

Hydrolytic Behavior of Isoalloxazines Related to Riboflavin III

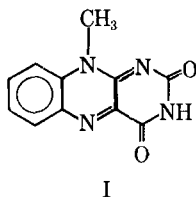
Covalent Hydration of

1,2-Dihydro-1-methyl-2-oxo-quinoxaline-3-carboxyureide

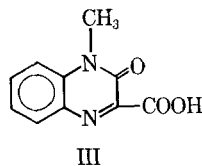
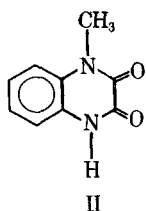
By DAVID E. GUTTMAN and THOMAS E. PLATEK

Previous studies concerned with the base-catalyzed degradation of 9-methylisoalloxazine led to the isolation of an intermediate which was reported to be an alloxan anil. The identification was based on comparing the properties of the isolated compound with those of what was thought to be authentic anil prepared by reacting *o*-methylaminoaniline with alloxan. Evidence has been obtained to indicate that an incorrect structure assignment was made and that the so-called anil is a compound which resulted from the covalent hydration of 1,2-dihydro-1-methyl-2-oxo-quinoxaline-3-carboxyureide. The hydrate is apparently formed by the addition of water across the double bond between N₄ and C₃ of the quinoxaline compound. It was found that in acid media, anhydrous and hydrated species coexisted in equilibrium while in neutral media, very little anhydrous form was detectable. The kinetics of attainment of equilibrium were studied at a number of different hydrogen-ion concentrations. Rate constants for forward and reverse reactions were determined. The influence of temperature on the rates of hydration and dehydration and on the equilibrium constant was also investigated.

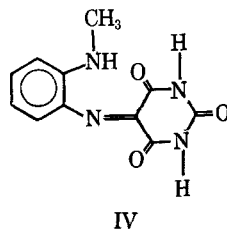
RECENT PUBLICATIONS from this laboratory (1, 2) described the results of studies concerned with the base-catalyzed degradation of 9-methylisoalloxazine (I). It was reported that a white crystalline material was isolated from par-



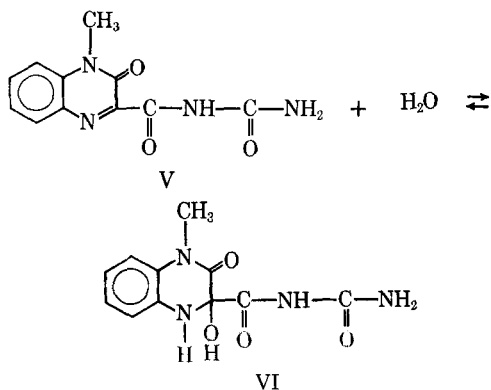
tially degraded solutions of the isoalloxazine and that the compound could undergo further reaction in basic solution to yield products which were identified as 1,2,3,4-tetrahydro-1-methyl-2,3-dioxoquinoxaline (II) and 1,2-dihydro-1-methyl-2-oxo-quinoxaline-3-carboxylic acid (III). The properties and behaviors of the isolated material were shown to be identical to those exhibited by a product formed by the reaction in neutral medium



of alloxan and *o*-methylaminoaniline and reported in the literature (3, 4) to be the alloxan anil (IV). This report will present evidence to suggest that



the compound formed as an intermediate in the base-catalyzed decomposition of 9-methylisoalloxazine and which can be synthesized by the reaction of alloxan with *o*-methylaminoaniline is not the anil (IV), as previously suggested, but rather a product (VI) which is formed through the covalent hydration of the double bond between N₄ and C₃ of 1,2-dihydro-1-methyl-2-oxo-quinoxaline-3-carboxyureide (V). (Scheme I.)



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King and Clark-Lewis (5, 6) and Clark-Lewis (7) have previously questioned the existence of alloxan anils such as IV. They reported that the condensation of *o*-methylaminoaniline with alloxan in neutral medium yielded a yellow crystalline compound with a characteristic spectrum which was identified as the ureide (V). That a white crystalline compound can also form under similar reaction conditions was not discussed by these authors. They did, however, refer to the work of Rudy and Majer (8) who obtained, from the reaction of 2-alkylamino-3-amino-pyridines with alloxan, two series of products, one yellow and unstable and the other colorless. The two different products were found to be interconvertible.

A number of considerations support the suggestion that the white compound in question is the covalent hydrate VI. These include: (a) the formation of products II and III can be readily explained on the basis of structure VI; (b) the compound on heating is converted to a yellow material with chromatographic and spectral characteristics of the ureide (V); (c) the equivalent weight of the compound, as determined by non-aqueous titration, corresponds to that of the hydrate (VI); (d) the ureide (V) in acidic and neutral media undergoes a rapid transformation to form a compound with spectral and chromatographic characteristics which are identical to those of the compound in question. Similarly, treatment of the latter compound with acid results in the formation of an equilibrium mixture composed of starting compound and ureide. Such facile transformations are readily explainable on the basis of a hydration-dehydration phenomenon.

The kinetics of the transformations described above were studied in some detail and, as will be seen, were found to be consistent with an acid-base catalyzed reversible covalent hydration.

RESULTS

Two different products were obtained when alloxan and *o*-methylaminoaniline were allowed to react in neutral alcoholic medium under slightly different conditions. One was the ureide (V) which is a yellow crystalline compound with the characteristic spectrum shown in Fig. 1. The other was a white crystalline compound which exhibited a much different spectrum, which is also shown in Fig. 1. This product, when heated above 100°, was converted to the ureide (V). The results of elemental analysis (C, 52.22%; H, 4.77%; N, 21.70%; O, 21.31%) were not consistent with structures IV (C, 43.6%; H, 4.10%; N, 22.8%; O, 19.5%) or VI (C, 50.0%; H, 4.58%; N, 21.20%; O, 24.2%) but were intermediate to the two possibilities. Equivalent weight determinations by nonaqueous titration yielded an average value of 265 which

agreed with structure VI (theory 264). The white compound, in subsequent discussions, will be referred to as compound VI and the yellow compound as the ureide.

The spectrum of the ureide in essentially aqueous medium, buffered in the neutral range of pH, was found to rapidly change with time. The resulting spectrum appeared to be the composite of two spectra, that of the ureide and compound VI. Thin-layer chromatography showed the presence in such solutions of two components, a minor amount of ureide and a major amount of compound VI. The results suggested that an equilibrium was established between the two compounds. The differences in the spectral characteristics between the two were used to follow the kinetics of equilibration. A wavelength of 380 m μ was used for this purpose and a typical result is shown in Fig. 2. Two different data plots are contained in this figure. Decrease in absorbance as a function of time is shown by the open circles. The closed circles represent a plot of $\log(A - A_\infty)$ versus time, where A is absorbance at time, t , and A_∞ is the constant absorbance value which was finally attained. The function $(A - A_\infty)$ reflects the concentration of ureide and the plot thus demonstrates that the rate of equilibration was first order with respect to ureide. The results of studies conducted at a number of different pH values are presented in Table I and Fig. 3. The A_∞ values shown in the table indicate that the equilibrium position was essentially independent of pH and that the equilibrium concentration of ureide was small. The plot of Fig. 3 yielded a slope of 1.03 and shows that the rate of attainment of equilibrium

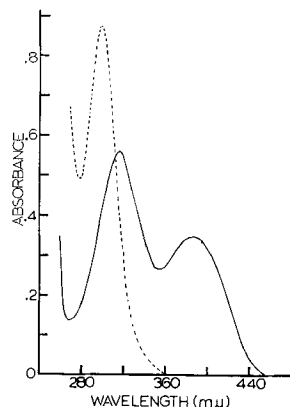


Fig. 1—Spectra exhibited by freshly prepared aqueous solutions of compounds V (—) and VI (---). The concentration of V was 6×10^{-5} M and that of VI was 1.97×10^{-4} M. The solutions contained 2% dimethylformamide.

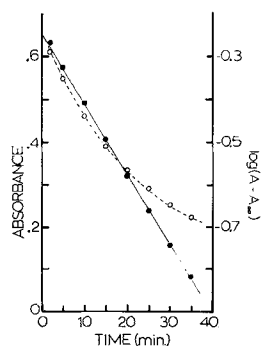


Fig. 2—A plot showing the rate of loss of absorbance at 380 m μ exhibited by a 1.22×10^{-4} M solution of the ureide (V) at pH 7.0 ($\mu = 1.0$) at 35°. Key: ---, absorbance vs. time plot; —, obtained by plotting $\log(A - A_\infty)$ vs. time. The solutions contained 2% dimethylformamide.

TABLE I—OBSERVED RATE CONSTANTS AND A_{∞} VALUES FOR THE TRANSFORMATION OF UREIDE IN BUFFERED AQUEOUS SOLUTION CONTAINING 2% DIMETHYLFORMAMIDE

pH	A_{∞}	$k_{\text{obs.}}$ (min. ⁻¹)
6.20	0.05	0.0062
6.40	0.05	0.0151
6.86	0.05	0.0288
7.00	0.05	0.0388
7.40	0.05	0.0890
7.60	0.05	0.147
7.80	0.03	0.220
7.90	0.04	0.262
8.00	0.02	0.389

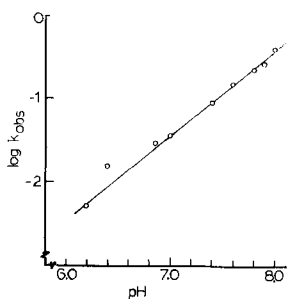


Fig. 3—A plot showing the relationship between observed rate constant and pH for the transformation of the ureide (compound V) at 35°.

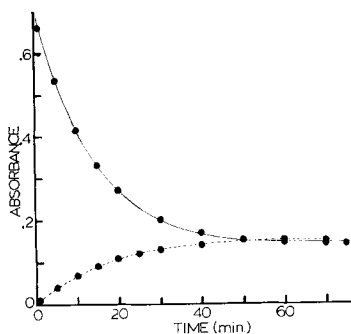


Fig. 4—A plot showing the time-dependent decrease in absorbance at 380 $m\mu$ for a 1.22×10^{-4} M solution of the ureide (V) (—) and the time-dependent increase in absorbance at 380 $m\mu$ for a 1.22×10^{-4} M solution of compound VI (---). The acid concentration was 0.58 N, $\mu = 1.0$, $T = 30^\circ$, and the solutions contained 2% dimethylformamide.

was first order with respect to hydroxide ion. The tabulated rate constants characterize rates of equilibration and are, therefore, sums of forward (k_f) and reverse (k_r) rate constants. The low equilibrium concentration of ureide precluded the accurate determination of an equilibrium constant and of the individual constants, k_f and k_r .

A similar transformation of the ureide, manifested by a loss of absorbance at 380 $m\mu$, was observed in acidic media. Treatment of compound VI with acid, in contrast, resulted in a time-dependent generation of a 380 $m\mu$ absorbing species. A typical result is shown in Fig. 4 where absorbance loss of a ureide-containing system and absorbance gain of a compound VI-containing system are plotted as a function of time. It is seen that, within experi-

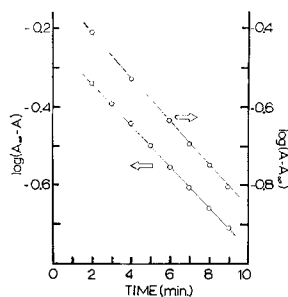


Fig. 5—A plot showing appearance ($A_{\infty} - A$) of 380 $m\mu$ absorbing species in solutions of compound VI and disappearance ($A - A_{\infty}$) of 380 $m\mu$ absorbing species from solutions of the ureide (V). The acid concentration was 0.58 N, $\mu = 1.0$, $T = 35^\circ$. The initial concentration of VI was 6×10^{-4} M. The initial concentration of V was 1.22×10^{-4} M. The solutions contained 2% dimethylformamide.

mental error, the same asymptotic absorbance value was approached by the two systems. Thin-layer chromatography of equilibrated solutions confirmed the presence of two components, the ureide and compound VI. Both solutions on long standing exhibited a gradual increase in absorbance at 380 $m\mu$. Spectrophotometric and chromatographic examination of aged solutions indicated the formation of the ketocarboxylic acid (III) which has significant absorbance at 380 $m\mu$. The semilog plots of Fig. 5 show that the rate of equilibration was first order with respect to starting compound. The rate of attainment of equilibrium was studied at a number of different acid concentrations and from both directions. In all cases, a first-order approach to equilibrium was observed. Rate constants and A_{∞} values are tabulated in Table II for systems in which the ureide was the starting material and in Table III for systems in which compound VI was the starting material. The results obtained with ureide systems are considered to be more accurate than those of the compound VI systems. A much lower concentration of ureide could be used to generate absorbance versus time plots. The compound VI systems were, by necessity, more concentrated and more susceptible to error because of the slow side-reaction which resulted in the formation of another compound which absorbed at 380 $m\mu$.

It is seen from both tables that the rate of equilibration was first order with respect to the concentration of hydrogen ion, that the equilibrium absorbance value was independent of the concentration of hydrogen ion, and that at a particular concentration of hydrogen ion, essentially the same rate constant was obtained regardless of whether the starting material was ureide or compound VI.

TABLE II—OBSERVED FIRST-ORDER RATE CONSTANTS AND A_{∞} VALUES OBTAINED IN FOLLOWING THE DECREASE IN ABSORBANCE AT 380 $m\mu$ EXHIBITED BY SOLUTIONS OF THE UREIDE IN ACID MEDIA^a

Normality of Acid	A_{∞}	$k_{\text{obs.}}$ (min. ⁻¹)
0.98	0.130	0.213
0.58	0.130	0.130
0.195	0.122	0.0452
0.097	0.125	0.0210

^a Temperature, 35°; μ , 1. The initial concentration of ureide was 1.22×10^{-4} M. The solutions contained 2% dimethylformamide.

TABLE III—OBSERVED FIRST-ORDER RATE CONSTANTS AND A_{∞} VALUES OBTAINED IN FOLLOWING THE INCREASE IN ABSORBANCE AT 380 $m\mu$ EXHIBITED BY SOLUTIONS OF COMPOUND VI IN ACID MEDIA^a

Normality of Acid	A_{∞}	$k_{obs.}$ (min. ⁻¹)
0.98	0.630	0.209
0.58	0.620	0.120
0.195	0.620	0.0403
0.097	0.600	0.0204

^a Temperature, 35°; μ , 1. The initial concentration of compound VI was 6.00×10^{-4} M. The solutions contained 4% dimethylformamide.

Equilibrium constants describing the ureide-compound VI interconversion could be calculated with reasonable accuracy from the data obtained with systems in which the ureide was the starting material. Here, the molar absorptivity of the ureide was calculated from absorbance values which were obtained by extrapolating the semilog plots to zero time. The concentration of ureide at equilibrium was then calculated from the observed A_{∞} value. The concentration of compound VI was obtained by difference between initial and final concentrations of ureide. The molar absorptivity was found to be independent of temperature and to have a value of 5750 (average of 11 determinations). The kinetics of ureide transformation was studied in 0.58 N hydrochloric acid at three different temperatures. Rate constants and equilibrium constants are presented in Table IV. As expected an increase in temperature resulted in an increase in the equilibrium concentration of ureide. The standard enthalpy change was estimated from these data to be approximately 4 Kcal. As can be seen, temperature had a much more pronounced influence on the rate of equilibration than on the equilibrium position.

DISCUSSION

Albert and Armarego (9) in their review of covalent hydration stated, "As chemists become more aware of the circumstances in which (covalent) hydration occurs and the means for detecting it, many new examples will probably be discovered and many puzzling discrepancies solved." A hydration-dehydration equilibrium such as depicted in Scheme I does provide a logical basis for explaining behaviors which previously had indeed been puzzling. Thus the spectral changes exhibited by the ureide in aqueous medium can be explained by the addition of water across the N_4-C_3 double bond to result in a reduction in the conjugation pathway of the molecule. Similarly, the conversion by heat of the white compound to yellow ureide is logically explained by a solid-state thermal dehydra-

tion. The anomalous elemental analysis could have resulted from partial dehydration by drying, to yield a mixture of hydrated and dehydrated product. In addition, Albert (9) reported that carbinolamines formed by the hydration of C=N bonds are readily oxidized by mild oxidizing agents to form oxo compounds and he suggested mild oxidation as a test for hydration and location of added water. That the dioxo compound (II) is formed by autoxidation of compound VI is added evidence to support hydration and the postulated structure. The kinetic data are consistent with those of other systems which were recently reviewed by Perrin (10).

Although the evidence strongly supports a hydration-dehydration phenomenon, the exact nature of the species undergoing reaction is not known. It is possible, for example, that in acid media both the ureide and the corresponding hydrate are protonated and that it was protonated species which underwent transformation. Similarly, at higher pH the reacting species could have been either neutral molecules or anions derived from the ionization of the imido hydrogen. Under all of the pH conditions studied, however, it was the hydrate that was the predominant species at equilibrium.

The identification of compound VI as a covalently hydrated ureide is strongly supported by the results of this investigation. The previously proposed scheme which summarized the degradative pathway of 9-methylisoalloxazine must then be revised and such a revision is presented in Scheme II.

It is also possible that the parent isoalloxazine might, in alkaline solution, undergo an equilibration to form a finite concentration of a hydrate resulting from the addition of water across the $N_{10}-C_9$ double bond and that this species, by attack of hydroxide ion at C_6 , directly formed the hydrated ureide. Although no confirmatory evidence of this possibility has yet been obtained, studies with 3,9-dimethylisoalloxazine, which will be reported in a future publication, do support such a possibility.

EXPERIMENTAL

Materials—1,2-Dihydro-1-methyl-2-oxo-quinoxaline-3-carboxyureide (V) and the compound (VI) which is now thought to be the ureide formed by the covalent hydration of V were synthesized by methods which were previously described (1).

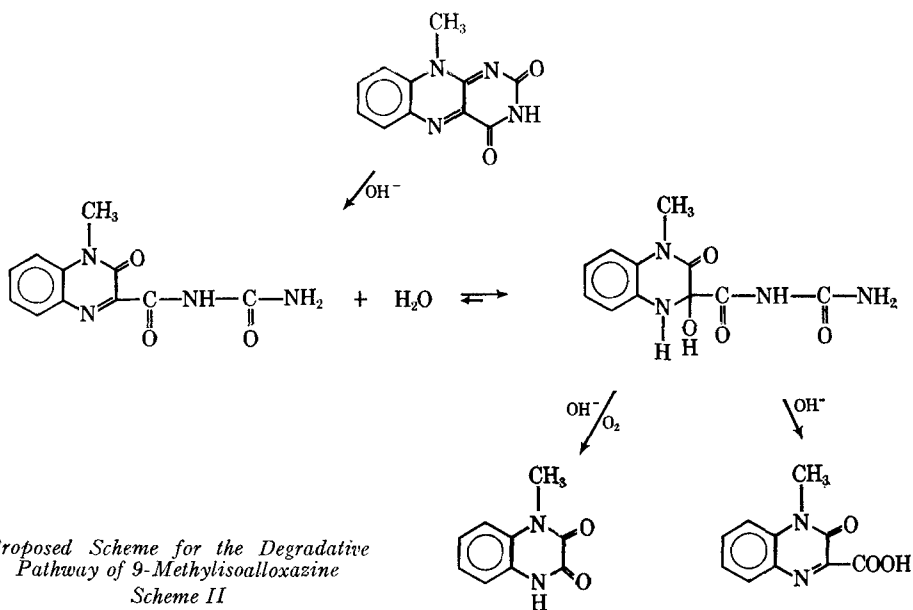
Thin-Layer Chromatography—Ten-microliter samples were applied on 300- μ silica gel plates and developed with a solvent system composed of chloroform-methanol-pyridine-water (180:15:1:1). After development, the plates were heated and examined under long wavelength ultraviolet light. The R_f value for the quinoxaline-3-carboxyureide was 0.90 and that for compound VI was 0.50.

Nonaqueous Titration—An accurately weighed

TABLE IV—INFLUENCE OF TEMPERATURE ON THE RATE OF EQUILIBRATION AND EQUILIBRIUM POSITION OF SYSTEMS INITIALLY CONTAINING 1.22×10^{-4} M UREIDE^a

Temp., °C.	A_{∞}	$K_{equil.}$	$k_{obs.}$ (min. ⁻¹)	k_r (min. ⁻¹)	k_f (min. ⁻¹)
10	0.080	7.75	0.00930	0.00106	0.00824
35	0.130	4.55	0.130	0.0234	0.107
50	0.172	3.07	0.413	0.101	0.312

^a The acid concentration was 0.58 N and the ionic strength was 1.0. The solutions contained 2% dimethylformamide. The equilibrium constant was calculated as the ratio (compound VI)/(ureide).



sample of compound VI was dissolved in 5 ml. of dimethylformamide and titrated with 0.1 *N* sodium methoxide in methanol to a thymol blue end point.

Spectra—All spectra were obtained with a Beckman model DB recording spectrophotometer.

Kinetic Studies—Stock solution of the ureide and of compound VI were prepared in dimethylformamide. Reaction mixtures were prepared by adding an aliquot of a stock solution to a volumetric flask containing a required volume of buffer or hydrochloric acid solution and at the desired temperature. The solution contained sufficient potassium chloride to make an ionic strength of 1.0. The solution was quickly mixed and a portion introduced to a cell which was placed in the cell compartment of a Beckman model DB spectrophotometer set at a wavelength of 380 $m\mu$ and equipped with a Sargeant SR recorder. The cell compartment of the spectrophotometer was maintained at the desired temperature by circulation of constant-temperature water from a P. M. Tamson

N.V. circulating bath. Absorbance *versus* time plots were traced by the recorder. Zero time was taken to be the time at which the aliquot of stock solution of reactant was introduced to the volumetric flask. Limited studies showed that, in the concentration range encountered, changes in the dimethylformamide concentration of the reaction mixture had no effect on the observed rate.

REFERENCES

- (1) Wadke, D. A., and Guttman, D. E., *J. Pharm. Sci.*, **55**, 1088(1966).
- (2) *Ibid.*, **55**, 1363(1966).
- (3) Kuhn, R., and Reinmund, K., *Chem. Ber.*, **67**, 1932 (1934).
- (4) Tishler, M., Wellman, J. W., and Ladenburg, K. J., *J. Am. Chem. Soc.*, **67**, 2165(1945).
- (5) King, F. E., and Clark-Lewis, J. W., *J. Chem. Soc.*, **1951**, 3080.
- (6) *Ibid.*, **1951**, 3379.
- (7) Clark-Lewis, J. W., *Chem. Ind.*, **1966**, 241.
- (8) Rudy, H., and Majer, O., *Ber.*, **71B**, 1323(1938).
- (9) Albert, A., and Armarego, W. L. F., in "Advances in Heterocyclic Chemistry," vol. IV, Katritzky, A. R., Boulton, A. J., and Lagowski, J. M., eds., Academic Press Inc., New York, N. Y., 1965, chap. 1.
- (10) Perrin, D. D., in *ibid.*, chap. 2.