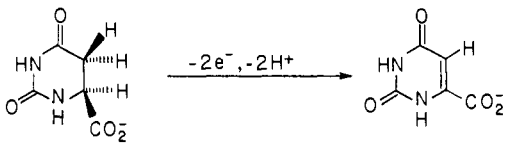
Stereoselectivity in the Enzymatic Oxidation and Nonenzymatic Hydrogen-Exchange Reactions of Dihydroorotate

Loren D. Keys III and Michael Johnston*

Contribution from the Departments of Chemistry and Biochemistry, The Searle Chemistry Laboratory, The University of Chicago, Chicago, Illinois 60637. *Received June 11, 1984*

Abstract: (*S*)-5,6-Dihydroorotate (**1**) undergoes a stereoselective hydrogen-exchange reaction with solvent deuterons in deuterium oxide buffer (100 mM NaP_i,[1] pD 7.8, 37 °C). High-field ^1H NMR analysis of the exchange reveals the formation of (5*R*,6*S*)-[5-^2H]dihydroorotate (**3**) in an apparent first-order process ($k_{obsd} = 5.4 \times 10^{-3}$ h^{-1}, 37 °C). Deuterium incorporation into the 5-*pro*-*S* position of **1** is also observed; *pro*-*S* exchange, however, occurs considerably more slowly ($k_{obsd} = 4.8 \times 10^{-4}$ h^{-1}, 37 °C) than does *pro*-*R* exchange. Stereoselective exchange was also seen for methyl (*S*)-dihydroorotate (**6**) and for 6-methyl-(*R*,*S*)-dihydrouracil (**7**), although these reactions are only modestly stereopreferential. We have capitalized on this stereochemical preference for 5-*pro*-*R* hydrogen exchange by preparing two diastereomers of deuterium-enriched dihydroorotates, **3** (from **1** in D_2O buffer) and **5** (from (*S*)-[5,5-$^2H_2$]dihydroorotate **4** in H_2O buffer). These chirally deuterated dihydroorotates were used to elucidate the stereochemical course of the reaction (**1** → **2**) catalyzed by the beef liver mitochondrial dihydroorotate dehydrogenase (EC 1.3.3.1). Enzymatic oxidation was readily followed by high-field ^1H NMR spectrometry; the data show that the enzyme is absolutely stereoselective with reference to the diastereotopic C_5 center. Only the *pro*-*S* hydrogen (or deuteron) is lost from C_5 during conversion of dihydroorotate to orotate, thereby describing a trans oxidation of substrate. Thus, we report the interesting observation that enzymatic processing of dihydroorotate (**1** → **2**) shows stereoselectivity at C_5 *opposite* to that of the nonenzymatic C_5-hydrogen exchange (**1** → **3**). These results are examined with reference to the conformational, stereoelectronic, and electrostatic factors that may influence the stereochemical course of both the enzyme-catalyzed and nonenzymatic reactions.

The single redox step in pyrimidine biosynthesis is the oxidation of (*S*)-5,6-dihydroorotate (**1**) to orotate (**2**), a reaction catalyzed

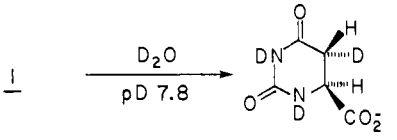


1 2

by dihydroorotate dehydrogenase [(*S*)-5,6-dihydroorotate; oxygen oxidoreductase, EC 1.3.3.1]. Dihydroorotate dehydrogenases have been partially purified from both bovine[2] and rat[3] liver mitochondria; these activities appear to be membrane-associated, multienzyme complexes linked functionally, and perhaps structurally, to the electron-transport proteins. The most well-characterized—and misnamed—dihydroorotate dehydrogenase is the crystallizable prokaryotic enzyme, induced by growth of *Clostridium oroticum* on orotic acid.[4] This protein is a soluble, nicotinamide-requiring enzyme, which probably functions in vivo as an orotate reductase on the biodegradative route toward aspartic acid.[5] Recently, Walsh, Cerami, and co-workers have purified to homogeneity the biosynthetic dihydroorotate dehydrogenases from *Crithidia fasciculata* and *Trypanosoma brucei*; these enzymes are flavoprotein oxidases.[6]

The chemical mechanism(s) of the dihydroorotate dehydrogenase reactions have not been detailed; but it is reasonable that substrate C_5-carbanion formation is involved, followed by C_6-hydride or C_6-radical transfer to an enzyme-bound electron acceptor. Enzymatic C_5-carbanion formation is a speculation in accord with model studies. Dihydroorotate, for example, undergoes sodium ethoxide catalyzed exchange of the C_5 hydrogens in refluxing ethanol-*d* without deuterium incorporation into the C_6 position.[7] Regardless of the precise mechanism for substrate oxidation, however, it seems likely that the dihydroorotate dehydrogenases will act stereoselectively at the diastereotopic C_5 position of the substrate heterocycle. Indeed, Blattmann and Retey[7] have shown that the biodegradative *C. oroticum* enzyme catalyzes the trans reduction of orotate. And very recently, Pascal and Walsh have determined that the crithidial enzyme catalyzes trans oxidation of dihydroorotate.[8] Predicting, therefore, cognate (although not necessarily *identical*) stereoselectivity for the mammalian biosynthetic reaction, we undertook to elucidate the stereochemical course of the oxidation catalyzed by the beef liver mitochondrial dihydroorotate dehydrogenase.

In the course of this work, we discovered that 6-substituted dihydrouracils undergo nonenzymatic, stereoselective exchange of the C_5 hydrogens in deuterium oxide buffer (eq 1). We have



1 3 (1)

attempted to rationalize this unexpected stereopreferential reaction in terms of the solution conformations of dihydroorotates. And we have further evaluated the stereochemical course of the enzymatic reaction with respect to the physicochemical data for the nonenzymatic exchange.

Experimental Section

Ultraviolet and visible spectrophotometric analyses were carried out using a Beckman DU monochromator equipped with a thermostated cell compartment and a strip chart recorder. Proton NMR spectra were obtained at 270 and 500 MHz using the Bruker HX 270 (modified with the Nicolet 1180 computer) and the DS-1000 instruments, respectively,

(1) Abbreviations: KP_i, potassium phosphate; DCIP, 2,6-dichlorophenolindophenol; NaP_i, sodium phosphate.
(2) (a) Miller, R. W.; Massey, V. *J. Biol. Chem.* **1965**, *240*, 1453, 1466. (b) Miller, R. W.; Kerr, C. T.; Curry, J. R. *Can. J. Biochem.* **1968**, *46*, 1099. (c) Miller, R. W.; Curry, J. R. *Can. J. Biochem.* **1969**, *47*, 725.
(3) Forman, H. J.; Kennedy, J. *Prep. Biochem.* **1977**, *7*, 345. Forman, H. J.; Kennedy, J. *Arch. Biochem. Biophys.* **1978**, *191*, 23.
(4) Friedmann, H. C.; Vennesland, B. *J. Biol. Chem.* **1958**, *233*, 1398. Friedmann, H. C.; Vennesland, B. *J. Biol. Chem.* **1960**, *235*, 1526. Udaka, S.; Vennesland, B. *J. Biol. Chem.* **1962**, *237*, 2018.
(5) Lieberman, I.; Kornberg, A. *Biochim. Biophys. Acta* **1953**, *12*, 223. Lieberman, I.; Kornberg, A. *J. Biol. Chem.* **1954**, *207*, 911.
(6) Pascal, R. A., Jr.; Trang, N. L.; Cerami, A.; Walsh, C. *Biochemistry* **1983**, *22*, 171.
(7) Blattmann, P.; Retey, J. *Eur. J. Biochem.* **1972**, *30*, 130.
(8) Pascal, R. A.; Walsh, C. T. *Biochemistry* **1984**, *23*, 2745.

0002-7863/85/1507-0486$01.50/0

with 3-(trimethylsilyl)propionic acid, sodium salt, or tetramethylsilane as an internal reference. Chemical shift values (δ) are given in ppm.

(*S*)-Dihydroorotic acid, DCIP, phospholipase C, Triton X-100, ubiquinone-30, deuterium oxide (99.8 atom % ^{2}H), and ethanol-*d* (99.5 atom % ^{2}H) were purchased from Sigma Chemical Co. Distilled, deionized water was used throughout. All other chemicals were reagent grade or better and were purchased from commercial sources.

Enzyme Purification. Dihydroorotate dehydrogenase was partially purified from beef liver mitochondria, isolated as described by Kearney and co-workers.[9] The mitochondria were disrupted, and the membrane fragments were subjected to digestion with phospholipase C followed by extraction with Triton X-100, according to the procedures developed by Salach[10] for purification of monoamine oxidase B. The Triton-solubilized enzyme was then loaded onto a DE-52 column (540 mL, 4 × 43 cm), and the enzyme was eluted with a KCl gradient (30–100 mM) in 20 mM Tris-HCl buffer, pH 9.0, containing 0.1% Triton X-100.[2] Active fractions from the DE-52 column were pooled and concentrated (15–20-fold) by ultrafiltration, using an Amicon filtration unit fitted with an XM-50 membrane. These procedures gave enzyme preparations with a specific activity of 290 units/mg,[11] representing a 30-fold purification over the activity assayed in disrupted mitochondria. Enzyme solutions of specific activity around 300 units/mg were routinely used, without further purification, for the NMR studies described here.

Enzyme Assay. Dihydroorotate dehydrogenase activity was assayed at 37 °C, using DCIP as an electron acceptor, by monitoring the loss of DCIP absorbance at 600 nm. It has been shown that the rate of DCIP reduction in the assay is equal to the rate of enzymatic oxidation of (*S*)-dihydroorotate and that orotate and reduced DCIP form stoichiometrically.[2b] A typical 1.0-mL assay contained 0.50 mM (*S*)-dihydroorotate, 68 μM ubiquinone-30, 50 μM DCIP, 0.1% Triton X-100, and 4.9 units of enzyme in 100 mM KP_i buffer, pH 7.8. Reaction was initiated by addition of substrate to a thermally equilibrated assay solution.

Quantitative Determination of (*S*)-Dihydroorotate. The enzyme assay described above was adapted for the quantitative determination of unknown concentrations of (*S*)-dihydroorotate. A 10-mL stock solution was prepared containing DCIP, sodium salt (0.29 mg, 1.01 μmol), ubiquinone-30 (0.40 mg, 0.68 μmol), Triton X-100 (10 mg), and 1000 units of dihydroorotate dehydrogenase, all in 100 mM KP_i buffer, pH 7.8, 20 °C. Measured aliquots (1.0 mL) of the stock solution were placed in a quartz cuvette and blanked at 600 nm (for DCIP, $\epsilon = 1.6 \times 10^4$ cm^{-1} M^{-1}). (*S*)-Dihydroorotate (1–25 nmol), in volumes from 1 to 10 μL of 100 mM KP_i buffer, pH 7.8, was then added to the cuvette, and the net absorbance decrease was measured. In this way, a standard curve of ΔA_{600} vs. [dihydroorotate] could be constructed for use in analysis of unknowns. We found that a minimum 5-fold molar excess of DCIP over dihydroorotate was required to give reproducible linearity of the standard curves.

Kinetics of Nonenzymatic Hydrogen Exchange. Nonenzymatic C_5-hydrogen exchange reactions of (*S*)-dihydroorotate, methyl (*S*)-dihydroorotate, and 6-methyl-(*R,S*)-dihydrouracil were followed by ^{1}H NMR spectrometry, by monitoring the integrated signal intensities of the C_5 and C_6 protons. A typical NMR experiment was conducted in a 10 mM solution of the specific dihydroorotate or dihydrouracil in 100 mM NaP_i deuterium oxide buffer, pD 7.8. Pseudo-first-order rate constants for C_5-hydrogen loss were obtained from semilog plots of integrated C_5-signal intensity vs. time.

Analysis of the Stereochemical Course of the Enzymatic Reaction. A 1.0-mL solution containing deuterium-enriched dihydroorotate (5.6 mM **3** or **5**), 20 mM DCIP, 68 μM ubiquinone-30, and 0.1% Triton X-100 in 100 mM NaP_i deuterium oxide buffer, pD 7.8, was placed in a 5-mm NMR tube. Immediately prior to obtaining the ^{1}H NMR spectrum, 0.8 mL of dihydroorotate dehydrogenase (360 units), which had been dialyzed previously against 100 mM NaP_i D_2O buffer, pD 7.8, containing 0.1% Triton X-100, was added to the tube. Spectra (500 MHz) were obtained at timed intervals; the C_5- and C_6-proton resonances of substrate dihydroorotate, and the C_5-vinyl signal of product orotate, were integrated against trimethylsilyl propionate (0.53 mM). The probe temperature of the spectrometer was 36 °C.

Preparation of Deuterium-Labeled Compounds. [5-^{2}H]Orotic Acid. Orotic acid (**2**, 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 stirrer, was heated for 12 days at 120 °C in an oil bath. After the solution had cooled to room temperature, the resulting suspension was transferred to a suitable vessel and was frozen and lyophilized. The residue was redissolved in a minimal volume (~100 mL) of hot water, cooled to 0 °C, and the solid precipitate was collected by vacuum filtration. The resulting filtrate was, in turn, evaporated in vacuo (to about 25 mL) and cooled to 0 °C, which afforded additional solid material. The solids were combined, dissolved in a minimum volume of hot water, decolorized with Norit A, and hot filtered. After cooling the filtrate to 0 °C, an off-white, crystalline product was obtained, filtered, and dried in vacuo over P_2O_5. Yield, 0.35 g (35%); mp 347–348 °C dec; mass spectrum, *m/z* 157 (M^+); MS analysis reveals 94.6% ^{2}H incorporation; ^{1}H NMR (Me_2SO-d_6) δ 11.22 (s, 1 H, NH), 10.72 (s, 1 H, NH), 5.99 (s, 0.03 H, C_5 vinyl); NMR analysis reveals 97% ^{2}H incorporated at C_5.

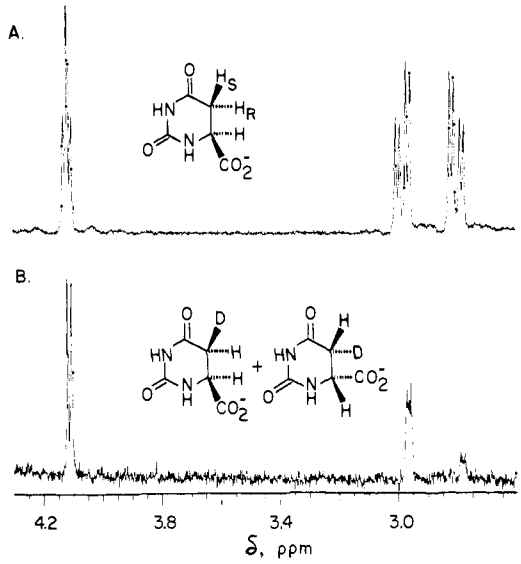


Figure 1. ^{1}H NMR spectra (500 MHz) (pD 7.8, 100 mM NaP_i, D_2O) of (A) authentic (*S*)-dihydroorotate (**1**), and (B) the reaction product obtained from catalytic reduction of [5-^{2}H]orotic acid.

A control sample of orotic acid was similarly incubated in 150 mL of D_2O at room temperature and recrystallized from hot water. This sample, together with authentic **2**, gave a mixed mp 347–348 °C dec; mass spectrum, *m/z* 156 (M^+); ^{1}H NMR (Me_2SO-d_6) δ 11.22 (s, 1 H, NH), 10.72 (s, 1 H, NH), and 5.99 (s, 1 H, C_5 vinyl).

Catalytic Reduction of [5-^{2}H]Orotic Acid. [5-^{2}H]Orotic acid (11.3 mg, 72 μmol, 95% ^{2}H at C_5, vide supra) was dissolved in ethanol, and PtO_2 (18.4 mg, 81 μmol) was added. The suspension was pressurized to 1500 psi of H_2 and heated to 70 °C for 21.5 h. The hot suspension was then filtered and the filtrate evaporated to dryness in vacuo. The residue was dissolved in 2 mL of hot D_2O, filtered, frozen, and lyophilized to yield a white powder. Thin-layer chromatography (silica gel; butanol/acetic acid/water, 4:1:1) revealed dihydroorotate (R_f 0.63) and traces of orotic acid (R_f 0.36). The ^{1}H NMR spectrum of the product mixture in buffered D_2O (pD 7.8, 100 mM NaP_i) gave resonances in the region of authentic dihydroorotate (2.8–4.2 ppm, vide infra, Figure 1): δ 4.11 (d, J = 6.77 Hz, 1.0 H, C_6 H), 2.96 (distorted d, J = 6.76 Hz, 0.8 H, C_5 H), and 2.80 (distorted d, 0.2 H, C_5 H).[12]

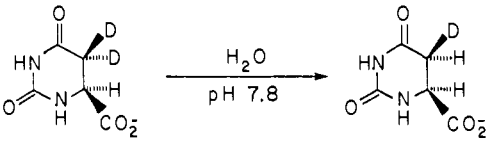
(5*R*,6*S*)-[5-^{2}H]Dihydroorotate (3). (*S*)-Dihydroorotic acid (100 mg, 0.63 mmol), dissolved in 10 mL of 0.5 M NaP_i buffer, pH 7.8, was frozen and lyophilized. The residue was dissolved in 10 mL of D_2O, and the lyophilization was repeated. The residue was dissolved in 50 mL of D_2O, and the solution was maintained at 37 °C, pD 7.8, for 264 h, after which time the ^{1}H NMR spectrum showed δ 2.80 (d, 0.83 H, 5-*pro*-*S* H),[13] 2.98 (dd, 0.19 H, 5-*pro*-*R* H),[13] and 4.11 (d, 1 H, C_6 H). Thus, NMR integration shows that [5-^{2}H]dihydroorotate prepared in this fashion was approximately 81% deuterium enriched at the *pro*-*R* position with attendant 17% deuterium incorporation into the *pro*-*S* position. The solution was lyophilized to dryness and used without further purification for the enzymatic studies described above.

(*S*)-[5,5-^{2}H$_2$]Dihydroorotate (4). (*S*)-Dihydroorotic acid (204 mg, 1.3 mmol) was dissolved in 25 mL of D_2O. The solution was then frozen, lyophilized, and dried in vacuo overnight. The residue was slurried in 100 g of ethanol-*d*, and sodium metal (3.5 mmol) was added at room temperature. This suspension was refluxed for 8 h. After cooling to

(9) Kearney, E. B.; Salach, J. I.; Walker, W. H.; Seng, R. L.; Kenney, W.; Zeszotek, E.; Singer, T. P. *Eur. J. Biochem.* **1971**, *24*, 321.
(10) Salach, J. I. *Arch. Biochem. Biophys.* **1979**, *192*, 128.
(11) One unit of activity corresponds to 1 nmol of (*S*)-dihydroorotate oxidized per minute, under standard assay conditions (vide supra).
(12) Cis reduction of [5-^{2}H]orotate will give both (5*S*,6*S*)-[5-^{2}H]dihydroorotate (**5**) and its enantiomer (5*R*,6*R*)-[5-^{2}H]dihydroorotate.
(13) The assignment of the two C_5-methylene resonances is discussed under Results.

ambient temperature, the solution was neutralized by the addition of aqueous HCl (3.5 mmol) and evaporated to dryness in vacuo. The residue was washed by slurrying in 3 mL of D_2O; the resulting white solid was collected by filtration at 0 °C and dried in vacuo. Yield, 145 mg (91%); mp 256–258 °C dec; TLC (silica gel; butanol/acetic acid/water, 4:1:1) gave a single spot, R_f 0.63, which comigrates with authentic (*S*)-dihydroorotic acid; ^1H NMR (D_2O) δ 2.81, 3.05 (dd, 0.15 H each, C_5 H_2), 4.37 (s, 1 H, C_6 H). Integration of the methylene resonances reveals 85% deuterium enrichment at C_5 with no detectable incorporation of deuterium into the C_6 position.

(5*S*,6*S*)-[5-^{2}H]Dihydroorotate (5). The (5*S*,6*S*)-5-deuteriodihydroorotate (**5**) was prepared as outlined above for **3**, except that the exchange was carried out by using (*S*)-[5,5-$^2H_2$]dihydroorotate (**4**) dissolved in 50 mL of NaP_i-buffered H_2O (0.1 M, pH 7.8). The reaction (eq 2) was monitored by the periodic removal of aliquots. These were,



(2)

4 5

in turn, lyophilized, redissolved in D_2O, and analyzed by ^1H NMR spectrometry. After 400 h of exchange at 37 °C, the ^1H NMR spectrum (pD 7.8, 100 mM NaP_i) showed δ 2.81 (dd, 0.37 H, 5-pro-*S* H),[13] 2.98 (d, 0.67 H, 5-*pro*-*R* H),[13] and 4.11 (d, 1 H, C_6 H). Thus, NMR integration shows that [5-^{2}H]dihydroorotate prepared in this fashion was approximately 63% deuterium enriched at the *pro*-*S* position with attendant 33% deuterium incorporation at the pro-*R* position. The product solution was lyophilized to dryness and was used without further purification for the enzymatic experiments described above.

It should be noted that the preparation of the stereoselectively deuterium-enriched compounds **3** and **5** occurs without racemization at C_6. This was shown by enzymatic assay of solutions containing weighed amounts of dihydroorotates, according to the procedures outlined above. These solutions gave stoichiometric reduction of DCIP upon incubation with the beef liver dehydrogenase. The enzyme is absolutely specific for (*S*)-dihydroorotate; correspondingly, C_6 racemates give only 50% reduction of DCIP.

Results

Verification of the Assignment of the C_5-Methylene Hydrogens of Dihydroorotate (1). The 500-MHz ^1H NMR spectrum of authentic dihydroorotate is shown in Figure 1A. As is expected for a pair of diastereotopic protons, the two C_5-methylene hydrogens give rise to sets of resonances with distinct chemical shift values, each appearing as a doublet of doublets, centered around 2.80 and 2.98 ppm; the vicinal couplings ($J_{5,6}$) are 6.40 and 6.66 Hz, respectively, at 24 °C. The C_6 hydrogen (δ 4.11) appears as a triplet in our spectra of the free acid (**1**), and of the sodium salt, in D_2O at ambient temperature. However, as expected for nonequivalent $C_{5S,6}$ and $C_{5R,6}$ couplings, C_6 resonances are resolved (doublet of doublets [J = 4.65 and 7.10 Hz]) in spectra of **1** in methanol-*d* (27 °C, data not shown). A similarly large coupling constant difference is observed for the C_6-hydrogen signal of methyl dihydroorotate (**6**) in D_2O. We find that the magnitudes of the 5,6-coupling constants—and, consequently, the ability to resolve the C_6 splitting—of dihydroorotates are both solvent and temperature dependent (vide infra).

Blattmann and Retey[7] assigned the upfield resonance (2.85 ppm) of *S*-dihydroorotate (**1**) to the 5-*pro*-*S* hydrogen and the downfield signal (3.05 ppm) to the 5-*pro*-*R* hydrogen. This correlation involved the synthesis of a chirally deuterated dihydroorotate from authentic *N*-[(benzyloxy)carbonyl]-(2*S*,3*R*)-[3-^{2}H]asparagine. However, sodium ethoxide catalyzed ring closure, to afford the desired, chirally deuterated heterocycle, was observed to proceed with loss of deuterium. The product obtained was only 39% ^2H enriched at C_5.

We chose to attempt to verify the Retey assignment employing a strategy that avoids deuterium exchange. This involved the PtO_2-catalyzed hydrogenation of [5-^{2}H]orotic acid (95% C_5-deuterium enrichment) to give a chirally deuterated dihydroorotic acid.[12] The reduction gave dihydroorotic acid as the major product (as evidenced by TLC, vide supra); the 500-MHz ^1H NMR spectrum of the product mixture in buffered D_2O (pD 7.8, 100 mM NaP_i, Figure 1B) showed two major resonances, each a doublet (J = 6.76 Hz) at 4.11 and 2.98 (distorted) ppm and integrating to 1.0 and 0.8 proton, respectively. The high-field resonance seen for diprotiodihydroorotate is nearly absent in the spectrum of Figure 1B and integrates to only 0.2 proton. These observations are consistent with the expected cis reduction (predominantly) of [5-^{2}H]orotic acid. Thus, the upfield resonance is allocated to the 5-*pro*-*S* hydrogen of (*S*)-dihydroorotate, and the downfield signal belongs to the 5-*pro*-*R* hydrogen. This assignment is in agreement with that made previously by Blattman and Retey.[7,14]

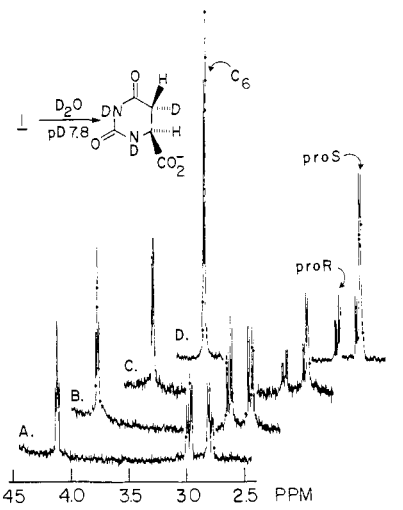
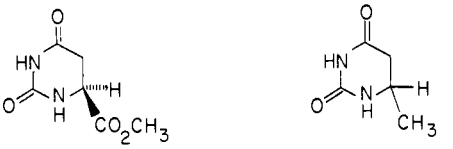


Figure 2. ^1H NMR spectra (500 MHz) of (*S*)-dihydroorotate (**1**) in 100 mM NaP_i buffer, pD 7.8, 37 °C, at (A) 0, (B) 34, (C) 172, and (D) 288 h.

Nonenzymatic C_5-Hydrogen Exchange of Dihydroorotate. We have found that three dihydrouracils [(*S*)-dihydroorotate (**1**), methyl (*S*)-dihydroorotate (**6**), and 6-methyl-(*R*,*S*)-dihydrouracil (**7**)] undergo a stereospecific exchange of the C_5-hydrogens with



6 7

solvent deuterons in NaP_i–deuterium oxide buffers. The exchange reaction is easily followed by ^1H NMR spectrometry; Figure 2 shows the typical spectral changes observed during the course of incubation of **1** in 100 mM NaP_i buffer, pD 7.8, at 37 °C. Figure 2 clearly shows that, as the exchange of C_5-hydrogens proceeds, the downfield (2.98 ppm) signal, which corresponds to the 5-*pro*-*R* hydrogen resonance (vide supra), collapses. After 288 h (Figure 2D), the integrated intensity of the 2.98 ppm signal is only 29% of those resonances at 2.80 (5-*pro*-*S* H) and 4.11 ppm (C_6 H), each of which integrates to one proton. Note further, from spectrum D of Figure 2, that the 2.80 ppm signal appears as a strong doublet—as would be expected for the C_5-hydrogen resonance of 100% *pro*-*R* deuterium-enriched **3**—which overlays an apparent doublet of doublets, representing the approximately 30% unexchanged **1** remaining. Further, the C_6-hydrogen resonance of Figure 2D is a doublet, consistent with monodeuteration of **1** at C_5. These observations demonstrate that *S*-dihydroorotate undergoes C_5 exchange, with stereoselective deuterium incorporation into the 5-*pro*-*R* position.

Solvent deuterons are seen to exchange with the C_5 hydrogens of **6** and **7** as well, but with stereoselectivities that are lower than

(14) Pascal and Walsh[8] have found that 5% rhodium on alumina similarly affords about 90% cis reduction of orotate in D_2O. This result, together with the data of Figure 1B, leaves little doubt that the original correlation of C_5 chemical shift values with C_5 stereochemistry is correct.

Table I. Exchange of C_5 Hydrogens of Dihydrouracils with Solvent Deuterium[a]

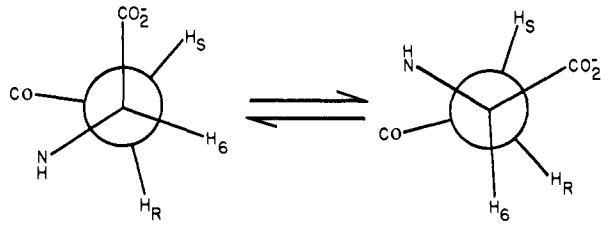
	k_{obsd}, $h^{-1} \times 10^3$ [b]								
	(*S*)-dihydroorotate (**1**)			methyl (*S*)-dihydroorotate (**6**)			6-methyldihydrouracil (**7**)		
T, °C	*pro-R*	*pro-S*	*R/S*	*pro-R*	*pro-S*	*R/S*	2.7 ppm	2.5 ppm	2.7/2.5
37	5.40	0.48	11.3	9.35	2.99	3.13	2.25	1.12	2.0
54	40.0	7.75	5.2	*c*	*c*	*c*	*c*	*c*	*c*
73	338.0	57.6	5.9	*c*	*c*	*c*	*c*	*c*	*c*
95	1940	420.0	4.6	*c*	*c*	*c*	1050	664.0	1.6

[a] Conditions: D_2O solvent, pD 7.8, 100 mM NaP_i. Initial concentration of dihydrouracils was 12 mM (30 mM at 54, 73, 95 °C). [b] Rate constants were obtained from semilog plots of integrated ^1H intensity vs. time (see Experimental Section). [c] Value not determined.

those for exchange of the C_5 protons of **1**. Table I shows, for example, that the pseudo-first-order rate constant for *pro-R* loss on **1** is roughly 10-fold greater than that of *pro-S* removal, at 37 °C. However, deuterium incorporation into the C_5 positions of dihydroorotate methyl ester (**6**) and of 6-methyldihydrouracil (**7**) is only modestly stereopreferential; at 37 °C, the rates for *pro-R*-exchange are only 2–3-fold greater than those for *pro-S* exchange. The data of Table I show also that the stereoselectivity of the proton-exchange reactions is temperature dependent. The rate differences between *pro-R* and *pro-S* loss are smaller at higher temperatures, at least for **1** and **7**.

Arrhenius plots (not shown), constructed from the rate data of Table I, for the two C_5-exchange reactions of **1** are linear. The activation energies, calculated from the Arrhenius plots, for 5-*pro-R* and 5-*pro-S* hydrogen abstraction are indistinguishable within experimental error ($E^{\ddagger}_{pro\text{-}R}$ = 22.5 kcal/mol; $E^{\ddagger}_{pro\text{-}S}$ = 22.8 kcal/mol).

Conformational Analysis. Dihydroorotates, and other 6-substituted dihydrouracils, can assume one of two limiting conformations; the C_6 carboxylate may be axial (**8**) or equatorial (**9**).[15]



8 **9**

Katritzky and co-workers have argued that **8** is the equilibrium-favored conformer of dihydroorotic acid, based on $C_{5,6}$ coupling constant differences for **1**, in unbuffered D_2O at ambient temperature.[16] The larger observed value, J = 6.9 Hz, was assigned to $C_{5R,6}$(ae) coupling in **8**; the smaller, J = 5.1 Hz, was attributed to $C_{5S,6}$(ee) coupling. Apparently these workers concluded that a value approaching 11–12 Hz, arising from trans-diaxial coupling for $C_{5S,6}$, would have been seen if **9** were the major solution conformer.

It occurred to us that the stereoselectivity of the C_5-hydrogen-exchange reaction might result from stereoelectronic control of exchange from differing equilibrium populations of the two dihydroorotate conformers, **8** and **9**. However, it was not clear from our data that either of the two structures predominates in aqueous solution since, unlike the Katritzky report, we observed very small coupling constant differences for **1** in buffered D_2O at pD 7.8 (vide supra).[17] Thus, we conducted a number of experiments designed to assess the thermodynamics of the process **8** ⇌ **9**.

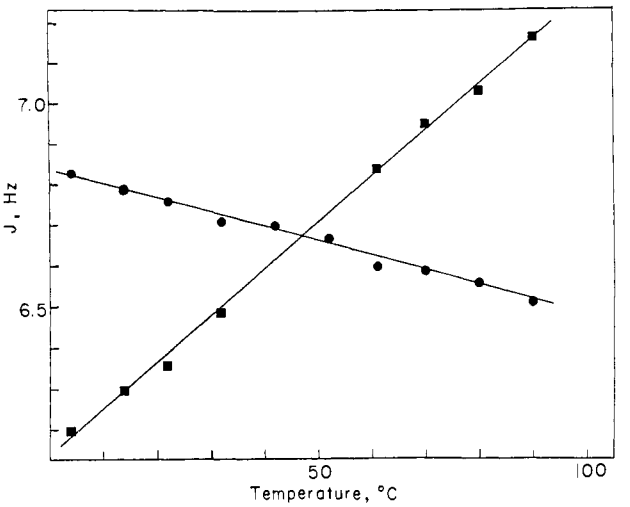


Figure 3. Temperature dependence of $J_{5S,6}$ (■) and $J_{5R,6}$ (●) for (*S*)-dihydroorotate (**1**) in 100 mM NaP_i buffer, pD 7.8, D_2O. Data obtained at 270 MHz.

Figure 3 shows the temperature dependence of the $C_{5,6}$ coupling constants of dihydroorotate (**1**) in buffered D_2O. It is of particular interest to note the "reciprocal" relationship of the observed J values with temperature: above about 46 °C, $J_{5S,6}$ is the larger constant; below 46 °C, $J_{5R,6}$ is greater. This finding could reflect a temperature-dependent shift in the equilibrium position for **8** ⇌ **9** (vide infra).[18]

The obvious linearity of the plots of Figure 3 demonstrates that **1** exists as an equilibrating mixture of conformers between 4 and 90 °C in D_2O buffer. Over the range of temperatures (37–95 °C) at which we observe stereoselective H_R exchange for **1**, the differences between $J_{5S,6}$ and $J_{5R,6}$ are very small (about 0.2–0.7 Hz); thus, it is not obvious from the data of Figure 3 alone that either **8** or **9** is a major equilibrium contributor at any temperature. However, these data can be used to calculate the equilibrium constants for **8** ⇌ **9**. This requires the assumption, as has been made by Katritzky et al.,[16] that $J_{ee} \sim 2.0$ Hz for **8** and $J_{aa} \sim 11.5$ Hz for **9**. Then $J_{5S,6}$ can be used to calculate each equilibrium constant for **8** ⇌ **9**, according to the following relationship:

$$J_{5S,6} = f_8 J_8 + f_9 J_9 \qquad (3)$$

where f_8 and f_9 are the fractions of **8** and **9**, respectively, at equilibrium ($f_8 + f_9 = 1$); and J_8 and J_9 are the theoretical $C_{5S,6}$ coupling constants for **8** (J_{ee} = 2 Hz) and **9** (J_{aa} = 11.5 Hz), respectively. Rearrangement of eq 3 gives eq 4; and the equilibrium constant (K_{eq}), at any single temperature, is obtained from eq 5.

$$f_8 = \frac{J_{5S,6} - J_9}{J_8 - J_9} = \frac{J_{5S,6} - 11.5}{-9.5} \qquad (4)$$

$$K_{eq} = \frac{1 - f_8}{f_8} \qquad (5)$$

(15) These relationships are actually quasi-axial and quasi-equatorial, with reference to, for example, cyclohexanyl systems. The dihydrouracil heterocycles are all variously distorted (half-chair) planar structures, wherein the C_5 and C_6 atoms are out of plane on opposite sides of the ring.

(16) Katritzky, A. R.; Nesbit, M. R.; Kurtev, B. J.; Lyapova, M.; Pojarlieff, I. G. *Tetrahedron* **1969**, *25*, 3807.

(17) Our studies were done at a single pH; we have not yet investigated the effects of pH, or of ionic strength, on the magnitudes of the coupling constants.

(18) Indeed, the authors of ref 16 might have concluded that **9** was the preferred solution conformation had they determined the $C_{5,6}$ coupling constants at temperatures above 46 °C.

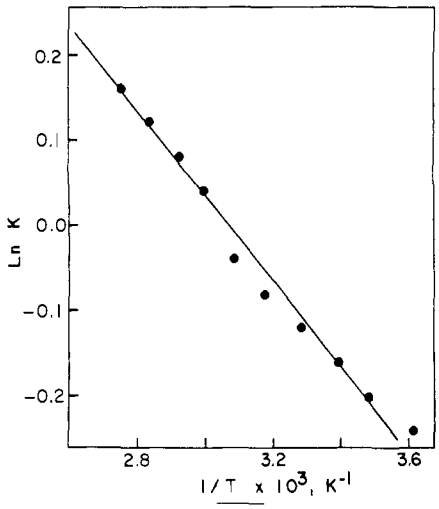


Figure 4. Temperature dependence of the equilibrium constants (K_{eq}) for the conformational rearrangement **8** ⇌ **9** in 100 mM NaP_i buffer, pD 7.8, D_2O.

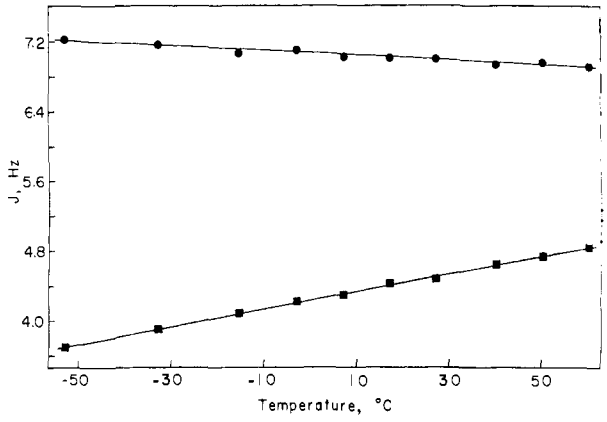


Figure 5. Temperature dependence of $J_{5S,6}$ (■) and $J_{5R,6}$ (●) for (*S*)-dihydroorotic acid (**1**) in CD_3OD. Data obtained at 270 MHz.

Figure 4 confirms the expected linear relationship between temperature and the calculated values for ln K_{eq} for the process **8** ⇌ **9**, between 4 and 90 °C. It is worth noting that the values for K_{eq} do not substantially deviate from unity over a rather wide range of temperatures; at 4 °C, K_{eq} = 0.79 (f_8 = 0.56), while at 90 °C, K_{eq} = 1.17 (f_8 = 0.46). Insofar as the estimates for J_8 and J_9 used in these calculations are accurate, the data indicate that **1** (in D_2O at pH 7.8, 4–90 °C) is a roughly 1:1 equilibrating mixture of conformers **8** and **9**. The ΔH value (calculated using the equilibrium constants of Figure 4) for the conformational rearrangement (**8** ⇌ **9**) is 0.92 kcal/mol. Thus, it appears that the energy difference between **8** and **9** is small and that the two conformers readily interconvert.

Figure 5 shows that the $C_{5S,6}$- and $C_{5R,6}$-coupling constants for **1** in deuterated methanol are also linearly dependent upon temperature over a wide (120 °C) range. Comparison of Figures 3 and 5 reveals that there are substantial solvent effects on the observed *J* values. At ambient temperature, for example, the difference between $C_{5S,6}$ and $C_{5R,6}$ coupling in pH 7.8 buffer is only 0.4 Hz, while this difference in methanol is 2.50 Hz. It is clear from these observations that the equilibrium position of **8** ⇌ **9** is affected by solvent. Indeed, when the data of Figure 5 are used to calculate the individual equilibrium constants, we find that K_{eq} ranges from 0.43 (f_8 = 0.70) at 60 °C to 0.22 (f_8 = 0.82) at −60 °C. The solvent effect is expressed only as a change in the equilibrium position **8** ⇌ **9**; as expected, the calculated ΔH (0.89 kcal/mol) in methanol is essentially identical with that in water (0.92 kcal/mol). Thus, dihydroorotate in methanol appears also to be a rapidly equilibrating mixture of conformers; but structure **8** is favored at equilibrium over a wide range of temperatures.

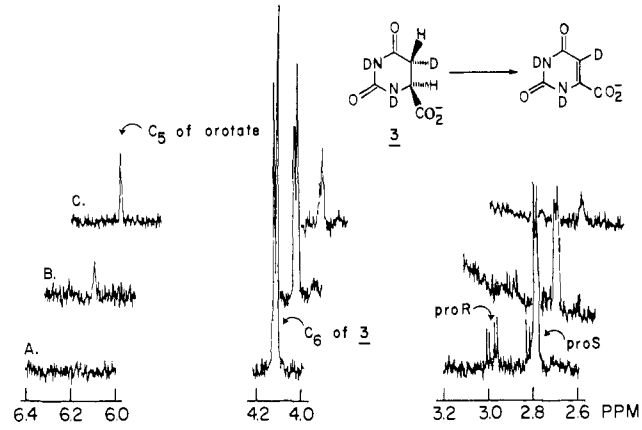


Figure 6. ^1H NMR spectra (500 MHz) of the progress of the enzymatic oxidation of (5*R*,6*S*)-[5-^2H]dihydroorotate (**3**) at (A) 0, (B) 3.0, and (C) 14.8 h. Experimental conditions are described in the text.

Stereochemistry of the Enzymatic Oxidation of Dihydroorotate (1). The ^1H NMR spectral changes occurring during the oxidation of the deuterium-enriched dihydroorotate **3** by beef liver mitochondrial dihydroorotate dehydrogenase are presented in Figure 6. The spectrum recorded prior to the addition of the enzyme (Figure 6A) has several features of note. There is a doublet at 2.8 ppm which integrates to 0.83 proton, given by the C_5 resonance of **3**. This doublet appears to overlay a doublet of doublets, indicative of the *pro-S* hydrogen of the small amount of unexchanged diprotiodihydroorotate. Correspondingly, the typical 5-*pro-R* doublet of doublets appears, centered at about 3.0 ppm, but integrates to only 0.20 proton. The C_6-hydrogen signal at 4.1 ppm integrates to 1.0 proton. These spectral features are consistent with a dihydroorotate sample (**3**) which is approximately 80% ^2H enriched at the 5-*pro-R* position and 17% ^2H enriched at the 5-*pro-S* site (as described in the experimental section, above). The resonances for Triton X-100 (which appear as a broad multiplet between 3.5 and 3.7 ppm) and those for DCIP (which occur in the aromatic region below 7.0 ppm) have been omitted from Figure 6.

The progress of the enzymatic reaction was readily monitored by integration of the C_6-methine dihydroorotate and C_5-vinyl orotate resonances (4.1 and 6.2 ppm, respectively; note especially Figure 6B,C). After nearly 15 h of incubation (Figure 6C), the reaction was observed to be 77% complete by integration of the C_6-methine signal.[19] Figure 7A plots the integrated intensity of the orotate vinyl resonance vs. percent conversion during the enzymic oxidation of **3**. The solid upper and lower lines in the figure indicate the results predicted for 5-*pro-S*- and 5-*pro-R*-proton (or deuteron) retention, respectively, at the C_5-vinyl position of the product orotate. The figure clearly shows that the integrated intensity of the developing orotate vinyl signal is that expected for enzymatic removal of the 5-*pro-S* proton (or deuteron) of **3**, with the 5-*pro-R* proton remaining in the orotate formed.

This experiment was repeated using **5**, which was deuterium enriched with stereochemistry opposite that of **3**; specifically, at the start of the enzymatic reaction, the C_5-*pro-R* signal integrated to 0.67 proton while the C_5-*pro-S* signal integrated to 0.39 proton. Figure 7B shows the expected and observed results during the enzymatic oxidation of **5**. Like the result obtained with **3**, the oxidation of **5** proceeded giving integrated orotate vinyl intensities corresponding to enzymatic removal of a 5-*pro-S* proton; the 5-*pro-R* proton remained in the product orotate.

Enzymatic oxidation of (*S*)-dihydroorotate (**1**) was also monitored by ^1H NMR, as a control against the reactions using **3** and **5** as substrate. In this experiment it was observed that the rates

(19) At this point, an aliquot of the reaction solution was removed and assayed for remaining dihydroorotate using the enzymatic DCIP reduction method described under Experimental Section. This result gave a value for remaining dihydroorotate of 13%. Thus, the percent reaction after about 15 h determined by the spectrophotometric technique (87%) agrees fairly well with that obtained by C_6-proton integration (77%).

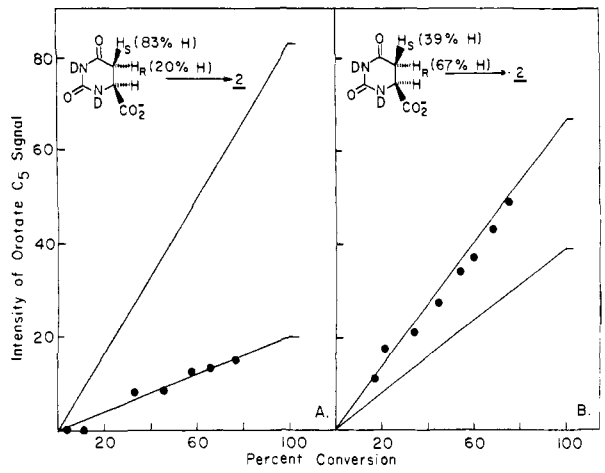


Figure 7. Predicted (solid lines) and observed (●) integrated orotate vinyl signal intensities during enzymatic oxidation of (A) deuterium-enriched (5*R*,6*S*)-[5-^{2}H]dihydroorotate (**3**) and (B) deuterium-enriched (5*S*,6*S*)-[5-^{2}H]dihydroorotate (**5**). See details in text.

of disappearance of the 5-*pro-R*, 5-*pro-S*, and 6 hydrogens, and the rate of appearance of the 5-vinyl orotate proton, were equal to one another, within experimental error.

Discussion

Stereoselectivity of the C_5-Hydrogen-Exchange Reaction. The stereoselectivity that is observed in the C_5-hydrogen exchange reactions of the dihydrouracils **1**, **6**, and **7** (Table I) is somewhat reminiscent of the stereopreferential abstraction of an axial α-proton seen for enolization of conformationally locked cyclohexanones.[20] It is generally agreed that the stereochemical course of these reactions is directed by stereoelectronic effects.

A similar explanation for the stereoselectivity of 5-*pro-R* hydrogen exchange in dihydrouracils is possible. The p-orbital of the *quasi*-axial C_5–H_R bond of **8** is nearly parallel to the p-orbitals of the C_4-carbonyl π-system. Thus, the incipient 5-*pro-R* carbanion will experience stabilization by orbital overlap. The corresponding alignments for the equatorial bonds of **8** and **9** are, by contrast, orthogonal to the carbonyl. The axial C_5–H_S bond of **9** is, of course, equivalent to C_5–H_R of **8**. But, since preferential 5-*pro-R* exchange is observed, the stereoelectronic argument implies that **9** is not the favored conformer for reaction. Accordingly, the previous literature[16] suggests that **8** is the preferred conformer of dihydroorotates. And similarly, 6-hydroxy-substituted dihydrouracils are believed to exist predominantly in the conformation that places the hydroxyl group in an axial position.[16,21] 6-Hydroxy-5,6-dihydrocytidine-5-phosphate, for example, gives a very small 5,6-vicinal coupling constant of 1.9 Hz in D_2O,[22] consistent with (vide infra) diequatorial coupling in a "pure", substituent-axial conformer, such as **8**. The 6-hydroxydihydrocytosines undergo stereoselective 5-*pro-R* hydrogen exchange,[22] consonant with the stereoelectronic principles discussed here. Given these apparent correlations between stereoselectivity and molecular conformations, we undertook to assess more thoroughly the equilibrium process **8** ⇌ **9**.

Conformational Analysis. Molecular models suggest that the $C_{5S,6}$ coupling constant for **9** should be large ($J_{5S,6}$ = 11–12 Hz), approximating that for diaxial coupling in cyclohexane ring systems. This prediction is underscored by the observation of $J_{5,6}$ = 11.1 Hz for a trans-fused dihydrouracil (*trans*-5,6-tetramethylenedihydrouracil), where H_5 and H_6 are clearly trans-axial to one another.[16] Correspondingly, the $C_{5S,6}$ coupling constant for **8** should be small (≃2–3 Hz), approaching that for diequatorial coupling. We have used these theoretical values (11.5 and 2.0 Hz), together with the data of Figures 3 and 5, to determine fractional solution populations of **8** and **9** as well as the equilibrium constants at various temperatures for the conformational rearrangement **8** ⇌ **9**.

Analysis of the data for **1** in methanol shows that **8** is, indeed, the preferred solution conformation (f_8 = 0.7–0.8) over a nearly 100-deg temperature range. On the other hand, dihydroorotate in buffered D_2O (100 mM NaP_i, pD 7.8) appears to exist as a rapidly equilibrating, nearly equimolar mixture of both **8** and **9**, at those temperatures (37–95 °C) where stereoselective 5-*pro-R* exchange is observed. At 37 °C, where the rate difference between H_R and H_S removal is greatest (R/S = 11.3, Table I), the estimated f_8 is 0.52 and the K_{eq} is 0.9. Clearly, the observed stereoselectivity of the exchange reaction cannot be attributed to any factor that requires a predominance of conformer **8** in buffered deuterium oxide solutions.

This finding is not necessarily incompatible with a stereoelectronic effect that gives rise to H_R stereoselectivity, even in a solution mixture of **8** and **9**. But this result would obtain only if the exchange reaction proceeds through a transition state that resembles **8** and is of lower energy than that arising from **9**; H_S abstraction from **9** appears to be stereoelectronically equivalent to H_R removal from **8**. It is not obvious that an "**8**-like" transition state should be of appreciably lower energy than one that is "**9**-like" when the ground-state energy difference between **8** and **9** is so small (ΔH = 0.92 kcal/mol for **8** ⇌ **9**).

It is also possible to rationalize the enhanced H_R exchange on (*S*)-dihydroorotate as arising from neighboring-group electrostatic repulsions. Table I shows that the C_5-exchange reaction is considerably more stereoselective for **1** than for both the dihydroorotate methyl ester (**6**) and 6-methyldihydrouracil (**7**). These reactions must all involve development of a C_5-carbanion equivalent. Exchange on the carboxylate salt **1**, however, is unique among the three by requiring the formation of two formal negative charges in close proximity (the C_6 carboxylate and the C_5 carbanion). It may be that neighboring electrostatic repulsions would be minimized if the 5-*pro-R* proton, as opposed to the 5-*pro-S* proton, is removed from **8**. In the limiting case of a tetrahedral carbanion, the p-orbital bearing the electron pair at C_5 and the C_6 carboxylate would exist in a trans-diaxial relationship, separated at greatest distance across the ring plane. In contrast to **8**, a Dreiding model of **9** suggests that tetrahedral 5*R*- and 5*S*-carbanions are equivalent structures, with reference to $C_{5,6}$-electrostatic interactions. Nonetheless, since conformers **8** and **9** are equally represented at equilibrium, electrostatic destabilization of a developing 5*S*-carbanion of **8** could give rise to the observed H_R-selective exchange reaction. The magnitude of this effect will clearly depend on the degree to which electron density is actually localized at C_5 in the transition state.

Table I also shows that the absolute rates of both H_R and H_S abstraction are each substantially greater for **6** than for **1**. This observation clearly underscores the hypothesis that neighboring electrostatic repulsions pose, at least, a kinetic barrier to C_5-carbanion formation at both the 5-*pro-R* and 5-*pro-S* positions. It is intriguing to consider whether the observed 2–3-fold stereoselective bias, in favor of the pseudo-axial hydrogens of **6** and **7**, represents the intrinsic stereoelectronic control of proton removal in systems where electrostatic influences are equivalent.

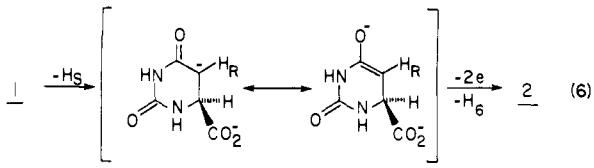
Stereochemistry of the Enzymatic Oxidation. The NMR studies reported here, using stereoselectively deuterium-enriched dihydroorotates **3** and **5**, show that beef liver dihydroorotate dehydrogenase catalyzes the anti elimination of hydrogens from substrate (Figures 6 and 7). The precise mechanism by which the enzyme accomplishes this stereoselective oxidation remains unclear, especially since we have not yet identified the redox-active cofactor(s). The facility with which dihydroorotates undergo nonenzymatic C_5-hydrogen exchange strongly suggests the intermediacy of a C_5 carbanion during enzymatic catalysis. C_6-Proton abstraction cannot be ruled out, but this seems unlikely for several reasons. First, resonance stabilization of a C_5 carbanion,

(20) Corey, E. J.; Sneen, R. A. *J. Am. Chem. Soc.* **1956**, *78*, 6269. Trimitsis, G. B.; Van Dam, E. M. *J. Chem. Soc., Chem. Commun.* **1974**, 610. Fraser, R. R.; Champagne, P. J. *Can. J. Chem.* **1976**, *54*, 3809. Fraser, R. R.; Champagne, P. J. *J. Am. Chem. Soc.* **1978**, *100*, 657. Ferran, H. E.; Roberts, R. D.; Jacob, J. N.; Spencer, T. A. *J. Chem. Soc., Chem. Commun.* **1978**, 49.

(21) Rouillier, P.; Delmau, J.; Nofre, C. *Bull. Soc. Chim. Fr.* **1966**, 3515.

(22) Liu, F.-T.; Yang, N. C. *Biochemistry* **1978**, *17*, 4877.

but not a C_6 carbanion, is possible (eq 6). Moreover, in none



(6)

of the exchange reactions studied, including exhaustive C_5 deuteration in refluxing ethoxide-*d*, do we observe C_6 exchange. Finally, preliminary experiments show that the dehydrogenase will catalyze C_5-deuterium exchange on substrate **1** when DCIP is removed from the assay system.[23] The lack of any detectable exchange when the enzyme is provided with a competent electron acceptor suggests that oxidation of an intermediate carbanion occurs rapidly during catalysis.

The data presented here (Figure 7) show unambiguously that the beef liver dihydroorotate dehydrogenase reaction proceeds with loss of only the 5-*pro-S* hydrogen from C_5 during oxidation of **1**. Thus, the stereochemical course of this reaction is identical with that recently determined by Pascal and Walsh for the dehydrogenase from *C. fasciculata*[8] as well as to that seen for the *C. oroticum* orotate reductase, characterized by Blattmann and Retey.[7] It is perhaps curious that all three of these enzymes promote stereopreferential removal of the 5-*pro-S* hydrogen, observed in the nonenzymatic exchange reactions to be less labile than the 5-*pro-R* hydrogen.

We suspect that dihydroorotate dehydrogenase binds only the conformer of **1** in which the carboxylate group is equatorial (**9**). In this case, abstraction of the axial 5-*pro-S* hydrogen becomes stereoelectronically, if not electrostatically, favored over removal of the equatorial 5-*pro-R* proton. This might appear to be energetically costly, insofar as we have tacitly suggested (vide supra) that H_S abstraction from **9** could involve a higher energy transition state than does H_R abstraction from **8**. However, any potential energy difference[24] between a transition state like **8** and one resembling **9** is likely to be insignificant when weighed against the very large intrinsic activation energies calculated for both H_S and H_R abstraction (22.5 and 22.8 kcal/mol, respectively).

There are other advantages gained by "freezing-out" **9** at the active site of the enzyme. One informative view of the dihydroorotate oxidation is provided by comparison to α,β-elimination reactions. These often proceed at maximal rates when the proton abstracted and the leaving group (C_5H^+ and C_6H:, if oxidation of **1** can be considered a cognate reaction) are antiperiplanar to one another.[25] If the enzyme were to bind **8**, a trans elimination could be accomplished only by removal of the apparently less labile 5-*pro-S* proton. But abstraction of the 5-*pro-R* proton would lead to the less favorable gauche oxidation. Moreover, 5-*pro-R* activation will necessarily place the enzymic base and the electron acceptor (perhaps a flavin, as has been suggested for the beef liver enzyme[2]) on the same surface of the substrate heterocycle, separated from one another by perhaps as small a distance as the $C_{5,6}$ bond.

In contrast to the foregoing predictions, we believe that utilization of **9** as the preferred substrate conformation could afford the following catalytic advantages. (1) Abstraction of the 5-*pro-S* proton is stereoelectronically favored and leads to an antiperiplanar oxidation. (2) A "less-hindered" active-site architecture is defined, wherein the enzymic base and the electron acceptor are placed on opposite faces of the substrate ring.

We are aware that these conclusions are highly speculative. We do not claim, for example, to be able to define the active-site structure of dihydroorotate dehydrogenase based solely on a small amount of stereochemical data and a large amount of chemical intuition. Furthermore, we are alert to the fact that the nonenzymatic hydrogen-exchange reactions are imperfect models for the enzymatic oxidation of dihydroorotate; 5-*pro-S* proton abstraction will be perhaps one of several steps in the conversion **1** → **2**.

Acknowledgment. We are indebted to Professors David Lynn and David Gorenstein for many enlightening discussions about this work. We also thank Professors Christopher Walsh and Robert Pascal for sharing with us their prepublication data on the crithidial dihydroorotate dehydrogenase. Victoria Hines Squire, of our laboratory, generously provided samples of the beef liver enzyme used in several experiments. This work was supported by PHS CA-19265 and by grants from both the NIH (CA 14599) and from the NSF (CHE 8206978) for the purchase of NMR equipment.

Registry No. **1**, 5988-19-2; **2**, 65-86-1; **3**, 39681-14-6; **4**, 93527-45-8; **5**, 93527-46-9; **6**, 39681-15-7; **7**, 93404-43-4; EC 1.3.3.1, 9029-03-2; [5-^{2}H]orotic acid, 90109-17-4; hydrogen, 1333-74-0.

(23) Keys, L. D., III; Johnston, M. A., unpublished results.

(24) This may be no more than about 2.0 kcal/mol at 37 °C, including the approximate 0.9 kcal/mol required for interconversion of **8** to **9**.

(25) Bartsch, R. A.; Zavada, J. *Chem. Rev.* **1980**, *80*, 453. Saunders, W. H.; Cockerill, A. F. "Mechanisms of Elimination Reactions"; Wiley: New York, 1973; pp 105–164, 205–219.