The Addition of Hydroxylamine to the Halogenated Uracils

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Hydroxylamine adds reversibly to C-6 of uracil bases *via* a multistep mechanism. Equilibrium constants measured at several *p*H values, 25°, μ = 3.0 *M* are: uracil, 0.25 M^{-1}; 5-iodouracil, 0.30 M^{-1}, 5-bromouracil, 0.24 M^{-1}, 5-chlorouracil, 0.06 M^{-1} and 5-fluorouracil, 5.11 M^{-1}. The kinetics of hydroxylamine addition to both 5-bromo-and 5-iodouracil are complex. At low hydroxylamine buffer concentrations, the rate constants are second-order with respect to hydroxylamine buffer but at higher concentration a first-order dependence is approached. Hydroxylamine elimination from 5-iodo-6-hydroxylamino-5,6-dihydrouracil is subject to general base catalysis by tris(hydroxymethyl)aminomethane but at higher concentrations the rate constants are not proportional to the concentration of general base. This reaction is subject to solvent effects where increasing ethanol concentration depresses the rate when measured at constant *p*H. These kinetics can be rationalized in terms of a multistep reaction pathway in which hydroxylamine addition to C-6 of the halogenated uracil precedes general acid catalyzed proton transfer to C-5 yielding the final 6-hydroxylaminodihydropyrimidine product.

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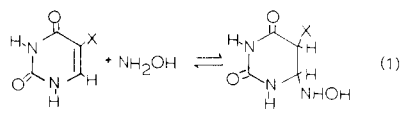
Hydroxylamine, a mutagenic agent, (1,2), is a strong α effect nucleophile which adds across the 5-6 double bond of pyrimidine rings thus promoting such reactions as the formation of 5,6 dihydro-N^4-hydroxy-6-hydroxylaminocytosine from cytosine, (3,4), both urea and isoxazoles from uracil derivatives, (2,5,6), and uracil and halide ions from halogenated uracils, (2,7,9). This latter reaction goes by a two step pathway which involves the formation of 5-halo-6-hydroxylamino-5,6-dihydrouracil (Equation 1) followed by its dehalogenation (Equation 2). Since hydroxylamine addition to the halogenated uracils is rapid relative to dehalogenation it is possible to separate these two reactions. The objectives of this report are to present the equilibrium constants for the reversible addition of hydroxylamine to the halogented uracils (Equation 1) and to show that this addition reaction goes *via* a multistep pathway in which proton transfer to C-5 is discrete from hydroxylamine addition to C-6. A preliminary report of this work has appeared in abstract form (10).



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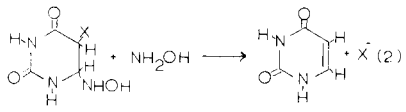

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EXPERIMENTAL

Hydroxylamine hydrochloride (Eastman Organic Chemicals) was twice recrystallized from 95% ethanol and dried *in vacuo* prior to use. Uracil and its 5-bromo, 5-chloro, 5-iodo and 5-fluoro analogs were used as received from Sigma Chemical Co. as was tris(hydroxymethyl)aminomethane (Tris). Reaction mixtures were prepared in capped 3.0 ml. cuvettes using glass distilled water for the preparation of all reagents and reaction mixtures. In addition to the uracil which was generally present in a 10^{-3} to 10^{-4} *M* concentration range, reaction mixtures contained varying amounts of hydroxylamine buffers of varying degrees of neutralization. Ionic strength was maintained at 3.0 *M* by the addition of potassium chloride. Reactions were monitored spectrophotometrically at appropriate wavelengths with either a Cary 14 or a Cary 118C recording spectrophotometer each of which was equipped with a cell compartment thermostatted at 25.0°.

Equilibrium constants (K_{eq}) for the formation of 6-hydroxylamino-5,6-dihydropyrimidines (adduct) from the reaction of hydroxylamine base (NH_2OH) with various uracils (ura) were calculated from the relationship, K_{eq} = [adduct]/[NH_2OH] [ura] where [adduct] = ΔA_λ and [ura] = A_λ. Equilibrium constants were measured over a reasonable concentration range using hydroxylamine buffers of varying degrees of neutralization.

The rate constants for adduct formation were measured under pseudo-first order conditions using the following relationships: $k_{obsd} = k_f + k_r$; $K'_{eq} = k_f/k_r$ = [adduct]/[ura] = $\Delta A_\lambda/A_\lambda$. In these equations; k_{obsd} is the psuedo-first order rate constant for hydroxylamine addition, k_r is the rate constant for the elimination of hydroxylamine to yield the uracil and K'_{eq} is the ratio of adduct to uracil at equilibrium. Combining these expressions yields the following equation which was used to calculate values of k_f $k_f = k_{obsd}/(1 + [1/K_{eq}])$. Values of k_{obsd} were determined from semi-logarithmic plots of extent reaction against time using the relationship $k_{obsd} = 0.693/t_{1/2}$.

In studies of buffer catalyzed elimination of hydroxylamine from the 5-iodouracil adduct, the adduct was prepared by mixing equal volumes of 0.01 *M* 5-iodouracil and 3.0 *M* hydroxylamine base 1 hour prior to use. Reactions were initiated by diluting the concentrated adduct solution into buffer solutions contained in capped 3.0 ml cuvettes. Reactions were followed spectrophotometrically and values of k_{obsd} calculated as previously described.

Results and Discussion.

The addition of hydroxylamine to halogenated uracils is

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rapid relative to the subsequent dehalogenation of the 6-hydroxylamino aduct. Figure 1 shows the time course for the addition of hydroxylamine to 5-iodouracil and Figure 2 shows both that the reaction is fully reversible and that essentially all of the 5-iodouracil added to the reaction mixtures is recovered upon dilution. This indicates that

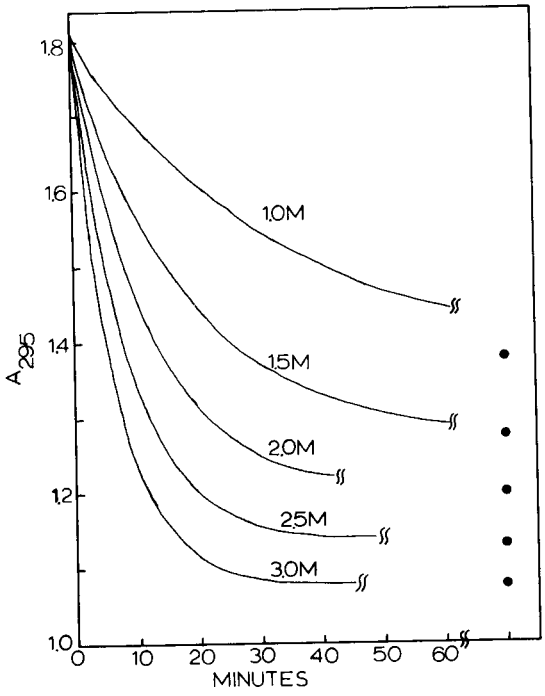


Figure 1. Time course for the approach to equilibrium for the reaction of increasing concentration of hydroxylamine buffers (82.4% free base) with 3.33×10^{-4} *M* 5-I-Uracil, μ = 3.0 *M*, 25°. Numbers refer to hydroxylamine buffer concentration. Solid circles (•) show the A_{295} absorption of each reaction mixture at a time where equilibrium had been achieved without appreciable dehalogenation of the adduct. These times were 112, 88, 82, 53, and 46 minutes for 1.0, 1.5, 2.0, 2.5 and 3.0 *M* hydroxylamine buffers respectively.

hydroxylamine promoted dehalogenation (9,10) of the adduct has little if any significance during the time required to reach equilibrium. Hence, it is possible to measure the equilibrium constants for the 6-hydroxylamino adduct formation using spectrophotometric methods similar to those used to measure equilibrium constants for nucleophile addition to carbonyl compounds (11). The equilibrium constants for the addition of hydroxylamine to uracil and its 5-halo derivatives measured at several *p*H values are shown in Table 1. There exists little difference in the magnitude of these equilibrium constants for hydroxylamine addition to either uracil, 5-bromouracil or 5-iodouracil (0.20 to 0.33 M^{-1}). The observed values for 5-fluorouracil are about 5.0 M^{-1} while the equilibrium constant for hydroxylamine addition to 5-chlorouracil is about 0.060 M^{-1}. Due to the small observed absorbance changes, this latter value for 5-chlorouracil can be regarded only as

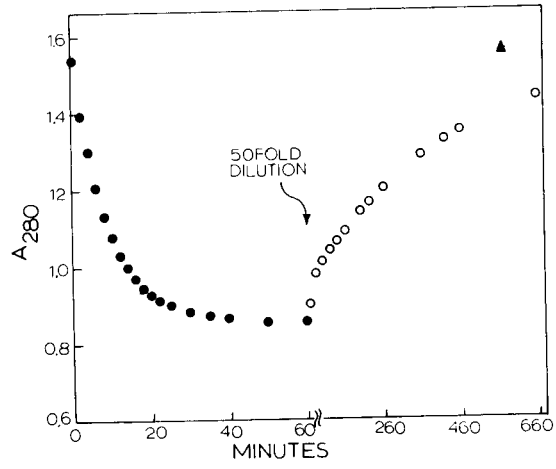


Figure 2. Reversibility of hydroxylamine addition to 5-iodouracil, 25°. Complete spectra of 3.0 *M* hydroxylamine buffer (90% free base) reacting with 6.25×10^{-3} *M* 5-iodouracil were measured as a function of time at 25° using 1.0 cm cells equipped with 0.98 cm spacers thus making the light path 0.02 cm. At equilibrium (t = 60 minutes) an aliquot of this reaction mixture was diluted 50-fold with water in 1.0 cm path length cells. Spectra were recorded at 25° until no further change in absorbance was observed. Closed circles (•), reaction mixture before dilution; open circles (○), reaction mixture after dilution; closed triangle (▲), diluted reaction mixture at t = ∞.

Table I.

Equilibrium Constants for the Addition of Hydroxylamine to the 5 Halouracils (a)

Halouracil	*p*H	No. of Determinations	K_{eq} (b) M^{-1}
Uracil	6.08	5	0.23
	6.87	5	0.27
5-Iodouracil	6.72	5	0.33
	6.31	5	0.31
	6.02	5	0.29
	5.65	5	0.28
5-Bromouracil	7.25	11	0.26
	6.30	14	0.24
	6.16	6	0.25
	5.45	9	0.20
5-Fluorouracil	6.58	6	5.13
	6.03	6	5.45
	5.61	6	4.76
5-Chlorouracil	5.56	5	0.06 (c)
	6.24	9	0.06

(a) 25°C, μ = 3.0 *M*. (b) Equilibrium constants calculated as described in the Experimental. (c) Due to the small observed absorbance changes, these values can only be regarded as estimates.

an estimate. Hence the values of K_{eq} for hydroxylamine addition to uracil and its halogenated analogues can be ranked: 5-F-Ura ≫ 5-I-Ura ≈ 5-Br-Ura ≈ Ura ≫ 5-Cl-Ura, a ranking similar to that observed for bisulfite addition to the same compounds.

The kinetics of hydroxylamine addition to both 5-iodo- and 5-bromouracil are complex. Values of k_f, the pseudo-first order rate constant for addition to the 5,6 double bond, have a greater than first power dependence upon the concentration of total hydroxylamine buffer, thus indicating that the buffer system may serve two functions in the reaction. Figure 3 illustrates this phenomenon for

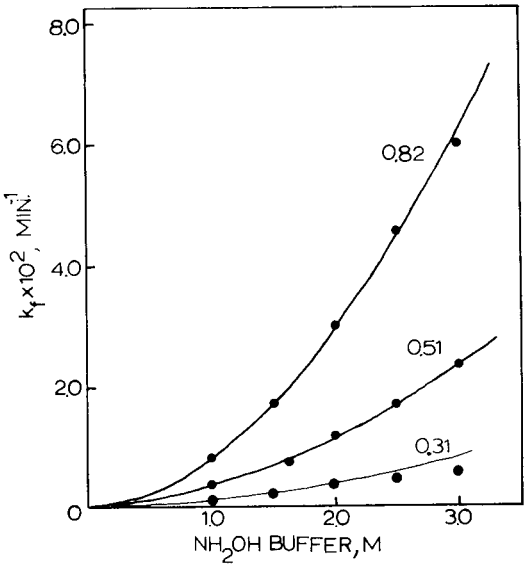


Figure 3. Non linear relationship between the observed first-order rate constants and hydroxylamine buffer concentration for the addition of hydroxylamine to 3.3×10^{-4} *M* 5-iodouracil, 25°, μ = 3.0 *M*. Numbers refer to the fraction NH_2OH base in each of the buffers. Solid lines were calculated from the relationship $k_f = k_l [NH_2OH\ Buffer]^2 + k_o [NH_2OH\ Buffer]$.

5-iodouracil, however, similar results were observed for 5-bromouracil. Secondary plots of $k_f/[NH_2OH$ buffer] against [NH_2OH buffer] (figure 4) were constructed. This representation of the data for 5-iodouracil indicates that in hydroxylamine buffers of greater than 50% free base, $k_f = k_l [NH_2OH]^2 + k_o [NH_2OH]$. At lower values of fraction hydroxylamine base, there is a change from second to first-order dependence on the hydroxylamine buffer. This result is again illustrated (Figure 4) for 5-iodouracil (31% hydroxylamine base) but is even more dramatic with 5-bromouracil in hydroxylamine buffers of both 30 and 50% hydroxylamine base. Thus, hydroxylamine addition to both 5-bromo- and 5-iodouracil is a multistep process which undergoes a change in rate determining step as a function of increasing hydroxylamine buffer concentration. Such a reaction mechanism is shown below where 1 mole of hydroxylamine acts as nucleophile to attack C-6 of the pyrimidine yielding an enolate anion which is subsequently protonated by hydroxylamine hydrochloride acting as a general acid catalyst of proton transfer. Such a mechanism is consistent with the data since the general acid catalyzed step would be second-order while the nucleophilic addition step work have only a first-order dependence on total hydroxylamine buffer. The observation of this phenomenon only in buffers of lower fraction hydroxylamine base (30-50% base) is also consistent with the scheme as these buffers contain higher concentration of general acid catalyst.

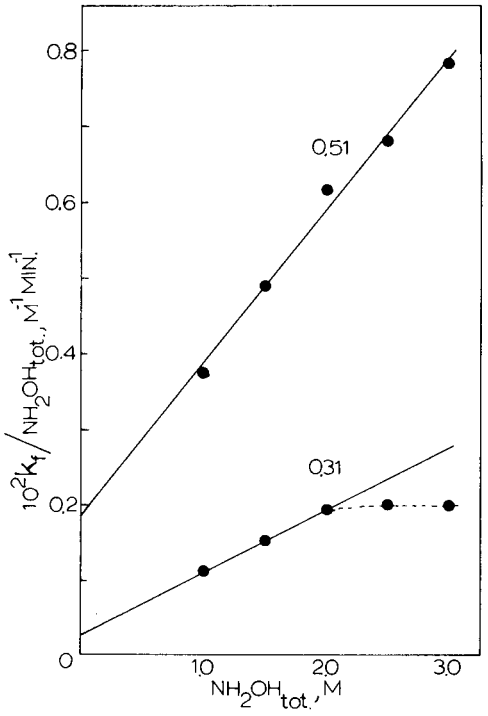
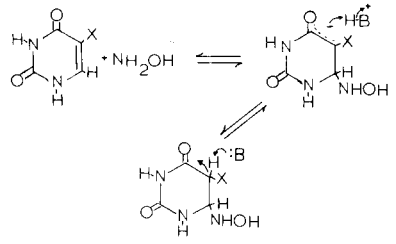


Figure 4. Relationship between the apparent second-order rate constants and total hydroxylamine buffer for the addition of hydroxylamine to 5-iodouracil, 25°, μ = 3.0 *M*. Numbers refer to the fraction hydroxylamine base in each series of reactions.



To further investigate the multistep nature of reversible hydroxylamine addition to 5-iodouracil, the elimination of

hydroxylamine from the freshly prepared adduct was examined as a function of increasing Tris buffer concentration using buffers of 0.30, 0.50 and 0.70 *M* Tris base. The results of these studies (Figure 5) indicate that the slopes of the three lines corresponding to the three different Tris buffers employed are equal. Hence, up to a concentration

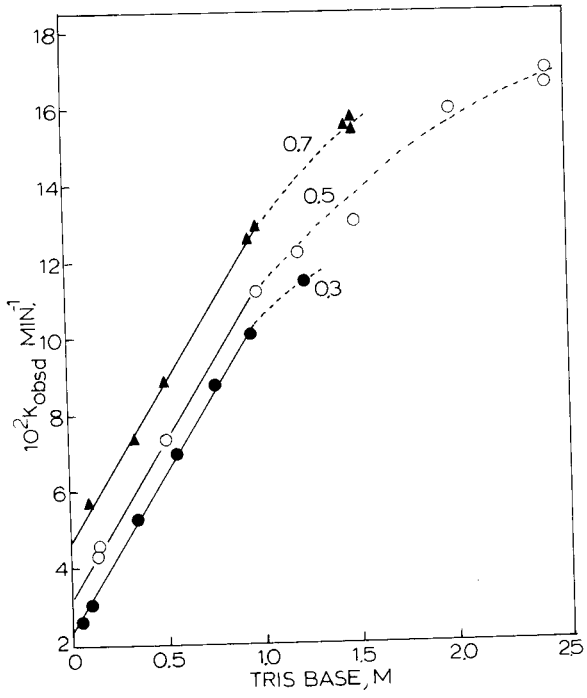


Figure 5. General base catalysis of hydroxylamine elimination from 5-iodo-6-hydroxylamino-5,6-dihydrouracil, 25°C, μ = 3.0 *M*. Numbers refer to the fraction tris(hydroxymethyl)aminomethane base in various buffers employed.

of about 1.0 *M* Tris base the reaction is subject to general base catalysis of proton transfer. Above 1.0 *M* Tris base there is a leveling in rate which could be interpreted as a change in the rate determining step from a general base catalyzed to a non-catalyzed reaction. By the principle of microscopic reversibility, this observation for hydroxylamine elimination from the hydroxylamino adduct is consistent with the above scheme. It should be noted however, that high Tris buffer concentrations may cause solvent induced rate depressions, as the addition of ethanol in the same concentration range (0-3.0 *M*) causes a significant reduction (~2X) in the observed rate constants. Thus, although the kinetics of general base catalyzed elimination of hydroxylamine from 5-halo-6-hydroxylamino-5,6-dihydrouracil are consistent with the above scheme, they must be interpreted in terms of the potential for buffer induced solvent effects.

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