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The Decarboxylation of 5-Azaorotic Acid (1)

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5-Azaorotic acid, *s*-triazine-2,4-dione (2,4-dehydro)-6-carboxylic acid, is one of a number of triazines that inhibit the growth of experimental neoplasms. It has been reported (3) to exert a carcinostatic effect on the adenocarcinoma-755. This compound, a competitive inhibitor of the synthesis *de novo* of pyrimidines (4) is a potential antineoplastic and antiviral agent but is ineffective when given orally to humans in a dose that will inhibit by 50 percent the conversion of a tracer injection of carboxyl-labelled orotic acid to C-14 carbon dioxide (5). This is presumably due to lability of 5-azaorotic acid in the acid conditions of the stomach.

Canellakis *et al.*, (6) have characterized the reaction product from the decarboxylation of 5-azaorotic acid in acid solution as 5-azauracil, *s*-triazine-2,4-dione (2,4-dehydro). The decarboxylation of a variety of carboxylic acids proceeds by essentially similar mechanisms (7). In particular, the decarboxylation of the quinaldic acids has been well documented (8) as proceeding through the intermediate $\backsim N\ H^+$ with the assumption that the complete sequence of events is as shown in Fig. 1. 5-Azaorotic acid may decarboxylate in acid solution by the same mechanism and it is presumed that only one of the various protonated forms is susceptible to decarboxylation.

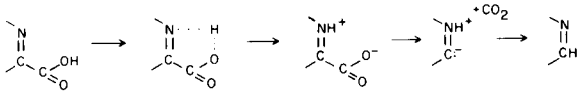


Fig. 1 The mechanism of the decarboxylation of quinaldic acid. (See text and reference 8).

Fig. 2 presents the change with time in the absorption spectrum of 5-azaorotic acid in 1.8 *M* sulfuric acid at 25° over a 12 hour period. The initial spectrum was identical to that of 5-azaorotic acid and at 12 hours to that of 5-azauracil. The single isosbestic point of these spectra suggests that the reaction proceeds with no intermediates detectable by spectrophotometric means. A wavelength of 260 mμ was chosen for the kinetic studies because it provided the large difference in extinction coefficients.

Fig. 3 shows the rate of decarboxylation of 5-azaorotic acid as a function of concentration of four acids. For each strong acid the rate of decarboxylation reaches a maximum ($k \sim 0.05\ min^{-1}$) and then decreased with increasing acid concentration. The rate of reaction increases with decreasing *p*H, the first detectable reaction at 50° ($k = 2 \times 10^{-5}\ min^{-1}$) occurring at *p*H 2.8-3.0. The weakest acid, phosphoric acid, never achieves the maximum rate of decarboxylation and also does not exhibit the subsequent reduction in reaction velocity at higher concentrations. It can be inferred from these data that there is a species of 5-azaorotic acid that decarboxylates and that either removal or addition of a proton from this species blocks decarboxylation. This concept is strengthened by the proposed structures associated with the various spectral shifts shown in table I. It may be seen that in this scheme all the possible protonated forms except that form with three protons (at N-1, 3, and 5) are found. For comparison, spectral data and proposed structures are presented for 5-azauracil in table II. Similarities are apparent between the properties of these two compounds.

(a) At high base concentrations (*p*H 13 to 6 *M* potassium hydroxide) there is no spectral change.

(b) Between *p*H 10 and 13 there is a hypsochromic shift.

(c) Between *p*H 8 and 10 there is no change in absorption wavelength.

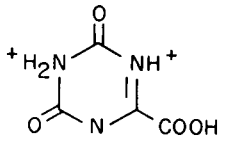
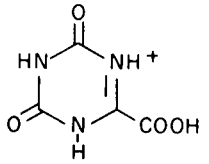
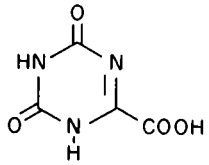
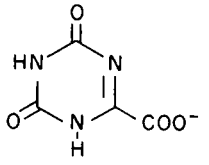
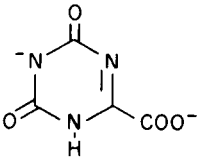
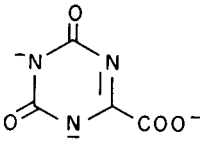
(d) Between *p*H 5 and 8 there is a bathochromic shift.

(e) Between *p*H 1.5 and 5 there is no change in absorption wavelength.

Under progressively more acidic conditions the spectral changes are quite different and it is assumed that protonation occurs with the ultimate formation of 5-azauracil^{+3} and 5-azaorotic^{+2}. The interpretation of the 5-azauracil spectra agrees with that of Jonas *et al.*,(9) for weakly acid and basic solutions. In the strong acid region both compounds exhibit a double shift between 1.8 *M* and 17.2 *M* sulfuric acid. These shifts are both bathochromic and with

TABLE I

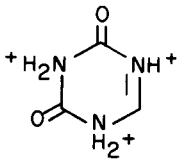
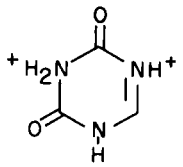
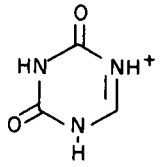
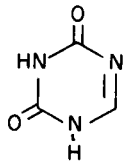
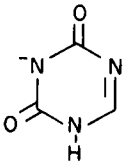
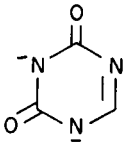
UV Spectral Characteristics of 5-Azaorotic Acid in Acid and Base

Molarity or *p*H	λ max	E max (a)	Isosbestic point (b)	Proposed Structure (c)
17.2*M* sulfuric acid	240	2.9×10^3	-	
10.8*M*	245	3.0×10^3	+	
1.8*M*	253	2.8×10^3	+	
0.04*M* (*p*H 1.5)	240	4.0×10^3		
*p*H 1.5 – 5.0	no change	-		
*p*H 5.0	240	-	+	
*p*H 7.5	254	4.3×10^3		
*p*H 7.5 – 10.0	no change	-		
*p*H 10.0	254	-	-	
*p*H 13	250	2.6×10^3		
*p*H 13 – 6*M* potassium hydroxide	no change	-		

(a) From E max = Absorbancy/molarity. (b) Refers to changes between a given molarity or *p*H and the next lower molarity or *p*H. (c) These structures are proposed as the predominant, though probably not sole species at the given molarity or *p*H.

TABLE II

UV Spectral Characteristics of 5-Azauracil in Acid and Base

Molarity or *p*H	λ max	E max (a)	Isosbestic point (b)	Proposed Structure (c)
17.2*M* sulfuric acid	222	4.4×10^3	-	
10.8*M*	225	4.2×10^3	+	
1.8*M*	230	3.8×10^3	-	
*p*H 1.5	235	3.1×10^3		
*p*H 1.5 – 5	no change			
*p*H 5	235		-	
*p*H 9	250	3.7×10^3		
*p*H 9 – 11.5	no change			
*p*H 11.5	250		-	
0.25*M* potassium hydroxide	247	3.0×10^3		
*p*H 0.25 – 5.7	no change			

(a) From E max = Absorbancy/molarity. (b) Refers to changes between a given molarity or *p*H and the next lower molarity or *p*H. (c) These structures are proposed as the predominant, though probably not sole species at the given molarity or *p*H.

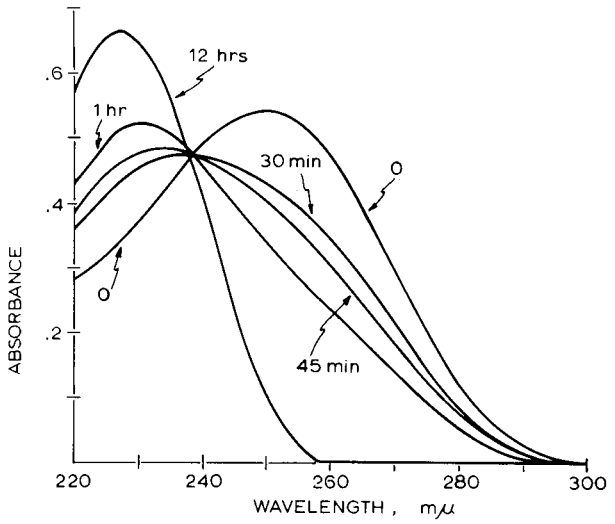


Fig. 2 The decarboxylation of 5-azaorotic acid as a function of time. 1.6 x 10^{-4} *M* 5-azaorotic and in 1.8 *M* sulfuric acid incubated at room temperature. Spectra were taken at times indicated.

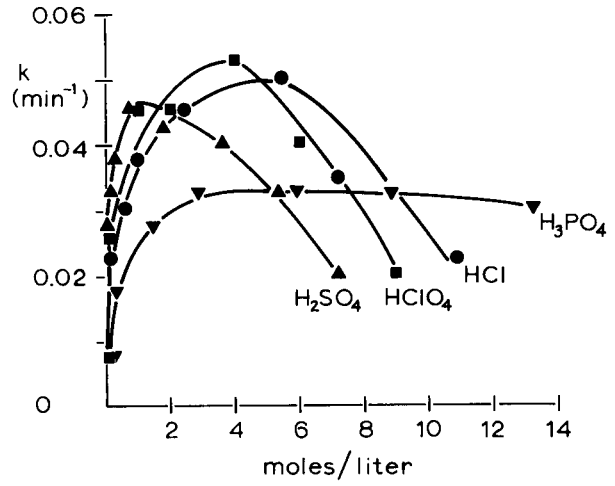


Fig. 3 The decarboxylation of 5-azaorotic acid as a function of acid concentration for several acids. 0.004 *M* 5-Azaorotic acid incubated at 40° in various acids as indicated by the abscissa. First-order rate constant for disappearance of 5-azaorotic acid calculated and shown on the ordinate.

the exception of the shift of 5-azaorotic acid at highest acidity show an increased E max and may represent successive addition of two protons to the ring. The shifts in spectra accompanied by a change in acidity from 0.04 *M* (*p*H 1.5) to 1.8 *M* sulfuric acid are markedly different for 5-azaorotic acid and 5-azauracil. It is proposed that the bathochromic shift for 5-azauracil corresponds to those observed at higher acidity and is, probably, an addition of a proton to the ring while the hypsochromic shift of azaorotate between 0.04 *M* and 1.8 *M* sulfuric acid is the result of protonation of the carboxylate group. Furthermore, measurements of the spectra of 5-azaorotic acid in solutions of phosphoric acid show changes in λ max and E max that correspond to the changes in decarboxylation rate. For reasons not apparent to us the spectral changes in phosphoric acid were not as distinctive as in sulfuric acid, but it was easily discerned that above 4 *M* phosphoric acid there were no regular changes in the spectrum.

These ionizations fit the pattern of decarboxylation as shown in Fig. 3. It is proposed that only ACOOH undergoes decarboxylation since $ACOO^-$ lacks the unionized carboxyl group and H^+ACOOH has a proton on N_5 that would block the transfer of the proton from the carboxyl group to N_5 (see Fig. 1). At higher *p*H values (*p*H 1-3) the rate of decarboxylation is controlled by the available concentration of ACOOH. In strong acid the second protonation occurs, and increases in the amount of ACOOH are offset by the production of H^+ACOOH. Finally, further formation of H^+ACOOH causes a decrease in the rate of decarboxylation. Dixon and Webb (10) consider in detail the effects on reaction rate of a progressive ionization of a compound from an inactive to an active and finally to an inactive form. From their theoretical discussion, changes in reaction velocity of the type observed would be anticipated if the separation of the two *p*Ka's were about one *p*H unit. The spectral data of Tables I and II and the kinetic data of Fig. 3 indicate that the ionizations involved in the protonation of the carboxyl group and the ring nitrogen, are separated by less than ten molarity units (or less than one *p*H unit). This view was substantiated by the experiments in which ionization of the carboxyl group was suppressed by ethanol as a replacement for the water component of the reaction solution. At 50° in acetate buffer (0.05 *M*) the relative rates of decarboxylation in 0, 20, 50, and 70 per cent ethanol were 1, 2, 5, and 14 respectively, despite the increase in apparent (11) *p*H from 2.5 to 3.2 over the range in ethanol concentrations. Thus, the limiting factor in the rate is the equilibrium between $ACOO^-$ and ACOOH, and an increase in the concentration of the ACOOH caused a marked increase in the reaction rate. In similar experiments with various concentrations of sulfuric acid the maximum rate of decarboxylation (sulfuric acid = 2 *M*) could be increased three-fold by the presence of 50 per cent ethanol in the reaction medium. It is well documented that ethanol and other low-dielectric solvents facilitate formation of the unionized carboxyl group and should decrease protonation of the ring nitrogens. Thus, ethanol should increase the spread between the *p*Ka's of the ionization of the carboxyl group and the ring nitrogens with a concomitant increase in concentration of ACOOH available for decarboxylation. Likewise, ethanol will decrease the ioniaztion of an acetic acid buffer and will

produce a rise in *p*H so that the increased rate of decarboxylation of 5-azaorotic acid in the presence of ethanol occurs in solution with fewer undissociated hydrogen ions.

The results with phosphoric acid are consistent with this interpretation since this weak acid would be incapable of maximally protonating a carboxyl group with a *p*Ka of about zero (1 *M* strong acid), and maximum velocity could not be achieved. Likewise, protonation of the ring believed to occur with 7 *M* strong acid would not occur and therefore the rate of decarboxylation would not be reduced below the maximum obtainable with this weak acid.

The possibility cannot be excluded, however, that the decarboxylating species is the zwitterion H^+ACOO^-. Green and Tong (12) have demonstrated the predominance of the zwitterion form of the structurally similar picolinic acid. Albert (13) has pointed out that a useful criterion for detection of zwitterion formation is hypsochromic shift in the long wave band of the suspected molecule upon the addition of acid. The bathochromic shift of 5-azaorotic acid between 0.05 *M* sulfuric acid (240 mμ) and 1.8 *M* sulfuric acid (253 mμ) suggests that the unionized species if predominant in this *p*H range. From the other two strong acid shifts for 5-azaorotic acid and the three acid shifts for 5-azauracil it would be predicted that addition of a proton to the ring of 5-azaorotic acid produces a hypsochromic shift; furthermore, the formation of a zwitterion would also involve addition of a proton to the ring. Moreover, it would be expected that addition of ethanol would not favor zwitterion formation but such addition did cause an increase in reaction rate.

It is concluded, therefore, that 5-azaorotic acid undergoes decarboxylation in acid solution by a mechanism similar to that for quinaldic acid and that the neutral (but not the monoprotonated or carboxylate) species is the active form in the decarboxylation. Less attractive alternatives are that it is the zwitterion which is the susceptible form or that the monoprotonated form has some susceptibility and the diprotonated species does not undergo decarboxylation.

EXPERIMENTAL

The absorption spectra were determined with the Bausch and Lomb 505 and Cary Model 15 spectrophotometers. Solutions used in these determinations were freshly prepared and kept at 0°. With these precautions no appreciable decarboxylation occurred prior to and during the spectral determinations. The *p*H was measured with a Leeds and Northrup *p*H meter.

The concentration of 5-azaorotate in all reactions was 4×10^{-3} *M*. Reaction mixtures were prepared by adding 0.5 ml. of 4×10^{-2} *M* 5-azaorotate solution to 4.5 ml. of the appropriate acid, buffer, or base solution. Aliquots of 0.5 ml. were removed at the indicated times, diluted with 9.5 ml. of an appropriate concentration of hydrochloric acid at 0° to give a final concentration of 1 *M*, and the absorbancy measured in a Beckman DU spectrophotometer at 280 mμ. 5-Azauracil in 1 *M* hydrochloric acid negligible absorption at 280 mμ compared with 5-azaorotate. 5-Azaorotate at 1 *M* hydrochloric acid did not decarboxylate during the period of absorbancy measurement when kept cold.

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(1) This research supported in part by a training grant from the USPHS (CA-5012).

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