gravimetrically prepared solutions of (4*S*)- and (4*RS*)-dihydroorotic acid.

Enzyme Assays. The following buffers were used for the assay of dihydroorotate oxidase at the indicated pHs: pH 9.0, 100 mM sodium pyrophosphate; pH 7.4 and 6.0, 100 mM potassium phosphate; pH 5.0, 50 mM sodium acetate. Assays were carried out at 30 °C in argon-saturated buffer, though no attempt was made to exclude oxygen during the reaction. For all kinetic experiments reported herein, the assay mixture (1 mL total volume) contained 6 pmol (0.36 μg) of dihydroorotate oxidase and 300 nmol of potassium ferricyanide (the K_M for ferricyanide at pH 6.0 is about 1–2 μM). The reaction was initiated by addition of dihydroorotate, and the decrease of ferricyanide concentration was monitored at 420 nm (ϵ = 1000 M^{-1} cm^{-1}). The reaction rates reported in this paper are given in nanomoles of ferricyanide reduced per minute. Naturally, the rates of dihydroorotate oxidation (a net two electron transfer) are half of these values.

Anaerobic Exchange Experiments. The dihydroorotate oxidase catalyzed exchange of solvent hydrogen into dihydroorotate was carried out as follows. Protic or deuterium-labeled (4*S*)-dihydroorotate (2.5–5 mg) and orotic acid (0.5–1 mg) were dissolved in 2 mL of the appropriate buffer. 2-Mercaptoethanol was added to a concentration of 1 mM, and the solution was saturated with argon. Dihydroorotate oxidase (2–6 μg) was added, and the solution was left under an argon atmosphere at 25 °C for 1–4 days. The reaction mixture was then acidified at pH 3 with HCl, and it was extracted 4 times with 1-mL portions of chloroform to remove the mercaptoethanol. The aqueous phase was concentrated to dryness, redissolved in D_2O, and filtered prior to NMR analysis.

Results

Syntheses of Deuterium-Labeled Dihydroorotates. Three reactions proved to be most useful in the syntheses of labeled dihydroorotates: (a) Enzyme-catalyzed hydrogenation of orotate by *Clostridium oroticum* dihydroorotate dehydrogenase gives overall trans addition of the elements of hydrogen to the olefinic carbons of orotic acid (Blattman & Retey, 1972). (b) Catalytic hydrogenation (Cohn & Doherty, 1956) of orotic acid over 5% rhodium on alumina gives overall cis addition of hydrogen. (c) Sodium methoxide treatment of dihydroorotate selectively exchanges the two C^5 hydrogens with the solvent.

Figure 1 outlines the syntheses of seven stereospecifically labeled dihydroorotates using these methods. In all cases, the solvent is the source of the newly introduced hydrogen atoms. In both the enzymic and rhodium-catalyzed hydrogenations the solvent is in facile equilibrium with the reduced form of the catalyst, and in the base-catalyzed exchange the solvent (methanol) is the only possible source of protons.

Compounds **1** and **4–10** represent the eight possible ways in which protium and deuterium may be combined on the three nonexchangeable positions of dihydroorotic acid. Particularly noteworthy are the 250-MHz proton NMR spectra of **4** and **5** (Figure 2) which clearly demonstrate the stereochemical complementarity of the enzymic and rhodium-catalyzed hydrogenations. Since the hydrogenation of orotate on a transition metal surface almost certainly proceeds with cis addition of H_2, the reduction of orotate by *Clostridium oroticum* dihydroorotate dehydrogenase must give overall trans addition, thus verifying the conclusion of Blattmann & Retey (1972). The NMR spectra of compounds **8** and **10** (Figure 2) testify to the high selectivity and efficiency of the methoxide-catalyzed exchange of the C^5 protons.

Steady-State Kinetic Isotope Effects. The maximal rate for the *Crithidia fasciculata* dihydroorotate oxidase reaction is observed at about pH 9 when molecular oxygen is the electron acceptor (Pascal et al., 1983). With potassium ferricyanide as the oxidant, the reaction is some 12 times faster at this pH. We initiated our study of deuterium kinetic isotope effects on dihydroorotate oxidase under the latter conditions using enzymically synthesized (optically pure) (4*S*,5*S*)-[4,5-$^2H_2$]DHO (**4**) and (4*S*,5*R*)-[5-^2H]DHO (**6**) as substrates. No isotope effect on V_{max} was observed with either substrate, though a small V/K isotope effect was observed with the trans-dideuterated compound **4** [see Table I; $^D(V/K)_{4,pH9}$ = 1.6). We reasoned, however, that substrate dehydrogenation might be rate determining at other pHs, so a survey of the kinetic isotope effects at pH 7.4, 6.0, and 5.0 was conducted. Figure 3 summarizes the pH-dependent variation of V_{max} with (4*S*)-DHO and the deuterium isotope effect on V_{max} observed with compound **4**. Enzyme activity was maximal at pH 7.4, but the largest isotope effect was observed at pH 6.0. Only small, presumably secondary isotope effects were observed with

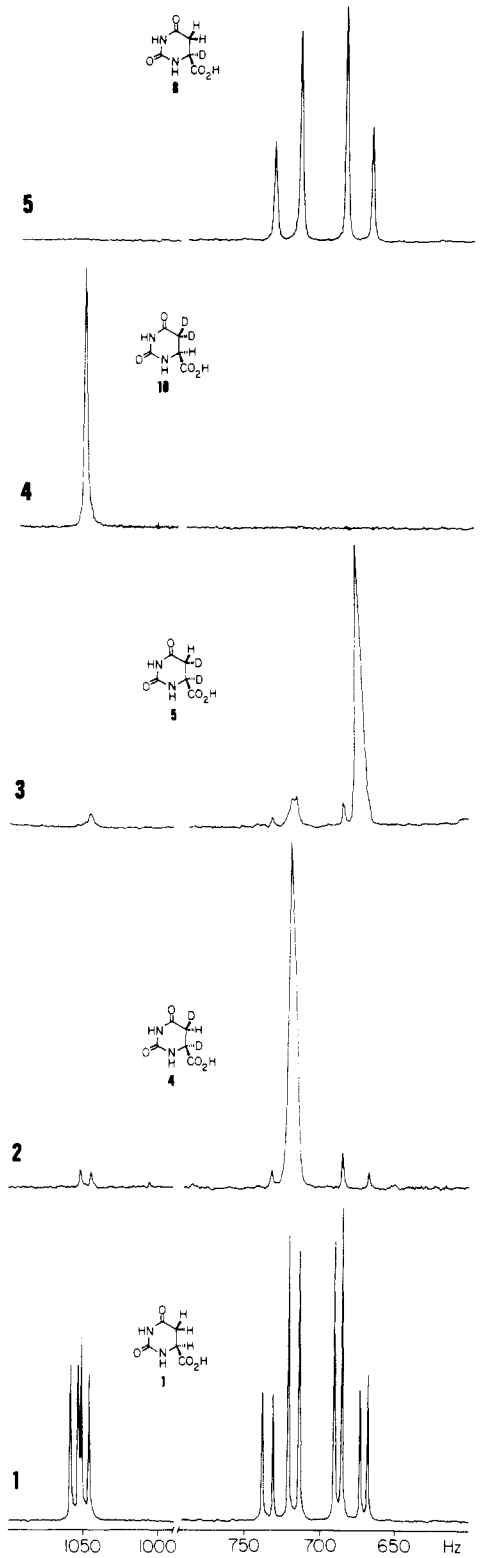


FIGURE 2: 250-MHz proton NMR spectra of deuterium-labeled dihydroorotates. Spectrum 1, (4*S*)-DHO (**1**); spectrum 2, (4*S*,5*S*)-[4,5-$^2H_2$]DHO (**4**); spectrum 3, (4*RS*,5*SR*)-[4,5-$^2H_2$]DHO (**5**); spectrum 4, (4*RS*)-[5,5-$^2H_2$]DHO (**10**); spectrum 5, (4*RS*)-[4-^{2}H]DHO (**8**).

compound **6** under any conditions (see Table I).

We therefore chose to examine the isotope effects more closely at pH 6.0. Thus far, only the enzymically synthesized 4*S* isomers of dihydroorotate had been employed. However, (4*R*)-DHO is not a substrate for dihydroorotate oxidase, nor does it significantly inhibit the enzyme. With an array of deuterium-labeled (4*RS*)-DHO's from which to select, we could expect to define the effect of deuterium substitution at

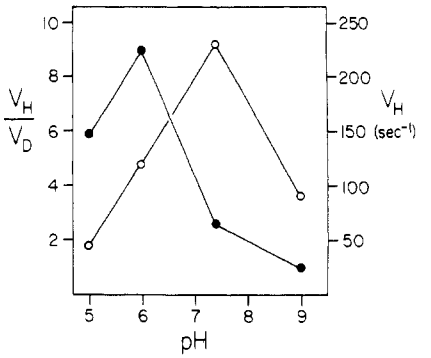


FIGURE 3: pH-dependent variation of dihydroorotate oxidase activity and deuterium isotope effects. (○) Turnover number (at V_{max}) for (4*S*)-DHO; (●) steady-state kinetic isotope effect on V_{max} ($V_H/V_D = {}^DV$) for (4*S*,5*S*)-[4,5-$^2H_2$]DHO (**4**).

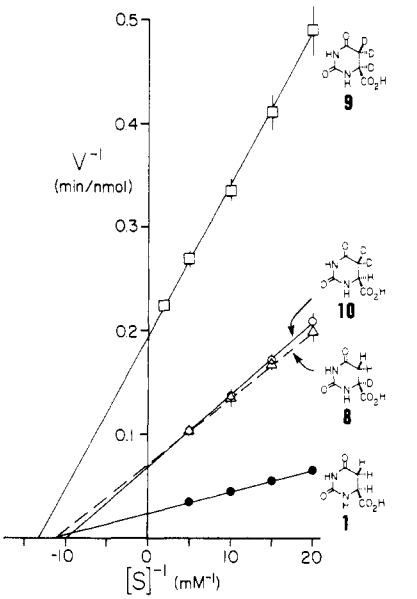


FIGURE 4: Double-reciprocal plots of dihydroorotate oxidase activity with various deuterium-labeled (4*RS*)-dihydroorotates. Assays were conducted at pH 6.0 as described under Materials and Methods. All points are the average of four to seven determinations with the standard deviations shown by the error bars. Straight lines were fitted by using the weighted least-squares method described by Roberts (1977).

each position of the molecule. Compounds **7** and **5** were not considered suitable for the study of isotope effects, the former because of low deuterium content (53%) and the latter because of the presence of some deuterium (~26%) in the position cis to the carboxyl group. Compounds **8**, **9**, and **10**, however, all contained >98% deuterium at specific sites and were ideal for these studies.

The data from the experiments with the (4*RS*)-DHO's are plotted in Figure 4, and the results are summarized in Table I. The V_{max} effects observed with (4*RS*)-[4-^{2}H]DHO (**8**) and (4*RS*)-[5,5-$^2H_2$]DHO (**10**) are similar ($^DV_{8,pH6}$ = 3.0 and $^DV_{10,pH6}$ = 2.8; corrected for a ca. 10% secondary deuterium isotope effect, the latter value will be ≈2.5). The isotope effect noted for the latter compound must be largely due to the C^5 deuterium cis to the carboxyl group, since (4*S*,5*R*)-[5-^{2}H]-DHO (**6**) shows only a small secondary isotope effect ($^DV_{6,pH6}$ = 1.1). Therefore, in (4*S*)-dihydroorotates primary isotope effects are produced by isotopic substitution at the 4- and 5-*pro-S* positions, and we conclude that these must be the carbon–hydrogen bonds broken during dehydrogenation. With (4*S*)-[4,5,5-$^2H_3$]DHO (**9**), which incorporates the labels of both **8** and **10**, the V_{max} isotope effect ($^DV_{9,pH6}$ = 8.0) is larger than the sum of $^DV_{8,pH6}$ and $^DV_{10,pH6}$ (5.8), but only slightly

Table I: Steady-State Kinetic Isotope Effects on Dihydroorotate Oxidase

substrate	pH	V_{max}[a]	turnover no. (s^{-1})	V/K[a]	K_M (μM)	^DV[b]	$^D(V/K)$[b]
(4*S*)-DHO	9.0	32.9 ± 2.0	91	1.9 ± 0.3	17		
	7.4	83 ± 5	231	1.47 ± 0.08	57		
	6.0	42.5 ± 1.0	118	0.485 ± 0.013	88		
	5.0	15.9 ± 1.2	44	0.115 ± 0.006	138		
(4*S*,5*S*)-[4,5-$^2H_2$]DHO (**4**)	9.0	32.1 ± 0.1	89	1.18 ± 0.13	27	1.0 (1.0)	1.6 (1.6)
	7.4	35 ± 4	96	0.42 ± 0.02	82	2.4 (2.5)	3.5 (3.9)
	6.0	6.5 ± 0.3	18.2	0.057 ± 0.002	115	6.5 (8.4)	8.5 (12.4)
	5.0	3.2 ± 0.2	9.0	0.024 ± 0.002	134	4.9 (5.9)	4.8 (5.7)
(4*S*,5*R*)-[5-^2H]DHO (**6**)	9.0	32.8 ± 1.0	91	1.94 ± 0.17	17	1.0 (1.0)	1.0 (1.0)
	7.4	75 ± 4	207	1.58 ± 0.09	47	1.1 (1.1)	0.93 (0.92)
	6.0	38.7 ± 1.4	107	0.494 ± 0.022	78	1.09 (1.11)	0.99 (0.99)
	5.0	not determined					
(4*RS*)-DHO	6.0	41.1 ± 0.4	114	0.475 ± 0.005	86		
(4*RS*)-[5,5-$^2H_2$]DHO (**10**)	6.0	14.5 ± 0.3	40.3	0.146 ± 0.002	99	2.83	3.24
(4*RS*)-[4-$^2H_2$]DHO (**8**)	6.0	13.8 ± 0.3	38.4	0.159 ± 0.004	87	2.97	2.99
(4*RS*)-[4,5,5-$^2H_3$]DHO (**9**)	6.0	5.12 ± 0.04	14.2	0.0700 ± 0.0014	73	8.0	6.8

[a] Velocities are reported as nanomoles of ferricyanide consumed per minute. All assays were conducted at 30 °C and contained 6 pmol (0.36 μg) of DHO oxidase. Double-reciprocal plots were constructed with the velocities determined at several substrate concentrations, and straight lines were fitted by using the weighted least-squares method described by Roberts (1977) to give $1/V_m$ (y intercept) and K_M/V_m (slope). [b] Values in parentheses are corrected for 100 atom % D, since compounds **4** (96 atom % D) and **6** (83 atom % D) were not fully deuterated.

smaller than their product (8.4). If we assume a 1.1-fold rate reduction caused by the secondary deuterium isotope effect from the 5*R* deuterium in compound **9**, the $^DV_{9,pH6.0}$ is ca. 7.2.

For most substrates at pH 6.0, the V/K isotope effects are similar to the V_{max} effects. However, note that, for trans-dideuterated **4**, $^DV_{4,pH6} < {}^D(V/K)_{4,pH6}$ but that for trideuterated **9**, $^DV_{9,pH6} > {}^D(V/K)_{9,pH6}$. The differences are substantial and reproducible, and the concentrations of the substrate solutions used in these experiments are accurate to within at least 2%, thus eliminating the most likely source of error in determining V/K (V_{max} measurements are, of course, insensitive to errors in substrate concentration). The reason for the fairly large effect on K_M produced by protium deuterium substitution at the 5-*pro*-*R*-H of (4*S*)-dihydroorotate is not obvious.

Enzyme-Catalyzed Exchange of Dihydroorotate Hydrogens with Solvent. The observation of primary kinetic isotope effects upon deuterium substitution at the 4- and 5-*pro*-*S* positions of (4*S*)-DHO suggests that *Crithidia* dihydroorotate oxidase catalyzes overall trans removal of H_2 from the substrate. However, we felt it imperative to validate this supposition directly. Additionally, we wished to design an experiment which might provide a clue as to the *order* of removal of the two hydrogen atoms—if in fact the dehydrogenation is stepwise, not concerted.

In the absence of oxidizing equivalents, the addition of dihydroorotate reduces the flavin moiety of dihydroorotate oxidase (Pascal et al., 1983). We reasoned that the protons from the substrate, while residing on the reduced flavin or other enzymic bases, might be accessible to the solvent. If so, reversible dehydrogenation of dihydroorotate under anaerobic conditions would lead to exchange of the transferred hydrogens with the solvent. This type of experiment has been successfully performed with other flavoprotein desaturases—succinate dehydrogenase (Retey et al., 1970) and fatty acyl-CoA dehydrogenase (Biellmann & Hirth, 1970).

Figure 5 illustrates such an experiment (reaction conditions are given in the figure legend). Protic (4*S*)-DHO was incubated with enzyme in deuterated buffer under an argon atmosphere. The 4- and 5-*pro*-*S* protons were slowly exchanged for deuterium as indicated by the disappearance of the multiplets at δ 4.19 (1132 Hz) and 2.70 (728 Hz). The appearance of a singlet at δ 2.85 (770 Hz) is due to the remaining 5-*pro*-*R*-H in the dideuterated product. Thus, the overall

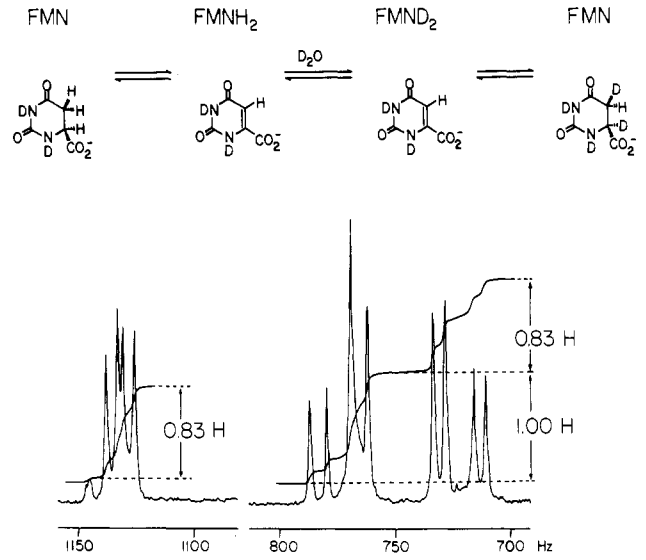


FIGURE 5: 270-MHz proton NMR spectrum of the product of enzyme-catalyzed exchange of protons from (4*S*)-DHO. The reaction mixture contained (4*S*)-DHO (5 mg), orotic acid (1 mg), and dihydroorotate oxidase (43 pmol, 2.6 μg) in 2.5 mL of deuterated potassium phosphate buffer (200 mM, pD 8.8, 1 mM 2-mercaptoethanol) under an argon atmosphere. The incubation was carried out for 36 h at 27 °C.

stereochemical outcome of enzymic dehydrogenation is indeed trans removel of H_2. In the absence of enzyme, no exchange is observed even after 4 days of incubation.

The exchange experiment illustrated in Figure 6 uses the reverse strategy. The 250-MHz proton NMR spectrum of (4*S*,5*S*)-[4,5-$^2H_2$]DHO (**4**, >98% D) is spectrum 1. Incubation of **4** with enzyme in protic buffer led to the exchange of the two deuterons for protons, as expected (spectrum 2). However, two important additional pieces of evidence were obtained. First, the 5*S* deuteron is exchanged twice as fast as the 4 deuteron. Second, the line pattern observed can be obtained by a summation of the spectra of (4*S*)-DHO, (4*S*)-[4-^2H]DHO, and (4*S*,5*S*)-[4,5-$^2H_2$]DHO. *There is no evidence for the presence of any 5S monodeuterated dihydroorotate.* If the enzyme-bound hydrogens derived from the substrate can exchange rapidly with the solvent, then this observation can only be explained by a stepwise desaturation

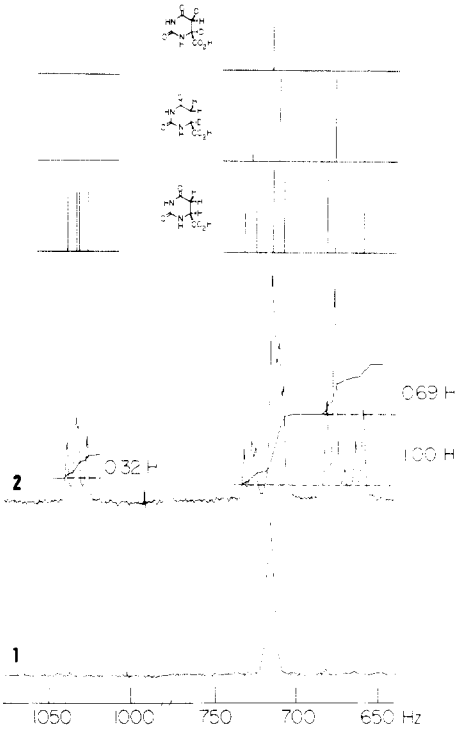


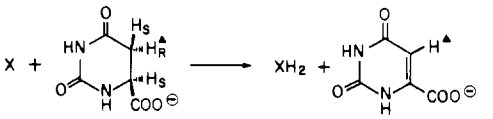
FIGURE 6: 250-MHz proton NMR analysis of the enzyme-catalyzed exchange of deuterons from (4*S*,5*S*)-[4,5-$^2H_2$]DHO (**4**). Spectrum 1, compound **4** (>98 atom % D); spectrum 2, product isolated after a 60-h incubation of **4** (2.5 mg), orotic acid (0.5 mg), and dihydroorotate oxidase (100 pmol, 6 μg), in 1.5 mL of protic potassium phosphate buffer (100 mM, pH 9.0, 1 mM 2-mercaptoethanol). The line spectra (top to bottom) of (4*S*,5*S*)-[4,5-$^2H_2$]DHO, (4*S*)-[4-^{2}H]DHO, and (4*S*)-DHO are displayed above to facilitate analysis of the product spectrum.

mechansim in which the 5-*pro-S* hydrogen is removed first to give a discrete enzyme-bound intermediate. Such an intermediate is likely to be the C^5 carbanion formed by abstraction of the 5-*pro-S* proton, which is considerably more acidic than the hydrogen at position 4, as witnessed by the base-catalyzed exchange reaction used to label some of the substrates described here.

Discussion

In this study we have prepared dihydroorotate substituted with one, two, or three deuterium atoms at C^4 and C^5 to probe mechanistic and stereochemical questions about this key redox transformation in pyrimidine ring biogenesis catalyzed by the pure dihydroorotate oxidizing enzyme from *Crithidia fasciculata*. The availability of all possible carbon-deuterated dihydroorotates of high isotopic and chiral purity allows NMR assignments which permit the unambiguous analysis of various dihydroorotate species generated in enzymatic turnover or exchange experiments.

The stereochemical course of desaturation could be deduced if specific substitution of deuterium for protium at the 5*R* or 5*S* locus of dihydroorotate was achieved and if only one deuterated species produced a primary deuterium kinetic isotope effect. In fact, at or near the pH optimum neither the C^4 nor C^5 C–H cleavage step is substantially rate determining in overall catalytic turnover, and we had to go to the acid side, to pH 6.0, to observe the enzyme under conditions where the transition state(s) for each C–H cleavage step is (are) substantially rate limiting for V_{max}. The (5*R*)-monodeuteriodihydroorotate, **6**, yields a ^DV of only 1.1 while the (5*R*,5*S*)-dideuteriodihydroorotate, **10**, yields a ^DV of 2.83. Clearly the 5*S* hydrogen is removed and the ^DV of 1.1 for **6** is the small value anticipated for a secondary deuterium effect at C^5.



Since only the 4*S* isomer of dihydroorotate is a substrate, the anti dehydrogenation stereochemistry obtains. The (4*S*)-monodeuteriodihydroorotate, **8**, also yields a substrate ^DV (2.97) at pH 6.0, and this fact is analyzed further below. The anti stereochemistry was also reported in an earlier NMR work by Blattman & Retey (1972) in a study not of a biosynthetic (dihydroorotate to orotate) dehydrogenase but of the degradative NADH-linked orotate reductase, from *Clostridium oroticum*, which is induced by growth of that organism on orotate as sole carbon source. In the Retey work no kinetic isotope effect data were recorded, and although the stereochemical conclusions are not in doubt, the interpretation was somewhat complicated and their experiments limited by the fact that their synthetic 5-monodeuteriodihydroorotate (nominally 5*R*-D_1) had only 29% D in the 5*R* position with 9% D in the 5*S* position as well. A lack of chiral purity is not a problem in the studies reported here. The anti dehydrogenation stereochemistry is also a feature of two other olefin-forming flavoenzymes, succinate dehydrogenase (Tchen & van Milligan, 1960) and the acyl-CoA dehydrogenases (Bucklers et al., 1970; Biellmann & Hirth, 1970; LaRoche et al., 1971). By contrast syn desaturation geometry is a hallmark of the O_2- and NADPH-linked monooxygenases working on such substrates as stearyl-CoA and Δ^7-cholesten-3β-ol (Schroepfer & Bloch, 1965; Paliokas & Schroepfer, 1968).

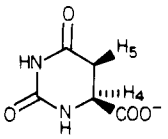
At the pH optimum (pH 7.4) for the crithidial dihydroorotate oxidase, only small ^DV isotope effects are observed. When enzyme assays are run at pH 6, where V_{max} is about 50% of that at pH 7.4, substrate isotope effects are maximal (Figure 3).

The kinetic isotope effect data determined with dihydroorotate species **10**, **8**, and **9**, documented in Figure 4 and Table I, reveal that at pH 6.0, where the effects are maximal, deuterium substitution at either the C^4 or the C^5-*S* proton locus leads to similar ^DV effects, 2.97 for **8** and 2.83 for **10**. Since we know from **6** that the 5*R*-D_1 contributes about a 1.1-fold retardation interpreted as a secondary deuterium effect, the corrected value for the 5*S*-D_1 contribution to ^DV in **10** may be 2.83 − 0.28 = 2.55. The fact that each carbon–deuterium bond cleavage step produces a 2.5–3-fold ^DV effect rules out the possibility that only one of the C^5–D or C^4–D cleavages cleanly limits V_{max}. Rather, these single isotope effects indicate that if this biosynthetic dihydrogenation is stepwise, then each C–H bond-breaking step has a kinetically significant transition state.

There is the possibility that the dehydrogenation may instead proceed by a concerted process where C^4–H and C^5–H_S cleavages occur in a single transition state. In such a case it may be that each site behaves independently with respect to isotopic substitution (Kresge, 1964; Hegarty & Jencks, 1975), and this property may be used as a test for concertedness.

Two recent enzymatic studies have also focused on the question of using double isotope substitutions and kinetic analyses to discriminate between concerted and stepwise processes. Hermes et al. (1982) have described studies on malic enzyme and on glucose-6-phosphate dehydrogenase in which measurement of the ^{13}C isotope effect on the $^D(V/K)$ values from substrate-specific deuteration may be used to indicate whether the ^{13}C-sensitive and deuterium-sensitive steps are one and the same. In essence, if ^{13}C substitution lowers

Table II: Effects of Adjacent Deuterium Substitution on ^{D}V and $^{D}(V/K)$ Isotope Effects with Crithidial Dihydroorotate Dehydrogenase



species compared	^{D}V[a]	$^{D}(V/K)$[a]
H_5, D_4 vs. H_5, H_4	$^{D_4}V = 2.97$	$^{D_4}(V/K) = 2.99$
D_5, H_4 vs. H_5, H_4	$^{D_5}V = 2.55$	$^{D_5}(V/K) = 2.93$
D_5, D_4 vs. H_5, H_4	$^{D_{4,5}}V = 7.2$	$^{D_{4,5}}(V/K) = 6.1$
D_5, D_4 vs. D_5, H_4	$^{D_{4,5}}(V)_{D_5} = 2.83$[b]	$^{D_{4,5}}(V/K)_{D_5} = 2.09$[c]
D_5, D_4 vs. H_5, D_4	$^{D_{4,5}}(V)_{D_4} = 2.42$	$^{D_{4,5}}(V/K)_{D_4} = 2.04$

[a] Observed values corrected for estimated 10% secondary isotope effects in **9** and **10** as noted in text. [b] Calculated from the ratio 7.2/2.55 = 2.83. [c] Calculated from the ratio 6.8/3.24 = 2.09.

a $^{D}(V/K)$ below the $^{D}(V/K)$ value seen with the ^{12}C substrate, Hermes et al. argue that a concerted process would be ruled out and come to this conclusion with both of the above enzymes. Belasco et al. (1983) have examined the enzymic process catalyzed by clostridial proline racemase where racemization involves breakage of a C–H substrate bond in one proline isomer and creation of a new C–H bond as the product enantiomer forms. That case differs from the DHO dehydrogenase case here in that the H delivered to the nascent proline product enantiomer comes from solvent so that there is a substrate deuterium rate effect coupled with a solvent deuterium rate effect; in the present example two substrate deuterium isotope effects at adjacent carbon loci are examined.

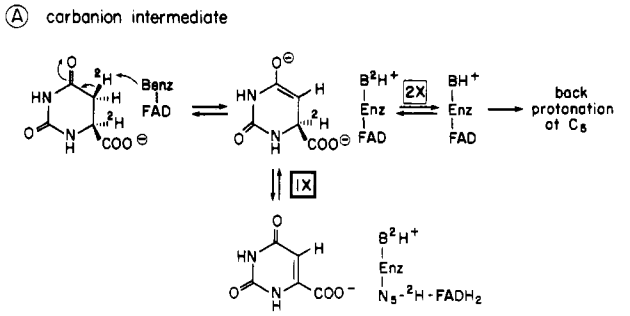
We have applied the nomenclature and approach of Hermes et al. (1982) to our kinetic data on ^{D}V, and $^{D}(V/K)$ values as summarized in Table II. Column one of this table shows the DHO substrate pair being compared to produce the values for the ^{D}V and $^{D}(V/K)$ values in the next two columns. The first two rows of data are the measured (corrected for secondary effect as noted above) ^{D}V and $^{D}(V/K)$ values for DHO species **8** and **10**, respectively, while the third row gives the values for the doubly deuterated (C^4, C^5) **9**. The fourth row, by comparing C^4,C^5-dideutero- vs. C^5-monodeutero-DHO values, isolates the effect of D substitution at C^5 on the ^{D}V and the $^{D}(V/K)$ contributed by deuterium at C^4. The fifth row reciprocally analyzes the effect of the deuterium at locus C^4 on the isotope effect contributed by deuterium at C^5.

We note first that $^{D_{4,5}}(V/K)_{D4}$ and $^{D_{4,5}}(V/K)_{D5}$ are effectively equal, again demonstrating the apparently symmetric rate retardation effect which deuterium at either carbon locus contributes to rate limitation. $^{D_{4,5}}(V/K)$ is less than $^{D_{4,5}}(V)$. Applying the test of Hermes et al. (1982), we note that $^{D_{4,5}}(V/K)_{D5} < {}^{D_4}(V/K)$, i.e., 2.09 < 2.99, and also $^{D_{4,5}}(V/K)_{D4} < {}^{D_5}(V/K)$, i.e., 2.04 < 2.92. From this inequality a concerted mechanism would be ruled out. An alternate statement is that $^{D_4}(V/K) \times {}^{D_5}(V/K) > {}^{D_{4,5}}(V/K)$, i.e., (2.99)(2.93) = 8.7 which is larger than 6.1. Clearly the product of the individual isotope effects on V/K values is greater than the effect with both deuteriums in place. We must however be cautious in applying the criterion of Hermes et al. which relies ultimately on the validity of the "rule of the geometric mean" (Bigeleison, 1955). According to this principle, for two hydrogens moving in a single transition state, $k^{HD} = (k^{HH}k^{DD})^{1/2}$. However, Limbach (1983) has noted that the rule of the geometric mean has not been rigorously validated by experiment, and he has found in some chemical cases that H + H may move faster than anticipated from rate comparisons of H + D and D + D substitutions, especially where quantum mechanical tunneling is involved in the transition state.[2] The observed isotope effects are therefore suggestive but not definitive evidence for a stepwise mechanism for crithidial dihydroorotate oxidase.

Studies with multiply deuterated substrates have been carried out previously for another flavoenzyme, general fatty acyl-CoA dehydrogenase (Murfin, 1974; Reinsch et al., 1980), which like dihydroorotate dehydrogenase carries out anti eliminations on substrates at sites adjacent to carbonyl groups. While both studies accumulated pre-steady-state kinetic evidence that both C^2–H and C^3–H cleavage steps appeared partially rate limiting in E–FAD reduction, they did not measure corresponding ^{D}V and $^{D}(V/K)$ effects for analysis of concerted vs. stepwise paths. The mechanistic path for succinate dehydrogenase has been analyzed with stereospecifically deuterated and tritiated succinates, but the symmetry of succinate has complicated the expression and analysis of large isotope effects. Intramolecular competition studies [see Retey & Robinson (1982) for a review] suggest a k_H/k_D of 5.3 for H_S removal of k_H/k_D of 1.35 for corresponding H_R removal in anti loss of the H_R,H_S pair.

One could argue for stepwise catalysis in the succinate dehydrogenase case, and exchange studies with succinate in D_2O confirmed partial exchange of solvent D into an H_R locus on succinate preferentially (Retey et al., 1970). However, small amounts of fumarate were required, and one must worry whether exchange monitored reversible formation of a bound succinate carbanion or instead an overall reaction with essentially complete sequestration of the H_S-derived proton while the H_R-derived one exchanges in the fumarate–enzyme complex.

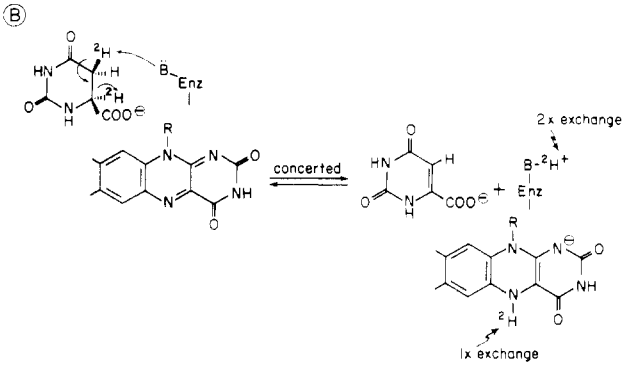
With this backdrop, we turn to our anaerobic exchange result with crithidial dihydroorotate oxidase, where long-term incubation of the (5*S*)-4,5-dideuteriodihydroorotate in H_2O in the absence of electron acceptor leads, at partial exchange, to about a 2-fold faster exchange at C^5 than at C^4. One must first be cautious in extrapolating this enzymic result under nonturnover conditions to that of normal turnover in the presence of O_2 or ferricyanide. With that in mind, one could interpret the exchange data in support of pathway A with a



C^5-carbanionic intermediate where the abstracted C^5–H, now on an enzymic base, exchanges with solvent protons on average two times for each time the C^4–H is ejected as a hydride to yield enzyme-bound orotate and $FADH_2$, whence both C^4-derived and C^5-H_S-derived hydrogens can exchange with solvent. Back-reaction would yield the all-protic dihydroorotate. Alternatively, and probably less likely from the isotope rate data analysis above, if one prefers concerted reaction

[2] A reviewer has noted that the rule of the geometric mean will not hold even if the reaction is concerted unless the concerted step is fully rate limiting. If this is not so, then the observed effect is a weighted average for a step with no effect (weight, *w*) and the concerted step (weight, 1 − *w*): $^{HD}v = w + (1 - w)^{HD}k$. Then even if $^{DD}k = (^{HD}k)^2$, $^{DD}v = w + (1 - w)(^{HD}k)^2$, which is not equal to $(^{HD}v)^2$.

pathway B, there is no selective exchange at an intermediate



stage (there would no be intermediate) but rather one would need to postulate 2-fold differential exchange rates for the C^5-derived D^+, located at some active base BD^+ compared to the C^4-derived D^+ presumed to be lodged at N^5 of $FMNH_2$. Evidence for partially sequestered protons at flavoprotein active sites has been gathered for such enzymes as D-amino acid oxidase (Walsh et al., 1973; Porter & Bright, 1976) and transhydrogenase (Louie & Kaplan, 1970).

This question of stepwise vs. concerted catalysis may in the end be of some practical relevance for specific blockage of dihydroorotate oxidation in the pyrimidine biosynthetic pathways of parasites and bacteria. In that regard Heidelberger and colleagues have recently reported the synthesis of 5-ethynylorotate and a preliminary finding of its inactivating activity toward the *Clostridium oroticum* degradative orotate reductase enzyme (Bhatt et al., 1981). If catalysis is stepwise and involves C^5 anionic intermediates, then in the back direction, reduced DHO dehydrogenase and 5-ethynylorotate could react, via initial hydride delivery to C^4 from $E\text{–}FMNH_2$, to produce the C^5 anion with allenic anion resonance contribution. Regiospecific protonation, not at C^5 but at the allenic terminus, would yield an electrophilic conjugated allene, a known suicidally uncovered functionality in many other enzyme cases (Walsh, 1982). It remains to be seen whether this acetylenic analogue inactivates the *C. fasciculata* dihydroorotate dehydrogenase.

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