Tautomerism of Orotic Acid Dianion. Effect of Calcium and Magnesium Cations on the Tautomeric Constant and on Tautomerization Dynamics

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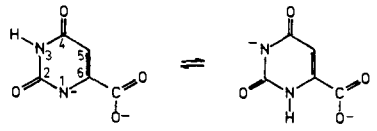
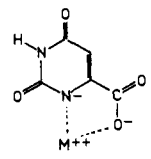
Abstract: Orotic acid dianion (HL^{2-}) exists in basic aqueous solutions as a mixture of two isomeric structures (K_T = [N(3)H]/[N(1)H] = 0.4 ± 0.08, ΔH_T = −0.3 ± 0.05 kcal, ΔS_T = −2 ± 0.4 eu). Its tautomeric interconversion as investigated by T-jump relaxation is fast (τ never exceeds 30 μs in D_2O) and is catalyzed by OD^- [k_{OD^-} = (3.3 ± 0.2) × 10^8 M^{-1} s^{-1}], by D_2O acting as an acid [k_{D_2O} = (3 ± 0.3) × 10^4 s^{-1}] and by orotate monoanion [$k_{H_2L^-}$ = (4 ± 0.7) × 10^6 M^{-1} s^{-1}]. The orientation of orotic acid methylation is discussed in terms of the change in tautomer populations caused by temperature. The tautomeric equilibrium constant is markedly affected by alkaline earth cations (Ca^{2+} and Mg^{2+}) owing to a specific complexation of the N(3)H form. Estimates of the complex formation constants, K_{comp}, are (1.7 ± 0.2) × 10^2 and (5 ± 0.5) × 10^2 M^{-1} for Ca^{2+} and Mg^{2+}, respectively. The relaxation time for the complexation process is shorter than 1 μs for Ca^{2+}, whereas it is in the millisecond range for Mg^{2+} with a second-order rate constant equal to (5.5 ± 0.6) × 10^5 M^{-1} s^{-1}.

Orotic acid (H_3L) is a key compound involved in the "de novo" biosynthesis of pyrimidines. The overall process of enzymatic attachment of a phosphoribosyl group from phosphoribosepyrophosphate (PRPP) is basically an electrophilic addition which, like methylation and protonation, ultimately requires an unsubstituted N(1) nitrogen atom. Between pH 3 and 9, orotic acid is present in aqueous solutions mainly as the orotate anion (HL^{2-}), N(3)H (structure I), in which the N(1) nitrogen is unsubstituted, is obtained by abstraction of a second proton (pK = 9.45)[1] and, according to previous observations on related uracil anion systems,[2-4] should be present together with N(1)H (tautomer structure II).



I ⇌ II

Orotic acid reportedly complexes strongly with divalent transition metal cations[5] and trivalent rare earth cations.[6] The structure of such a complex would be that of the chelated N(3)H dianion. However, this latter species might be present together with the N(1)H species in a ratio that is both pH dependent and a function of complex formation constant values.[7] By analogy with transition elements, alkaline earth dications may also be expected to form complexes with orotic acid, but to a lesser extent. Complexation of Ca^{2+} and Mg^{2+} merits special attention because of the high physiological importance of these ions. In previous studies of complex formation between calcium and magnesium cations and other ligands,[8,9] it has been observed that, although the value of complex formation constants is often within the same order of magnitude for both Ca^{2+} and Mg^{2+}, there is a striking difference (three orders of magnitude)[8,10] when the rate of complexation is considered. This difference has been tentatively advanced to account for the antagonistic biochemical behavior of these cations.[8]



III

The present study is partly designed to evaluate the ratio of tautomeric orotate dianions in aqueous solutions and to determine the kinetics of tautomeric interconversion. It also deals with the effects of Ca^{2+} and Mg^{2+} on both tautomerism equilibrium and kinetics in an attempt to gain information concerning orotic acid metabolism and/or alkaline earth cation transport in biological processes.

Experimental Section

The molecular weights of the purified compounds were determined by mass spectrometry. Analytical TLC on cellulose plates (Merck) was performed in solvents A (1-butanol-acetic acid-water, 4:1:5), B (2-propanol-aqueous ammonia-water, 7:1:2), and C (water-saturated 1-butanol). All compounds were found to be homogeneous.

Orotic acid (Aldrich) was recrystallized from water. 3-Methylorotic acid was prepared as previously described.[11] An alternative procedure for obtaining 3-methylorotic acid was given by Fox et al. based on direct methylation of orotic acid by dimethyl sulfate.[12] In an attempt to prepare 3-methylorotic acid in this way, puzzling results were observed: at the temperature of the steam bath (Fox's conditions) 3-methylorotic acid was obtained as described; however, at room temperature, 1,3-dimethylorotic acid was the main product and the 3-methyl derivative was not detected. A rationale for this observation is suggested in the Discussion section.

Kinetic Apparatus and Data Processing. Temperature-jump experiments were performed as described elsewhere.[13-15] The standard temperature conditions were $t_{initial}$ = 1 and t_{final} = 10 °C for kinetic runs in water and $t_{initial}$ = 7 and t_{final} = 15 °C in deuterium oxide. Relaxation amplitudes, as computed from the equation $\log y = y_0 - t/\tau$, were corrected for short relaxation times (cf. Experimental Section in ref 14).

pH Measurements. pH was monitored by a Radiometer PHM64 pH meter equipped with a Radiometer G202C glass electrode. pD values were calculated from pH values using the relationship pD = pH + 0.4, since the pH meter was standardized in water.[16] Orotic acid pK_a values in D_2O were estimated using the relationship p$K_a(D_2O)$ = p$K_a(H_2O)$ + 0.5.[17]

UV Spectra. UV spectra were recorded on a Cary 118 UV spectrophotometer fitted with thermostated cells. Since the experiments were designed for complex formation constant determination, buffer solutions leading to the complexation of metallic ions were undesirable; thus citrate and glycine buffers were deemed unsuitable. Moreover, use of buffers with constituents likely to produce insoluble salts with cations was excluded. It was also checked that at the pH values of the experiments no hydroxide precipitation nor appreciable reaction of the acidic metallic cations with water (according to $M^{2+} + H_2O \rightleftharpoons MOH^+ + H^+$) occurred.[18] The following buffers were finally chosen: for pH 7.32, primary NBS buffer KH_2PO_4 (0.025 M) + Na_2HPO_4 (0.025 M); for pH 9.18, primary NBS buffer $Na_2B_4O_7$ (0.01 M); for

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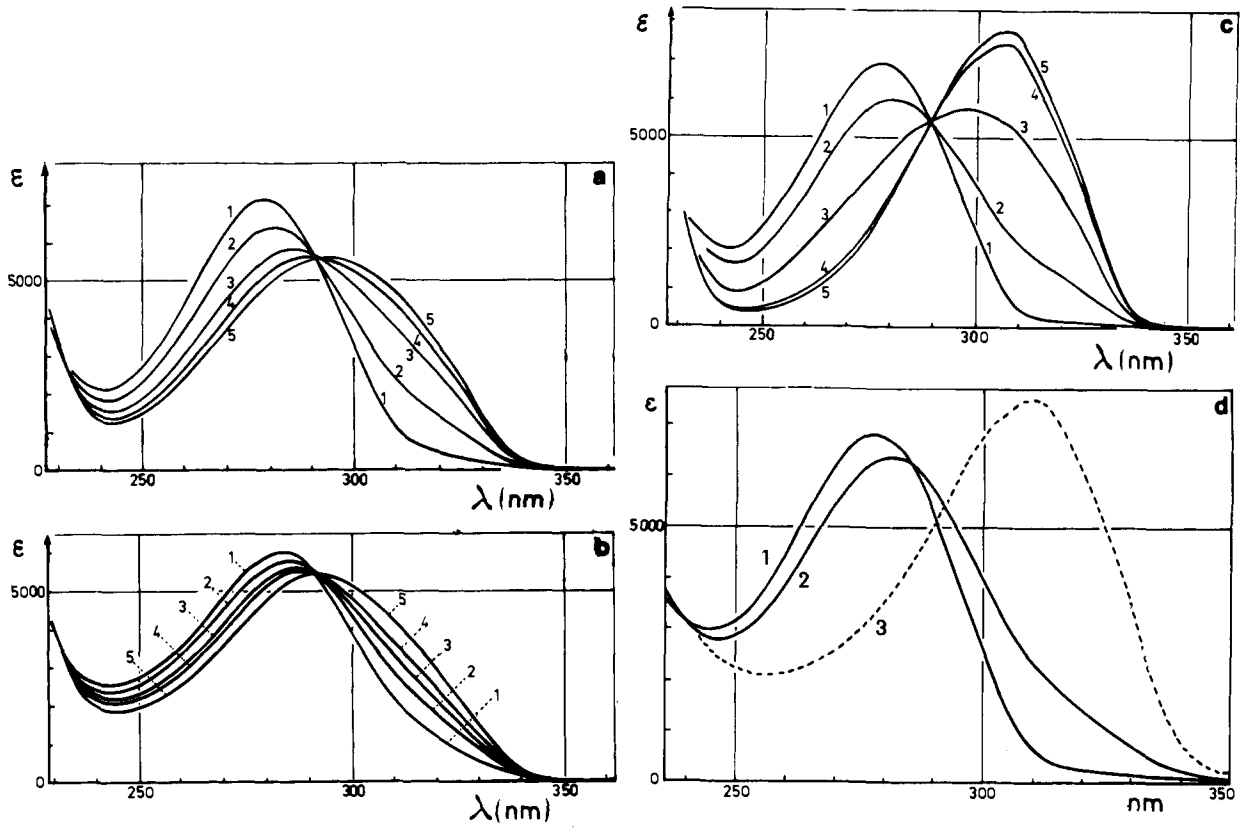


Figure 1. (a) UV spectra of orotic acid at pH 9.18 (20 °C, initial I = 0.03) as a function of $Mg(ClO_4)_2$ concentration ($C_{Mg^{2+}}$): (1) 0; (2) 2.2×10^{-2} M; (3) 4.4×10^{-2} M; (4) 8.8×10^{-2} M; (5) 13.2×10^{-2} M. (b) UV spectra of orotic acid at pH 11 (20 °C, initial I = 0.1) as a function of $CaCl_2$ concentration ($C_{Ca^{2+}}$): (1) 0; (2) 4.45×10^{-2} M; (3) 8.9×10^{-2} M; (4) 13.35×10^{-2} M; (5) 22.25×10^{-2} M. (c) UV spectra of orotic acid dianion (HL^{2-}) in methanol (20 °C) as a function of $Mg(ClO_4)_2$ concentration $C_{Mg^{2+}}$: (1) 0; (2) 3.27×10^{-5} M; (3) 8.7×10^{-5} M; (4) 1.42×10^{-4} M; (5) 1.75×10^{-4} M. (d) UV spectra of 3-methylorotic acid: (1) pH 7.4 (I = 0.1); (2) pH 10.01 (I = 0.1); (3) pH 10.01, $C_{Ni^{2+}} = 1.9 \times 10^{-4}$ M.

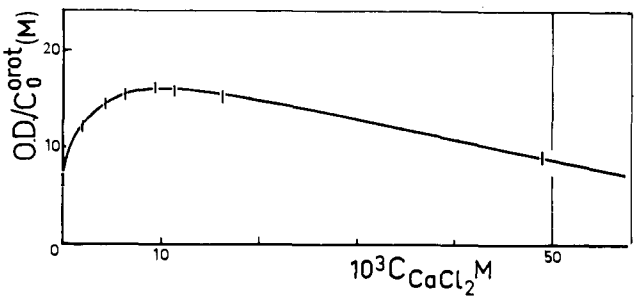


Figure 2. Variation of the relaxation amplitude per mole (in OD units per mole) at 14 °C in D_2O as a function of Ca^{2+} concentration (λ 326.5 nm).

pH 10.01, primary NBS buffer $NaHCO_3$ (0.025 M) + Na_2CO_3 (0.025 M); for pH 11, $Na_2B_4O_7$ (0.11 M) + NaOH; for pH 12 and 13, NaOH.

Titration of Ca^{2+} and Mg^{2+}. Ca^{2+} was titrated by EDTA (10^{-2} N) at pH 13 in the presence of calcon indicator. Magnesium solutions were titrated by EDTA (10^{-2} N) at pH 10 in the presence of Eriochrome indicator.

Results

UV Spectra. The UV spectrum of orotic acid monoanion (10^{-4} M) run at neutral pH (7.35) is not affected by addition of alkali metal (Na^+ and Li^+) or alkaline earth metal cations (Ca^{2+}, Mg^{2+}) at a concentration of 10^{-2} M. However, at pH values near and above pK (9.45), drastic changes in the spectrum are observed when $CaCl_2$ or $Mg(ClO_4)_2$ are added (Figure 1a and 1b). Sodium and lithium chloride do not induce significant spectral changes in orotate solutions at concentrations comparable to or higher than those of $CaCl_2$ and $Mg(ClO_4)_2$.

Table I. Kinetic Constants of Orotate Dianion Tautomerization in D_2O at 14 °C (I = 0.2)

$10^2 \times C_{Ca^{2+}}$, M	$10^{-8} \times k_{OD^-}$, $M^{-1}\ s^{-1}$	$10^6 \times k_{H_2L^-}$, $M^{-1}\ s^{-1}$	$10^{-4} \times k_0$, s^{-1}
0	3.3 (0.2)[a]	4.7 (0.7)	2.9 (0.1)
0.91	3.8 (0.1)	2.7 (0.3)	1.5 (0.1)
1.95	5.1 (0.3)		1.6 (0.3)
4.94	7.6 (0.3)		1.41 (0.3)
10.9	12.2 (0.1)		1.65 (0.2)

[a] Standard deviation.

In methanol free of basic impurities, the UV spectrum of orotate monoanion (H_2L^-) is unaffected by the addition of Mg^{2+} or Ca^{2+}. However, in methanolic solutions of orotate dianion (HL^{2-}) (1.2×10^{-4} M), the addition of Mg^{2+} up to a concentration of 1.7×10^{-4} M produces significant changes in the spectrum; no significant change occurs beyond this concentration (Figure 1c).

Kinetic Experiments. Without Divalent Cations. When a basic solution of orotic acid in D_2O is subjected to a fast temperature jump (5 μs), a relaxation phenomenon corresponding to a decrease in optical density is observed. The amplitude of the relaxation varies with pD around the pK(D_2O) (10) corresponding to dianion formation, and is constant beyond pD 10.5. The maximum relaxation amplitude observed at 308 nm is 20 OD units/mol of orotic acid; at 326.5 nm it is 7 OD units/M (Figure 2).

Relaxation time τ never exceeds 30 μs, is independent of the observation wavelength, and depends on pD and on the analytical orotic acid concentration in the "acidic" range of our study.[19] The experimental values of τ (Table I) are well fitted

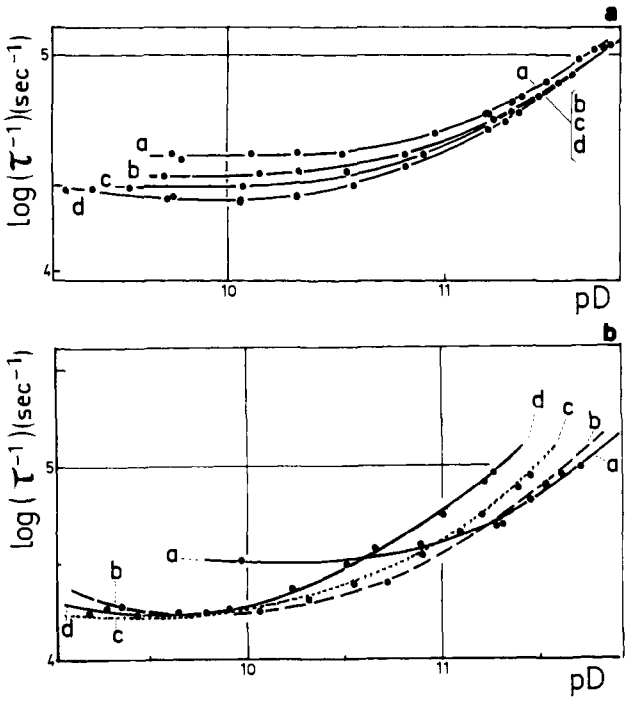


Figure 3. (a) Relaxation time of orotate dianion tautomerization as a function of pD in D_2O at 14 °C (I = 0.2) ($C_0^{or} = 7.84 \times 10^{-4}$ M) ($C_{Ca^{2+}}$): (a) 0; (b) 1.9×10^{-3} M; (c) 4.25×10^{-3} M; (d) 6.3×10^{-3} M. Dashed curves are obtained by fitting the experimental data with eq 1. (b) Relaxation time of orotate dianion tautomerization as a function of pD in D_2O at 14 °C ($I = 0.2$) ($C_0^{or} = 7.5 \times 10^{-4}$ M) ($C_{Ca^{2+}}$): (a) 0; (b) 1.95×10^{-2} M; (c) 4.94×10^{-2} M; (d) 1.09×10^{-1} M. Dashed lines are obtained by fitting the experimental data with eq 1.

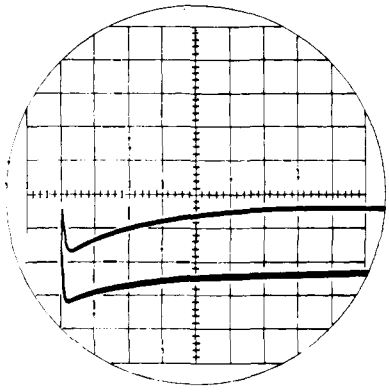


Figure 4. Oscilloscope trace of the relaxations in D_2O of orotic acid/Mg^{2+} systems: $C_0^{or} = 4.5 \times 10^{-4}$ M; $C_{Mg^{2+}} = 1.5 \times 10^{-3}$ M; λ 312.5 nm; 20 mV/div, 0.5 ms/div; upper trace, pD 10.43; lower trace, pD 10.81.

by the relation

$$\tau^{-1} = k_{OD}\text{-}[OD^-] + k_{H_2L}\text{-}[H_2L^-] + k_0 \qquad (1)$$

where $[H_2L^-]$ is the concentration of orotate monoanion.

Addition of Ca^{2+} and Mg^{2+}. Effect of Calcium Cations. The relaxation spectrum of orotic acid in the presence of Ca^{2+} ions depicts a time-resolved phase and a phase shorter than the heating time. The time-resolved process displays a marked dependence on $CaCl_2$ addition. When Ca^{2+} concentration is progressively increased, the minimum of the rate/pD profile first decreases (Figure 3a), but the plot is not significantly affected at high pD. As increasing amounts of Ca^{2+} are added, the minimum of the profile tends toward a constant value (Figure 3b) and the reaction rate in the high pD range increases. The amplitude of the time-resolved relaxation phenomenon is also affected by the presence of calcium ions (Figure 2).

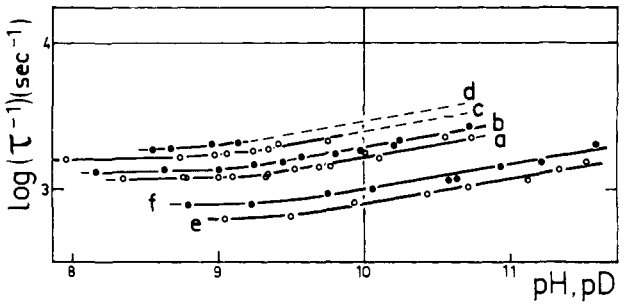


Figure 5. Relaxation time of orotate dianion complexation by Mg^{2+}. In H_2O (12 °C, $I = 0.2$) ($C_0^{or} = 7.78 \times 10^{-4}$ M) ($C_{Mg^{2+}}$): (a) 2.34×10^{-3} M; (b) 5.5×10^{-3} M; (c) 1.02×10^{-2} M; (d) $1.64 \times 10^{10-2}$ M. In D_2O (15 °C, $I = 0.2$) ($C_0^{or} = 4.5 \times 10^{-4}$ M) ($C_{Mg^{2+}}$): (e) 1.5×10^{-3} M; (f) 2.9×10^{-3} M. Dashed lines are obtained by fitting experimental data with eq 1.

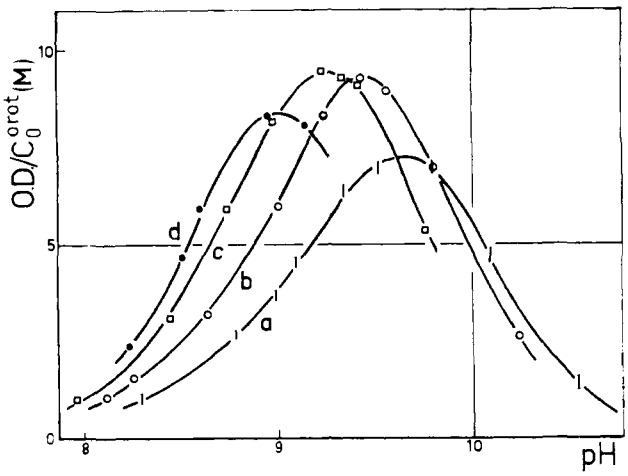


Figure 6. Relaxation amplitude, in OD units per mole, of N(3)H, $Mg^{2+} \rightleftharpoons N(3)H + Mg^{2+}$ in H_2O at 10 °C (λ 312.5 nm, $I = 0.2$) ($C_{Mg^{2+}}$): (a) 2.34×10^{-3} M; (b) 5.5×10^{-3} M; (c) 1.02×10^{-2} M; (d) 1.64×10^{-1} M.

Effect of Magnesium Cations. Orotic acid solutions (8×10^{-4} M) in D_2O relax in two time-resolved modes when subjected to a fast temperature jump (Figure 4). The fast phase displays a dependence on pD and on an orotic acid concentration which is qualitatively similar to that observed in cation-free solutions. This phase is not affected by addition of Mg^{2+} at a concentration of 10^{-2} M. The slow process observed in H_2O is also time resolved (whereas the rate of the fast one surpasses the heating time) and exhibits a slight dependence on pH and metal concentration (Figure 5). Its amplitude is also dependent on these same factors (Figure 6).

Discussion

Interpretation of UV Spectra of HL^{2-}. The UV studies of methylated orotic acid dianions and substituted uracil anions show that the spectrum of the N(1)-substituted derivative is blue shifted with respect to the N(3)-substituted one.[1,2,12,20] The spectrum of orotic acid dianion (HL^{2-}) has a λ_{max} that lies between that of 1-methyl- and 3-methylorotic acid dianions (Figure 1b) and exhibits a strong residual absorbance at 300 nm (A_{300}/A_{max}) ≃ 2:3, whereas for 1-methylorotate this ratio is only 0.1).[12] All this strongly suggests that, in water, both N(3)H and N(1)H are present.

If it is assumed that the absorbance at 320 nm is due only to the N(3)H form, and if the molecular extinction coefficient is that of 3-methylorotic acid dianion (ε 4200) at this wavelength, the following tautomeric equilibrium constant is obtained: $K_T = [N(3)H]/[N(1)H] = 0.4 \pm 0.08$ at 25 °C. It must be stressed that this value is only a rough estimate.

However, given the approximations made, this value compares satisfactorily with $K_T = 0.2$ which is inferred from the difference between the pK of 1-methylorotic acid (9.82) and that of 3-methylorotic acid (10.52).[12] The tautomeric constant is somewhat different from that observed in anionic uracil where N(3)H is slightly favored.[4] This suggests that some hydrogen bonding between N(1)H and the carboxylate group takes place, thereby increasing the stability of the N(1)H tautomers. The bathochromic shift of the UV spectra observed upon addition of divalent cations to orotic acid dianion suggests that the N(3)H structure becomes increasingly favored. Since addition of Na^+ or Li^+ to aqueous orotate dianion at similar ionic strengths does not cause changes in spectra, this shift cannot be ascribed only to ionic strength effects, and is likely to result from the association of the divalent cations specifically with the N(3)H tautomer (III).[21] This likelihood is strongly supported by the marked change in the spectrum of dianionic 3-methylorotic acid caused by addition of the strongly complexing Ni^{2+} cation (Figure 1d). Moreover, charge localization at N(1)H and at the carboxylate group indicates that it is reasonable to attribute structure III to the complex. That an unsubstituted nitrogen atom adjacent to the carboxylate group is required for complex formation is also supported by the fact that addition of divalent cations to a pH 10 solution of isoorotic acid (uracil 5-carboxylic acid) causes only slight changes in absorbance.[22] It thus seems that the spectral changes caused by alkaline earth cations result from a shift in the tautomeric equilibrium toward the N(3)H form and from complexation of this latter structure.

Tautomeric equilibrium shift has also been observed in uracil monoanions; however, in this case, adding divalent cations leads conversely to stabilization of the N(1) structure.[3,23] It is reasonable to assume that, in uracil anions, the metallic ion is more likely to be bonded to N(3) which has two adjacent electronegative oxygen atoms, than to N(1) which has only one neighboring oxygen atom. The occurrence of a uracil-like complexation in orotate dianion cannot be excluded; however, it is likely to be overshadowed by the formation of the $N(3)H{\cdot}M^{2+}$ complex (III) in which much stronger bonds are involved. The ionic nature of the bonds in this complex is confirmed by the strong complexation of HL^{2-} by Mg^{2+} in methanol where a stoichiometric concentration of cations leads to a nearly stoichiometric concentration of $N(3)H{\cdot}M^{2+}$ (Figure 1c).

Relationships between absorbance data and the various equilibria occurring in aqueous orotic acid solution can be readily derived (cf. Appendix I). From multilinear fits at constant pH and for given analytical orotic acid concentrations, C^{or}_o, we have estimated the complex formation constants, K_{comp}, of the dianion for Ca^{2+} and Mg^{2+} as $(1.7 \pm 0.2) \times 10^2$ and $(5 \pm 0.5) \times 10^2\ M^{-1}$, respectively. A typical consequence of the divalent cation effect is that the apparent pK of dianion formation decreases: e.g., at pH 8, the overall dianion concentration increases tenfold and the total concentration of dianions with structure I (free or complexed) is nearly a hundred times greater when 10^{-1} M of Mg^{2+} is added. This result should be kept in mind when dealing with the biochemistry of orotic acid, especially since there may be areas in the physiological medium in which there are low dielectric constants and in which complexation is greatly enhanced (as seen in methanol).[24]

Kinetics

Dynamics of Tautomerization without Added Divalent Cations. Due to the similarity between the dependence of relaxation amplitude on the wavelength and the differential spectrum of the N(1)H and N(3)H tautomers (as inferred from the spectra of 1-methyl- and 3-methylorotic acid dianions), the chemical relaxation is assigned to the equilibrium I $\rightleftharpoons$ II.

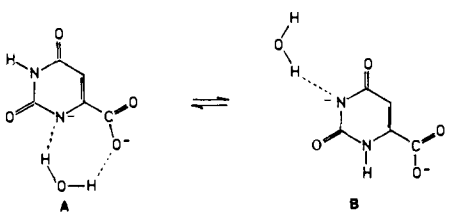
The relaxation amplitude, A, is related to the thermodynamic functions of the tautomeric equilibrium by

$$A = \frac{\Delta H_T}{RT^2}(\Delta\epsilon_\lambda) l C^{or}_o \frac{K_T}{(1+K_T)^2}\Delta T \qquad (2)$$

where ΔH_T and K_T are the tautomerization enthalpy and constant, $\Delta\epsilon_\lambda$ the difference between the molar extinction coefficients of N(1)H and N(3)H tautomers at the observation wavelength, l the optical path of the T-jump cell (0.7 cm), ΔT the magnitude of the temperature, and C^{or}_o the analytical concentration of orotic acid.

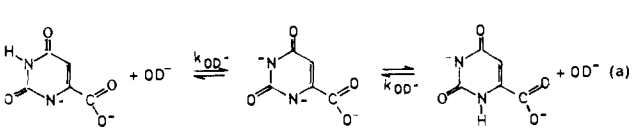
As previously postulated from the UV spectra of 1-methyl- and 3-methylorotic acid dianion, the absorbance of orotate dianion (HL^{2-}) at 310 nm is due only to the N(3)H tautomer. Since the observed relaxation signal in this wavelength range corresponds to a decrease in absorbance, it is concluded that N(3)H is the exothermic species (ΔH_T of the N(1)H $\rightleftharpoons$ N(3)H tautomeric equilibrium is negative) although it is slightly less favored than N(1)H ($\Delta G_T > 0$). By setting K_T equal to 0.4 (calculated from UV measurements) in eq 2, it is possible to estimate the enthalpy (-0.3 ± 0.05 kcal) and entropy (-2 ± 0.4 eu) of the tautomeric equilibrium.[25] The slight but significant decrease in entropy on going from N(1)H to N(3)H is understandable if it is assumed to arise from a more ordered water solution in N(3)H (A, more ordered solvation; B, less ordered solvation).

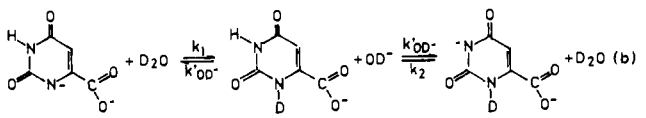
Positive ΔG_T and negative ΔH_T may account for the temperature dependence of the product distribution for orotic acid methylation (cf. Experimental Section). At room temperature, the free energy of the transition state for methylation of the N(3)H tautomer lies lower than that for the N(1)H one and, hence, the N(1)Me derivative would be formed preferentially. This compound would be subsequently methylated to the 1,3 derivative. When the temperature is raised (if the hypothesis that $\Delta H_T < 0$ is still valid at high temperatures), the energy of the N(3)H tautomer should increase with respect to that of N(1)H, whereupon inversion of the levels of the transition state free energies for methylation of N(1)H and N(3)H should occur. This would result in methylation of the N(1)H tautomeric dianion leading to 3-methylorotic acid which, as observed by Fox et al.,[12] is not further methylated under these conditions.



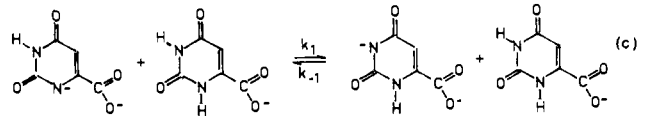
A B

The fact that the relaxation time for reequilibration of the tautomeric equilibrium is well fitted by eq 1 strongly suggests that the acid- and base-catalyzed mechanism, which seems to be general in the tautomerism of neutral,[13-15,26] cationic,[28] and anionic[4] heterocyclic compounds, is also valid here. Indeed, the dynamics of proton transfer in orotate dianion can be readily understood in terms of a dissociative mechanism involving an intermediate species. Three significant path mechanisms (a–c) can account for the tautomeric exchange:[27] base catalysis by OD^- (a), acid catalysis by D_2O (b), and acid





catalysis by H_2L^- (autocatalysis) (c). The relaxation time for



pathway a is $\tau_{basic}^{-1} = k_{OD^-}[OD^-]$. In pathway b, the relaxation time, $\tau_{D_2O}^{-1} = (k_1 + k_2)/2 = k_1(1 + K_T)/2$, can be rewritten as an expression of the pK_1' of dianion N(3)H formation, $\tau_{D_2O}^{-1} \simeq (k_{OD^-}'/10^{-pK_1'})K_{D_2O}$, where pK_1' is expressed in terms of the ionization constant of orotic acid dianion ($pK_1 = 9.95$ in D_2O)[1] and of the tautomeric constant K_T, and K_{D_2O} is the ionic product of D_2O. This equation becomes $\tau_{D_2O}^{-1} = (k_{OD}'K_{D_2O}/2K_2K_T)(1 + K_T)^2$. For pathway c, when $K_2 \ll D^+ \ll K_1$ (cf. Appendix **I**), a simple expression for the relaxation time is readily derived: $\tau_{H_2L^-}^{-1} = [(k_{-1} + k_1)/K_1]C^{cor}{}_o[D^+]$.

The experimental constants determined from eq 1 are satisfactorily consistent with these tautomerization mechanisms (Table I). The low value for the second-order rate constant of base catalysis, k_{OD^-}, arises both from the fact that formation of the intermediate trianion ($pK_2 > 13$) is only slightly exergonic (which means that the reaction is no longer diffusion controlled)[4] and from the fact that the encounter rate between two negatively charged species is low. For the same reasons, a similarly low value of k_{OD^-} (3.9×10^8 M^{-1} s^{-1}) was found in uracil monoanion exchange.[4] However, between anionic uracil and orotic acid, there are marked differences in rate constant values when dealing with the autocatalysis and the pH-independent pathway (water catalysis). These differences can be easily understood on the basis of the following considerations. The low value of the autocatalysis rate constant in orotic acid is, in all likelihood, related to a low encounter rate between *two anions*, whereas in uracil the reaction indeed occurs between an anion and a neutral species. Moreover, the expected contribution to the overall relaxation time from water catalysis can be evaluated from the relaxation time for acid catalysis by D_2O; taking $k_{OD^-} = 4 \times 10^9$ M^{-1} s^{-1} (a value inferred for base catalysis in fluorouracil which proceeds similarly, according to exergonic fixation of OH^- on a monoanion)[4] and $pK_1' \simeq 10.4$ in D_2O at 14 °C, we find $\tau_{D_2O}^{-1} \simeq 3 \times 10^4$ s^{-1} which compares well with the k_0 term of eq 1 (Table I). The difference between the k_0 term in the dynamics of uracil anion exchange can stem from the higher pK value of the latter compound and from the fact that the reaction involves OD^- fixation on a neutral molecule and results in a higher value of k_{OD^-}.[4] It must be stressed that, as in uracil and fluorouracil anions, water catalysis can, by itself, account for the pH-independent term in the kinetic law.

Addition of Divalent Cations. When divalent cations are added, the resulting coupling of the tautomeric equilibrium to the complexation equilibrium can be represented by eq 3 in which k_i's are composite rate constants:

$$N(3)H{\cdot}M \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} N(3)H + M \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} N(1)H + M \qquad (3)$$

Actually, this simple general scheme covers a rather complex mechanistic pattern. Indeed, tautomerization proceeds via the above-mentioned acid- and base-catalyzed pathways, and the base-catalyzed path involving formation of the intermediate monoanion might be affected by complexation of the monoanionic species.

Effect of Ca^{2+}. The time-resolved relaxation observed in this case is clearly tautomerization. The relaxation time for complexation, as expected from rates measured in other Ca^{2+}-ligand systems,[10,28] is in the submicrosecond range (i.e., beyond the resolution of our Joule-heated T-jump assembly). Experimental measurements of the tautomerization relaxation time reveal that (1) In the 9–10 pD range where base catalysis is weak, the relaxation time reaches a limiting value which corresponds to a decrease by a factor of 2 with respect to the relaxation in metal-free medium; (2) addition of calcium ions produces a decrease in relaxation time which is more marked at "low" than at high pD values where it is offset by a steady increase with increasing $[Ca^{2+}]$.

The first point is understood as follows: the decrease of the reciprocal relaxation time with addition of calcium results from the kinetic coupling of tautomerization with complexation. Assuming a rapid complexation equilibrium, we can readily show that the relaxation time for tautomerization is

$$(\tau^{-1})_T{}^M = k_{-2} + k_2 \frac{[N(3)H] + (K_{comp})^{-1}}{[M] + [N(3)H] + (K_{comp})^{-1}}$$

where [M] and [N(3)H] are the concentrations of metallic cations and of N(3)H tautomers, respectively, at the initial temperature. When $[M] \gg [N(3)H]$, the cation concentration is buffered and the preceding expression can be rewritten as

$$(\tau^{-1})_T{}^M = k_{-2} + \frac{k_2}{1 + C^M{}_o/(K_{comp})^{-1}}$$

where $C^M{}_o$ is the overall metal concentration. When the concentration of added metal is increased, the limiting relaxation time becomes

$$(\tau^{-1})_T{}^{lim} \simeq k_{-2}$$

If it is assumed that the N(3)H and N(1)H tautomers are in equal proportion ($k_2 \simeq k_{-2}$), comparison of $(\tau^{-1})_T{}^{lim}$ with $(\tau^{-1})_T{}^0 = k_2 + k_{-2}$ leads to $(\tau^{-1})_T{}^{lim}/(\tau^{-1})_T{}^0 \simeq 0.5$. This prediction is actually observed in the reaction rate of tautomerization with and without added calcium (Figure 3a,b) when pD <10. However, at high pD the decrease in reaction rate upon Ca^{2+} addition is apparently offset by an increase in base catalysis efficiency. This increase when $[Ca^{2+}] > 10^{-2}$ M is likely to arise from a decrease in the apparent pK of trianion formation ($pK_{2,app}$) upon metal addition. As a consequence, formation of the intermediate trianion involved in the basic pathway should be increasingly favored from a thermodynamic standpoint and its rate should tend to be diffusion controlled. The complex formation constant can be very roughly estimated by setting k_{OD^-} equal to 2×10^9 M^{-1} s^{-1}. [This is an arbitrary value based on the rate constant (3.9×10^9 M^{-1} s^{-1}) observed in 5-fluorouracil anion exchange where *two monoanions* are involved, as opposed to the base-catalyzed exchange of orotate dianion which involves a *monoanion* and *dianions*.] It can be further assumed that the limiting value of k_{OD^-} will be reached when $pK_{2,app}$ is 3–4 free energy units less than that of water, e.g., $pK_{2,app} = 12.5$.[29] On the basis of both of these assumptions, together with the data in Figure 7 and the expression of $pK_{2,app}$ (cf. Appendix I), we find that the complex formation constant $K_{comp}{}^{tri}$ equals 5×10^2 M^{-1}, which is not unrealistic when compared with that of K_{comp}.

After the fast phase (viz., complexation) has relaxed, the concentrations of complexed and free N(3)H reach pseudo equilibrium values. These concentrations then shift according to the slow relaxation mode. Since the pseudoequilibrium concentrations are not very different from the initial equilibrium values (in the case of a small temperature perturbation), the amplitude of the slow relaxation may be easily computed from the equilibrium concentrations of N(1)H and N(3)H (cf. eq. II-1 in Appendix II). The calculated amplitude accounts satisfactorily for these observed data.

Effect of Mg^{2+}. The "fast" relaxation is readily attributed to tautomerization since it presents a pD/rate profile and a rate/concentration dependence similar to those observed in

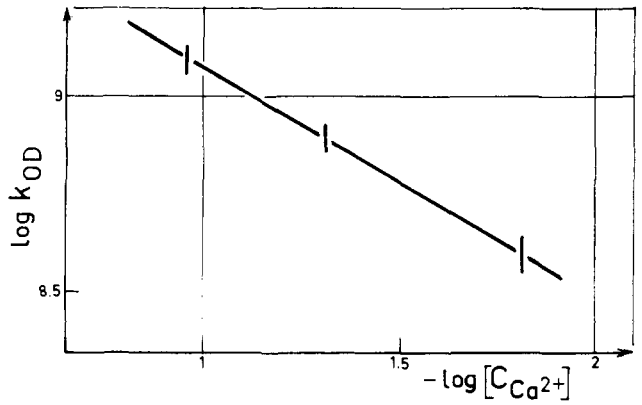


Figure 7. Relaxation of N(1)H ⇌ N(3)H in the presence of Ca^{2+} (I = 0.2): log k_{OD^-} (eq 1) vs. $-\log C_{Ca^{2+}}$.

metal-free orotate solutions. The slow relaxation is assumedly due to complexation. The two relaxation times computed for eq 3, where tautomerization is assumed to be much faster than complex formation, are $(\tau^{-1})_T = k_2 + k_{-2}$ *and* $(\tau^{-1})_{comp} = k_{-1}\{[N(3)H] + [M]\} + k_1 - [k_{-1}k_2[M]/(k_2 + k_{-2})]$ *or* $(\tau^{-1})_{comp} = [k_{-1}/(1 + K_T)]K_T[M] + k_{-1}[N(3)H] + k_1$. From the expression of [N(3)H] in Appendix I one obtains (when the metal concentration is buffered and $[H^+] \gg K_2$)

$$(\tau^{-1})_{comp} = \frac{k_{-1}}{1 + K_T} K_T C^M{}_o + k_{-1} C^{or}{}_o \times \frac{1}{(1 + K_T)\left(1 + \frac{[H^+]}{K_1}\right) + \frac{C^M{}_o}{(K_{comp})^{-1}}} + k_1 \quad (4)$$

The weak dependence of $(\tau^{-1})_{comp}$ on pH (pD) and on metal and orotic acid concentrations (Figure 5) indicates that k_1 in eq 4 is the significant kinetic term of the complexation process. A multilinear fitting of experimental data with eq 4 gives a value of k_1 equal to $(1.1 \pm 0.1) \times 10^3$ s^{-1}. Using k_1 and K_{comp} (5×10^2 M^{-1}) to calculate k_2 gives $(5.5 \pm 0.5) \times 10^5$ M^{-1} s^{-1}. This value compares satisfactorily with those observed in the reaction of magnesium with other ligands (oxine, IDP), thereby suggesting that the rate-limiting step in the complexation of N(3)H is the replacement of the water molecule(s) by the ligand.[10,28]

Equation III-2 in Appendix III predicts that the relaxation amplitude of complexation will reach a maximum at a pH value which decreases as $[Mg^{2+}]$ increases. Similarly, the variation of the amplitude with metal concentration, at a fixed pH, reaches a maximum for a given value of this concentration (eq III-3 in Appendix III). These trends are actually observed experimentally (Figure 6). The numerical values in Appendix III and Figure 6 allow the enthalpy of the complex dissociation to be roughly estimated (eq III-1 in Appendix III) as equal to -0.25 (± 0.07) kcal M^{-1}.

Conclusion

Even at pH values well below the pK of dianion formation, orotic acid, in the presence of dications, is stabilized mainly as the complexed N(3)H dianion tautomer. This may have two types of consequences on phosphoribosylation by orotidine pyrophosphorylase: either the lifetime of the complexed form is long as for Mg^{2+} (and for Ni^{2+}, Co^{2+}, and Cu^{2+} complexes as inferred from Eigen's experiments[8]) and, in this case, inhibition of phosphoribosyl group addition might occur; or the lifetime is short as for Ca^{2+}. To gain further understanding of the role played by alkaline earth cations and divalent transition metals in biochemical processes involving orotic acid, this question is currently being investigated in our laboratory.

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Appendix I

The following expressions for the concentrations of the various species present in aqueous orotic acid can be derived from constants K_1 and K_2 for dianion (HL^{2-}) and trianion (L^{3-}) formation, respectively, tautomeric constant K_T, constant K_{comp} of the equilibrium (I ⇌ II), constant $K_{comp}{}^{tri}$ of the trianion complexation, and the overall concentration of orotic acid, $C^{or}{}_o$, and of metallic cations, $C^M{}_o$:

$$K_1 = [HL^{2-}][H^+]/[H_2L^-]$$

$$[HL^{2-}] = [N(3)H] + [N(1)H]$$

$$K_2 = [L^{3-}][H^+]/[HL^{2-}]$$

$$K_T = [N(3)H]/[N(1)H]$$

$$[K_{comp}]^{-1} = [N(3)H][M]/[N(3)H{\cdot}M]$$

$$(K_{comp}{}^{tri})^{-1} = [L^{3-}][M]/[L^{3-}{\cdot}M]$$

Provided $C^M{}_o \gg [N(3)H{\cdot}M]$ (viz., $[M] \simeq C^M{}_o$), one finds

$$[N(1)H] = \frac{C^{or}{}_o}{K_T} \times \frac{1}{\left[\left(1 + \frac{1}{K_T}\right)\left(1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]}\left(1 + \frac{C^M{}_o}{(K_{comp}{}^{tri})^{-1}}\right)\right) + \frac{C^M{}_o}{(K_{comp})^{-1}}\right]} = \frac{C^{or}{}_o}{K_T}\frac{1}{D}$$

$$[N(3)H] = C^{or}{}_o \times \frac{1}{D}$$

$$[N(3)H{\cdot}M] = C^{or}{}_o \frac{1}{D} \times \frac{C^M{}_o}{(K_{comp})^{-1}}$$

$$[L^{3-}] = \frac{K_2}{[H^+]} C^{or}{}_o \frac{1}{D}\left(1 + \frac{1}{K_T}\right)$$

$$[L^{-3}{\cdot}M] = \frac{K_2}{[H^+]} C^{or}{}_o \frac{1}{D}\left(1 + \frac{1}{K_T}\right)\frac{C^M{}_o}{(K_{comp}{}^{tri})^{-1}}$$

$$[H_2L^-] = \frac{[H^+]}{K_1} C^{or}{}_o \frac{1}{D}\left(1 + \frac{1}{K_T}\right)$$

$$K_{T,app} = K_T\left[1 + \frac{C^M{}_o}{(K_{comp})^{-1}}\right]$$

$$K_{1,app} = \frac{[[N(1)H] + [N(3)H] + [N(3)H{\cdot}M]][H^+]}{[H_2L^-]}$$

$$K_{1,app} = K_1 \frac{(1 + K_{T,app})}{(1 + K_T)}$$

$$K_{2,app} = K_2 \frac{(1 + K_T)\left[1 + \frac{C^M{}_o}{(K_{comp}{}^{tri})^{-1}}\right]}{1 + K_{T,app}}$$

At pH $\ll$ pK_2 the absorbance at wavelength λ is

$$A_\lambda = \epsilon_{N(1)H}[N(1)H] + \epsilon_{N(3)H}[N(3)H] + \epsilon_{H_2L^-}[H_2L^-] + \epsilon_{N(3)H{\cdot}M}[N(3)H{\cdot}M]$$

$$A_\lambda{}^M = \frac{C^{or}{}_o}{\left(1 + \frac{1}{K_T}\right)\left(1 + \frac{[H^+]}{K_1}\right) + \frac{C^M{}_o}{(K_{comp})^{-1}}} \times \left[\frac{\epsilon_{N(1)H}}{K_T} + \epsilon_{N(3)H} + \epsilon_{N(3)H{\cdot}M}\frac{C^M{}_o}{(K_{comp})^{-1}} + \epsilon_{H_2L^-}\frac{[H^+]}{K_1}\left[1 + \frac{1}{K_T}\right]\right] \quad (I\text{-}1)$$

When $C^{M}_{o} = 0$

$$A_\lambda^0 = \frac{C^{or}_{o}}{\left(1 + \frac{1}{K_T}\right)\left(1 + \frac{[H^+]}{K_1}\right)} \times \left[\epsilon_{N(3)H} + \epsilon_{N(1)H}\frac{1}{K_T} + \epsilon_{H_2L^-}\left(1 + \frac{1}{K_T}\right)\frac{[H^+]}{K_1}\right]$$

Introducing A_λ^0 in the expression of A_λ gives

$$A_\lambda^M = \frac{1}{\left(1 + \frac{1}{K_T}\right)\left(1 + \frac{[H^+]}{K_1}\right) + \frac{C^{M}_{o}}{(K_{comp})^{-1}}} \times \left[A_\lambda^0\left(1 + \frac{1}{K_T}\right)\left(1 + \frac{[H^+]}{K_1}\right) + C^{or}_{o}\epsilon_{N(3)H\cdot M}\frac{C^{M}_{o}}{(K_{comp})^{-1}}\right]$$

$$A_\lambda^M = \frac{\alpha + \beta C^{M}_{o}}{\gamma + \delta C^{M}_{o}}$$

where $\alpha = \left(1 + \frac{1}{K_T}\right)\left(1 + \frac{[H^+]}{K_1}\right)A_\lambda^0$

$$\beta = C^{or}_{o}\epsilon_{N(3)H\cdot M}/(K_{comp})^{-1}$$

$$\gamma = \alpha/A_\lambda^0$$

$$\delta = 1/(K_{comp})^{-1}$$

Hence,

$$\Delta A_\lambda = A_\lambda^M - A_\lambda^0 = \frac{\beta}{\gamma}C^{M}_{o} - \frac{\delta}{\gamma}C^{M}_{o}A_\lambda \quad \text{(I-2)}$$

Appendix II

The overall equilibrium is

$$N(3)H\cdot M \rightleftarrows N(3)H + M \rightleftarrows N(1)H + M$$

After a temperature perturbation, the complexation tends to its equilibrium much more rapidly than tautomerization. While the slow process relaxes to its equilibrium, the variation of concentrations in the various species are related by

$$\Delta[N(1)H] = -\Delta[N(3)H]\left[1 + \frac{C^{M}_{o}}{(K_{comp})^{-1}}\right]$$

and, with buffered metal concentration,

$$\Delta[N(3)H\cdot M] = [N(3)\cdot M]\frac{\Delta[N(3)H]}{[N(3)H]}$$

When concentration changes are monitored by spectrophotometric absorption the observed amplitude of the slow relaxation, when $K_2 \ll [H^+] \ll K_1$, is

$$A_\lambda = [\epsilon_{N(3)H}\Delta[N(3)H] + \epsilon_{N(1)H}\Delta[N(1)H] + \epsilon_{N(3)H\cdot M}\Delta[N(3)H\cdot M]]\Delta Tl$$

where $\Delta[N(3)H]$, $\Delta[N(1)H]$, and $\Delta[N(3)H]$ are the differences between initial and final temperature concentrations. The amplitude is rewritten as

$$A_\lambda = l\left\{\epsilon_{N(3)H} - \epsilon_{N(1)H}\left[1 + \frac{C^{M}_{o}}{(K_{comp})^{-1}}\right] + \epsilon_{N(3)H\cdot M}\frac{C^{M}_{o}}{(K_{comp})^{-1}}\right\}\Delta[N(3)H]\Delta T$$

$\Delta N(3)H$ is readily obtained from the expression for N(3)H (cf. Appendix I); then

$$A_\lambda = l\left[\frac{\epsilon_{N(3)H} - \epsilon_{N(1)H}\frac{K_{T,app}}{K_T} + \epsilon_{N(3)H\cdot M}\frac{C^{M}_{o}}{(K_{comp})^{-1}}}{RT^2(1 + K_{T,app})^2}\right] \times C^{or}_{o}K_T\Delta H_T\Delta T \quad \text{(II-1)}$$

If we define the formal quantity $\Delta H_{T,app}$, A_λ may be rewritten as

$$A_\lambda = l\frac{C^{or}_{o}\Delta\epsilon\Delta H_{T,app}K_{T,app}\Delta T}{RT^2(1 + K_T)^2}$$

where $\Delta\epsilon = \epsilon_{N(3)H} - \epsilon_{N(1)H}$.

Identifying eq II-1 with the preceding equation leads to

$$\Delta H_{T,app} = \Delta H_T \times \left[\frac{\epsilon_{N(3)H} - \epsilon_{N(1)H}\left(1 + \frac{C^{M}_{o}}{[K_{comp}]^{-1}}\right) + \epsilon_{N(3)H\cdot M}\frac{C^{M}_{o}}{(K_{comp})^{-1}}}{(\epsilon_{N(3)H} - \epsilon_{N(1)H})\left[1 + \frac{C^{M}_{o}}{(K_{comp})^{-1}}\right]}\right]$$

If the wavelength of observation is chosen so that $\epsilon_{N(3)H} = \epsilon_{N(3)H,M}$ one obtains

$$\Delta H_{T,app} = \Delta H_T$$

Appendix III

Tautomerization (the fast process) is coupled to the complexation

$$N(3)H\cdot M \rightleftarrows N(3)H + M \rightleftarrows N(1)H + M$$

The relationships between the variations of the concentrations in the slow mode are

$$\Delta[N(1)H] = \frac{\Delta[N(3)H]}{K_T}$$

$$\Delta[H_2L^-] = \Delta[N(3)H]\frac{1}{K_1}\left(1 + \frac{1}{K_T}\right)[H^+]$$

and, since $\Delta[H_2L^-] + \Delta[N(1)H] + \Delta[N(3)H] + \Delta[N(3)H\cdot M] = 0$

$$\Delta[N(3)H\cdot M] = -\Delta[N(3)H]\left(1 + \frac{1}{K_T}\right)\left(1 + \frac{[H^+]}{K_1}\right)$$

$\Delta[N(3)H]$ is derived from the expression of $[N(3)H]$ given in Appendix I.

The amplitude of the slow relaxation is then

$$A_\lambda = l\frac{\alpha + \beta[H^+]}{\left[\left(1 + \frac{1}{K_T}\right)\left(1 + \frac{[H^+]}{K_1}\right) + \frac{C^{M}_{o}}{(K_{comp})^{-1}}\right]^2} \times \frac{\Delta H_{comp}}{RT^2(K_{comp})^{-1}}C^{M}_{o}C^{or}_{o}\Delta T \quad \text{(III-1)}$$

with

$$\alpha = \epsilon_{N(3)H} + \epsilon_{N(1)H}\frac{1}{K_T} - \epsilon_{N(3)H\cdot M}\left(1 + \frac{1}{K_T}\right)$$

and

$$\beta = (\epsilon_{H_2L^-} - \epsilon_{N(3)H\cdot M})\left(1 + \frac{1}{K_T}\right)\frac{1}{K_1}$$

The value of $[H^+]$ which makes the derivative of A_λ with respect to $[H^+]$ equal to 0 is

$$[H^+] = K_1\left[1 + \frac{C^{M}_{o}}{(K_{comp})^{-1}\left(1 + \frac{1}{K_T}\right)} - \frac{2\alpha}{K_1}\frac{1}{\beta}\right] \quad \text{(III-2)}$$

The derivative of A_λ with respect to C^M_o is 0 when

$$C^M_o = (K_{comp})^{-1}\left(1 + \frac{1}{K_T}\right)\left(1 + \frac{[H^+]}{K_1}\right) \qquad \text{(III-3)}$$

Convenient calculations may be performed with data from observations made at 312.5 nm since $\epsilon_{H_2L^-} = \epsilon_{N(1)H} \simeq 0$; $(\epsilon_{N(3)H\cdot M})_{312.5} = 6850$, $(\epsilon_{N(3)H})_{312.5} = 5100$, $K_T = 0.4$, $K_{comp} = 5 \times 10^2\ M^{-1}$ for Mg^{2+}.

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Dependence of Aryl Ether Acylation upon Lewis Acid Stoichiometry

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Abstract: Acylation of alkyl aryl ethers has been observed to be uniquely dependent on the stoichiometry of the Friedel-Crafts catalyst. With 100 mol % catalyst, acylation proceeds rapidly and in high yield; with large molar excesses of catalyst, the reaction is essentially completely arrested. This inhibition can be reversed by using sterically bulky alkyl groups which effectively prevent complexing between catalyst and aryl ether. Based on these observations, we have developed processes for regioselective intramolecular acylation of either a phenyl or an alkoxylated phenyl ring when both are present.

Introduction

The Friedel-Crafts reaction has been extensively studied and utilized for many years. A prodigious variety of catalysts and reaction conditions have been explored, demonstrating the tremendous scope of this reaction type.[1] Acylation reactions are of particular value owing to the selectivity and ease with which they are usually accomplished, thus providing useful routes to highly functionalized aromatic ring systems. Two comprehensive reviews[2,3] further illustrate and catalog the synthetic utility of this acylation process.

A large variety of Friedel-Crafts acylating agents also has been studied. The two most widely useful systems involve the reactions of acid anhydrides[4-11] and acid chlorides.[2,3,12,13] Accompanying ether cleavage by the Lewis acid catalyst also has been reviewed within the scope of these acylation reactions.[14-17] However, little study has been focused upon the effects of excess catalyst on the acylation reaction itself. The question of Lewis acid catalyst stoichiometry, particularly with respect to the acylation of aryl ethers, has been largely disregarded. For difficult cases, especially those involving simultaneous ether cleavage, the utility of several less common catalysts such as antimony pentachloride, gallium trichloride, and silver trifluoromethanesulfonate has been demonstrated.[18,19] High-temperature acylation conditions have also been invoked using only traces of Lewis acid catalyst;[20] however, such methods are not generally applicable to aromatic ethers. One recent, but unusual, example illustrating the effects which may be associated with catalyst stoichiometry deals with the acylation of phenyl methyl thioether.[21] The observation was made that, by using 100 mol % $AlCl_3$, acylation para to the methylthio group could be maximized. Deviation from this

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