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Hydrolysis of 2-Methyl-Δ^2-oxazoline. An Intramolecular O–N-Acetyl Transfer Reaction

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Like the corresponding thiazoline system, 2-methyloxazoline exhibits inhibition of hydrolysis in acid solutions. At equilibrium the N-acetylethanolamine to oxazoline ratio is only 18. Ring closure of O-acetylethanolamine to form a hydroxyoxazolidine intermediate is 140 times faster than for the corresponding thiol compound. The thiol ester undergoes acetyl transfer more rapidly, however, because the partitioning of the cyclic intermediate favors the oxygen ester 6×10^6 to 1 over the amide. The unusual value for the equilibrium constant for O–N transfer is discussed. In anhydrous formic acid, formylation of proteins occurs rather than the thermodynamically favored acyl transfer reaction because of the more rapid rate of the former.

The pK_a and rate of hydrolysis of 2-methyloxazoline from pH measurements and the evidence that it is not an intermediate in an O–N-acetyl transfer reaction have been reported.[1] In this paper we extend the measurements to include an evaluation of the equilibrium constants for the several species at equilibrium. In addition a detailed spectrophotometric analysis is made of the kinetics of oxazoline hydrolysis and comparison made with the results of the thiazoline analog.[2,3]

Experimental

O-Acetylethanolamine[4] and 2-methyloxazoline[5] were synthesized as described elsewhere. N-Acetylethanolamine was an Eastman Kodak Co. product, redistilled before use. Spectrophotometric measurements were made with a Cary 11 spectrophotometer in the 200 mμ region. Even in this low wave length region maxima are not observed; hence it is necessary to work on the slopes of the absorption curves which rise to a maximum further in the ultraviolet. Formate, acetate and phosphate buffers at about 10^{-2} M concentration were used to control the pH. Beckman model G and Radiometer 4 pH meters were used in pH meter experiments. For experiments at constant pH a Radiometer TTTl pH stat was employed. All the experiments were performed at 25° and 0.10 ionic strength controlled with KCl.

Results

We confirm the rate measurements previously reported[1] by following the initial rise in pH with time in solutions of 2-methyloxazoline which are 25%, 50% or 75% neutralized. The pH rises for about 4 hours due to the formation of O-acetylethanolamine after which it drops as the concentration of N-acetylethanolamine increases. The best composite values of several runs, 0.1 M or less in oxazoline, for the maximum and final equilibrium pH values, respectively, for the following percentages of free base oxazoline are: 25%, 6.6, 5.3; 50%, 7.1, 5.9; 75%, 7.8, 6.4. These results may be analyzed to yield the equilibrium constants for the three species present at equilibrium.

Let the following symbols represent the molar concentrations of the components of the system at equilibrium: (TH^+), oxazoline in cationic form; (T), oxazoline in basic form; (SH^+), O-acetylethanolamine in cationic form; (S), O-acetylethanolamine in basic form; and (N), N-acetylethanolamine. Also let C be total concentration of all the above components

$$C = (TH^+) + (T) + (SH^+) + (S) + (N)$$

If y is the number of equivalents of hydrochloric acid added originally, we may also write $y = (Cl^-)/C$. Define the ionization constants, $K_1 = (T)(H^+)/(TH^+)$ and $K_2 = (S)(H)/(SH^+)$; and the equilibrium constants, $K_{ST} = (SH^+)/(TH^+)$, $K_{NT} = (N)/(T)$ and $K_{NS} = (N)(H^+)/(SH^+)$. Only two of the last three equilibrium constants are independent because $K_1K_{NT} = K_{ST}K_{NS}$.

Electroneutrality demands that

$$(H^+) + (TH^+) + (SH^+) = (Cl^-) + (OH^-)$$

In all the cases to be considered (OH^-) is negligible. Also let $x = y - [H^+]/C$ where the brackets denote the concentration of hydrogen ion rather than the activity as measured on the pH meter. The last term need be considered only in acid solutions, since it is negligible in neutral solutions where $x = y$. From the above equations it may be shown that at equilibrium

$$\frac{x}{1 - x} = \frac{(H^+)(1 + K_{ST})}{K_1(1 + K_{NT}) + K_2K_{ST}} \qquad (1)$$

Thus measurements of the equilibrium pH values determine the ratios of equilibrium constants. For the oxazoline system the last term in the denominator is negligible because $K_2 = 8 \times 10^{-10}$ M. In addition, if K_{ST} and K_{NT} are both much greater than unity, which result implies no oxazoline of consequence at equilibrium, then eq. 1 reduces to $x/(1 - x) = (H^+)/K_{NS}$, and hence one equilibrium constant is uniquely determined.

In the oxazoline system the maximum in the curve of pH *versus* time is due to the formation of O-acetylethanolamine at a much greater rate than N-acetylethanolamine. At the maximum in the pH *versus* time curve an equilibrium does exist between oxazoline and O-acetylethanolamine, but N-acetylethanolamine is in equilibrium with neither. Let $a = 1 - (N)/C$ to allow for the small amount of N-acetylethanolamine formed at the maximum in the curve. If (S) is small compared with (SH^+), it may be shown that at the maximum in the *pH versus* time curve

$$\frac{x}{a - x} = \frac{(H^+)(1 + K_{ST})}{K_1} \qquad (2)$$

Hence K_{ST} may be independently evaluated if we know a or approximate it as unity.

We may also combine, for a single run, the pH measurements at the maximum and at equilibrium

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to obtain

$$K_{NT} = \frac{(H^+)_{equil}}{(H^+)_{max}}\left(\frac{1-x}{a-x}\right) - 1 \qquad (3)$$

Since only two of the equilibrium constants are independent, their separate evaluation by eq. 1 and 2 should be consistent with eq. 3.

The validity of the method has been checked by applying it to the 2-methylthiazoline system, where satisfactory agreement is observed with the equilibrium constants independently evaluated by other methods.[3] Since the equilibrium values of the thiazoline system are about pH 2 or less, $x \neq y$ in this case and the H^+ activity coefficient correction is important. The oxazoline system, on the other hand, yields equilibrium pH values near pH 6 so that $x = y$, which presents a more favorable case for analysis by the method outlined.

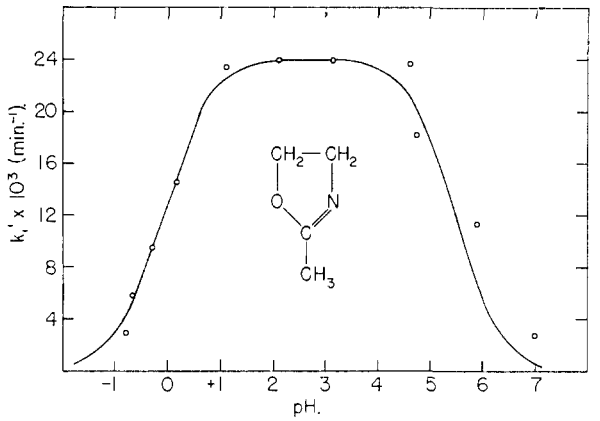

Fig. 1.—Initial rate of hydrolysis of 2-methyloxazoline as a function of pH from spectrophotometric measurements at 25° and 0.10 ionic strength. Circles are experimental points. Curve is drawn according to eq. 4 with $k_1 = 24 \times 10^{-3}$ min.$^{-1}$ and $(k_3 + k_5)/k_2 = 1.2$. The curve for 2-methylthiazoline, if drawn on the same scale, would be of the same shape and approximate position on the abscissa, but only $^1/_{28}$ as high.

Equations 1 and 2 indicate that for the results reported in the first paragraph of this section, where $x/(1-x)$ is $^1/_3$, 1 and 3, both the equilibrium and maximum pH values for the three successive solutions should be separated by 0.48 pH unit. Although each recorded value is the best value for several runs, the values are not wholly self-consistent. The pH values are in an unbuffered region and hence susceptible to contamination. Nonetheless the equilibrium constants evaluated by the methods just described give $K_{ST} = 45$, $K_{NT} = 18$ and $K_{NS} = 1.3 \times 10^{-6}$ to within 10%. These values are sufficiently accurate to be useful in the evaluation of rate constants and to provide a basis of comparison with other systems.

Figure 1 shows the initial rate of hydrolysis of 2-methyloxazoline *versus* pH as determined from spectrophotometric measurements at 205 or 210 mμ. Even though the reactant oxazoline and product N-acetylethanolamine absorb similarly in this region, the hydrolysis of oxazoline may be followed as an initial rate because non-absorbing O-acetylethanolamine is the main initial product. Figure 1 shows inhibition of hydrolysis in acid solutions as has been observed for thiazoline derivatives.[2,3] Due to this inhibition the mechanism of oxazoline hydrolysis proposed by Porter, Rydon and Schofield[1] cannot be correct because it contains no acid-inhibited step. Instead we apply the hydrolysis mechanism already indicated for thiazoline compounds.

$$TH^+ \rightleftharpoons T + H^+ \quad K_1 = (H^+)(T)/(TH^+)$$

$$TH^+ + H_2O \underset{k_2}{\overset{k_1}{\rightleftharpoons}} H^+ + D$$

$$N \underset{k_4}{\overset{k_3}{\rightleftharpoons}} D \underset{k_6}{\overset{k_5}{\rightleftharpoons}} S$$

$$SH^+ \rightleftharpoons S + H^+ \quad K_2 = (H^+)(S)/(SH^+)$$

where the symbols are the same as those already introduced in the equilibrium discussion and D is a hydroxyoxazolidine intermediate. Applying the steady state approximation to D yields for the initial rate of disappearance of oxazoline

$$-\frac{dC_T}{dt} = \frac{k_1 C_T(H^+)[(k_3 + k_5)/k_2]}{[K_1 + (H^+)][(H^+) + (k_3 + k_5)/k_2]} = k_1' C_T \qquad (4)$$

where $C_T = (T) + (TH^+)$.

Equation 4 provides a satisfactory fit to the experimental points with $k_1 = 24 \times 10^{-3}$ min.$^{-1}$, $pK_1 = 5.5$ and $(k_3 + k_5)/k_2 = 1.2$ M. The k_1-value is determined by the maximum, pK_1 by the mid-point of the right-hand descent and the last collection of constants by the left-hand ascent of the curve of Fig. 1. The k_1-value is the same as that determined by the initial rate of change of pH of partially neutralized oxazoline solutions on the pH meter, and the pK_1 value used in the smooth curve of Fig. 1 is that determined by extrapolation of the pH–time curve to zero time. These agreements of two independent methods and the observed acid inhibition in acid solutions provide strong support for the mechanism and the existence of a tetrahedral intermediate. Since the O-acetyl derivative is made much more rapidly than the N-acetyl, $(k_3 + k_5)/k_2 = k_5/k_2$ in the oxazoline system.

The equilibrium constants already defined may be expressed in terms of the rate constants to give $K_{ST} = k_1k_5/k_2k_6K_2$, $K_{NS} = k_3k_6K_2/k_4k_5$ and $K_{NT} = k_1k_3/K_1k_2k_4$. From the first equation we may calculate $k_6K_2 = 6.4 \times 10^{-4}$ min.$^{-1}$ M from the constants already evaluated.

According to the reaction scheme, the initial rate of disappearance of solutions originally containing O-acetylethanolamine is given by[2]

$$k_6' = \frac{k_6K_2[(H^+) + k_3/k_2]}{(H^+)[(H^+) + (k_3 + k_5)/k_2]} \qquad (5)$$

For $7.1 < p\text{H} < 7.7$ the disappearance of 10^{-3} M O-acetylethanolamine is inverse first order in (H^+) as followed on the pH stat or by the appearance of N-acetylethanolamine on the Cary 11 spectrophotometer. Since we already know that $k_3/k_2 << k_5/k_2 = 1.2$ M, eq. 5 can only exhibit $(H^+)^{-1}$ dependence when $k_3/k_2 > (H^+)$ so that eq. 5 reduces to $k_6'(H^+) = k_6K_2k_3/k_5$. No pronounced general catalytic action is observed in the $7.1 < p\text{H} < 7.7$ region where $k_6'(H^+) = 1.0 \times 10^{-10}$ min.$^{-1}$ M. Therefore $k_5/k_3 = 6.4 \times 10^6$, con-

sistent with the partitioning of the hydroxyoxazolidine intermediate strongly in favor of the O-acetyl derivative.

Porter, *et al.*, studied the *p*H decrease with time in half-neutralized solutions of O-acetylethanolamine hydrochloride.[1] In solutions of such high *p*H considerable intermolecular reaction effects occur and little can be inferred concerning intramolecular acetyl transfer. By carefully extrapolating such *p*H-time plots to zero time, however, we obtain $pK_2 = 9.1$. Thus $k_6 = 8 \times 10^5$ min.$^{-1}$. Controlled addition of base so that the *p*H is never greater than 7.5 yields equilibrium *p*H values similar to those obtained for oxazoline.

The remaining rate constants may be easily evaluated from the rate and equilibrium constants already determined. A summary of the results is given in Table I. The pK_2 value in the thiazoline system was taken in part from the one in the oxazoline system.[3] The k_4-value for oxazoline is computed from the other known constants of the oxazoline system. Direct determination of k_4 in strongly acidic solutions of N-acetylethanolamine is compromised by concomitant hydrolysis, but the directly determined k_4 value is consistent with the recorded value.

TABLE I

RATE AND EQUILIBRIUM CONSTANTS FOR OXAZOLINE AND THIAZOLINE SYSTEMS AT 25° AND 0.10 IONIC STRENGTH

	Oxazoline	Thiazoline
K_{ST}	45	11
K_{NS}, M	1.3×10^{-6}	4.5×10^{-2}
K_{NT}	18	8.5×10^4
pK_1	5.5	5.2
k_1, $\times 10^3$ min.$^{-1}$	24	1.05
$(k_3 + k_5)/k_2$, M	1.2	0.11
k_5/k_2, M	1.2	0.06
k_3/k_2, M	1.9×10^{-7}	0.05
k_5/k_3	6.4×10^6	1.2
k_6K_2, min.$^{-1}$ M	6.4×10^{-4}	4.5×10^{-6}
k_4, min.$^{-1}$	8×10^{-5}	10×10^{-5}
pK_3	9.1	9.1

Discussion

To the extent that the oxazoline system studied here may serve as a model system for oxazoline ring formation in proteins, the value of $K_{NT} = 18$ implies one oxazoline ring for every 18 serine residues. This number is considerably greater than the number of thiazoline rings indicated from cysteine residues.[2] Some proteolytic enzymes contain a sufficient number of serine residues so that 1 oxazoline ring might be considered thermodynamically probable. The thermodynamic argument should not be extended too far, however, since the equilibrium position for the components of a protein is the constituent amino acids.

Comparison of the k_6K_2 values reveals that ring closure is 140 times faster for O-acetylethanolamine than for the corresponding S-acetyl compound. It is unlikely that differences in K_2-values can account for all this difference which is opposite from the expected direction. The thiol ester still disappears much more rapidly than the carboxylic acid ester, however, because ring opening favors the oxygen ester 6 million to 1 over the amide whereas the thiol ester and amide are about equally probable. These results imply that amines react more rapidly with carboxylic acid esters than with thiol esters in the initial combination step, but that breakdown of the intermediate complex into products rather than reactants is much more likely in the case of the sulfur-containing intermediate. The partitioning of the cyclic intermediate is not so easily evaluated in other systems where no dehydration by-product of the acyl transfer cyclic intermediate exists.[6]

The equilibrium constant for the acetyl transfer reaction S-acetylmercaptopropanol to the O-acetyl derivative[7] is 56 at 39°. This value is probably quite temperature independent and is in agreement with a value of about 10^2 deduced from comparison of tabulated data of O and S esters.[8] Dividing the K_{NS} value for the thiazoline system by 56 should yield a value close to the K_{NS} value for the oxazoline system, hereafter called K_{NO}. However, $4.5 \times 10^{-2}/56 = 8 \times 10^{-4}$ is 620 times greater than the observed $K_{NO} = 1.3 \times 10^{-6}$ for the oxazoline system. If the thiazoline K_{NS} value is normal, evidently more O-acetylethanolamine or less N-acetylethanolamine is present at equilibrium than we might predict.

It is worth attempting some estimates of the K_{NS} and K_{NO} values in order to determine which value is unusual. At 25° and *p*H 7 the free energy change for S–N transfer is $\Delta G = -1.36 \log (4.5 \times 10^5) = -7.7$ kcal./mole. Under the same conditions the free energy of hydrolysis of a thiol ester may be taken[7] as -7.3 kcal./mole. The difference $+0.4$ kcal./mole should approximate the free energy of hydrolysis of the amide bond in N-acetyl-β-mercaptoethylamine. This value is more positive than expected by about 1 kcal./mole. Utilization of about -0.5 kcal./mole for the free energy of hydrolysis of an amide bond would predict a K_{NS} smaller than the observed value, but only by a factor of 4.5.

Similarly, the free energy change for O–N transfer from K_{NO} at 25° and *p*H is -1.5 kcal./mole. Taking the free energy of hydrolysis of an acetyl oxygen ester,[7] under the same conditions, as -4.8 kcal./mole yields -3.3 kcal./mole as the free energy of hydrolysis of the amide bond in N-acetylethanolamine. This high negative value is in the range observed for dipeptides where charge repulsion is usually invoked to account for the unusually large result. No such explanation is possible to account for the large negative value observed for N-acetylethanolamine, which value is responsible for K_{NO} being 10^{-2} times that expected. Of course, we could have proceeded from the other direction, assumed a normal free energy of hydrolysis for the amide bond and then found the free energy of hydrolysis of O-acetylethanolamine to be less negative than normal. Looking at it as above, however, the conclusion is that the OH group of N-acetylethanolamine activates the amide bond, whereas the SH group does not activate the amide bond in the corresponding thiol derivative. This activation should be interpreted in the thermo-

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dynaimic sense only because the k_4 values for the two systems are similar. Mechanistically the effect can be ascribed to the partitioning of the intermediate, already discussed, which so strongly favors the ester over the amide in the oxazoline system.

An N–O-acyl shift occurs when proteins are placed in concentrated mineral acids.[9] In anhydrous formic acid, however, formylation of the serine residues of proteins takes place with little acyl transfer.[10,11] In solutions of low water content the equilibrium constant for ethyl formate formation,[12] $K_E = (\text{ester})^2/(\text{acid})(\text{alc}) \simeq 5$. Combining this constant with $K_{NO} = (N)(H^+)/(OH^+)$, where (OH^+) is the concentration of O-acetylated residues, we obtain $K = (\text{ester})^2/(OH^+) = K_E K_{NO}(\text{acid})/(H^+)$. The acidity function[13] of anhydrous, 26 M, formic acid is 160. Substitution of these values with those of the equilibrium constants yields $K \simeq 10^{-6}$. This equilibrium constant indicates that N–O transfer is thermodynamically favored over formylation when proteins are placed in anhydrous formic acid. The small value of K is well beyond any uncertainties introduced by unusual values of equilibrium constants as discussed above. The conclusion is that formylation rather than transfer occurs because the former reaction is favored kinetically. This conclusion is borne out by a half-life for formylation of about 4 hours[11] as compared with longer times for transfer to occur.[9] Possibly the free amino acid serine does not formylate under the same conditions because the positive charge on the molecule inhibits the acid-catalyzed esterification.

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Dependence of the *cis* Effect on Ring Size. Magnitude of the Steric Interaction of the Phenyl Rings in *cis*-1,2-Diphenylcyclopentane and Demonstration of Restricted Rotation[1]

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Equilibration of *cis*- and *trans*-1,2-diphenylcyclopentane at 110° has given an equilibrium constant of 33 which is compared with data available for the stilbenes and the 1,2-dimethylethylenes, cyclopropanes and cyclopentanes. Evidence for restricted rotation of the phenyl groups of *cis*-1,2-diphenylcyclopentane (but not for the stilbenes, diphenylcyclopropanes or *trans*-1,2-diphenylcyclopentane) has been adduced from an unusually broad aromatic proton spectrum in the n.m.r., with narrowing when the sample is heated.

Some of the consequences of the steric interaction of substituents adjacent to one another in 2- (olefin-forming), 3-, 4- and 5-membered cyclic transitions states have been reviewed.[2] Even a semi-quantitative knowledge of the magnitudes of such *cis* effects has been almost entirely lacking, however. It has seemed likely that there should be significant variations of the *cis* effect with ring size in 2-, 3-, 4- and 5-membered cyclic compounds. The present investigation is concerned with the effect on the free energy of the *cis*-1,2-diphenyl interaction in *cis*-1,2-diphenylcyclopentane (*cis*-I) (relative to the *trans* isomer). A further consequence of the proximity of the two phenyl groups in this molecule is the restriction of the rotation of the phenyl rings around the single bonds joining them to the cyclopentane structure. Evidence from n.m.r. studies supporting such restricted rotation will be reported later in this paper.

Equilibration of *cis*- and *trans*-1,2-Diphenylcyclopentane (*cis*-I).—*cis*- and *trans*-1,2-diphenylcyclopentane had been prepared previously by Weidlich.[3] It was desired to equilibrate these two substances in order to have a direct measure of the free energy difference between the *cis* and *trans* isomer. Equilibrations were carried out at 110° using potassium in di-*n*-butylamine and also 5% palladium-on-charcoal in the presence of hydrogen to prevent dehydrogenation.[4] Equilibrium was approached from both the *cis* and *trans* side. Since preliminary experiments indicated that the equilibrium lay well toward the *trans* isomer a rather sensitive analytical method was needed and a differential infrared spectroscopic method employing differences in absorption at 690 and 750 cm.$^{-1}$ was found satisfactory. The results are summarized in Table I.

The average value of the results at 110° and the probable error are 2.91 ± 0.07 which corresponds to an equilibrium constant at this temperature of 33.4 ± 0.8 and a free energy difference of 2.68 ± 0.16 kcal. It is striking that measurements of the equilibrium constants[5] of the reactions of *meso*- and *dl*-hydrobenzoin with the *cis*- and *trans*-cyclic acetals (*cis*- and *trans*-II), respectively, combined with an estimated free energy difference

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