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Hydrolysis of 2-Methyl-Δ^2-thiazoline and its Formation from N-Acetyl-β-mercaptoethylamine. Observations on an N–S Acyl Shift[1]

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The rate of hydrolysis of 2-methyl-Δ^2-thiazoline has been studied as a function of pH, in media ranging from concentrated HCl to buffer solutions at pH 9. The reaction was followed by measurement of ultraviolet absorption at 260 mμ and also by Raman spectroscopy. The rate shows a maximum at about pH 3, the molecule being quite stable in concentrated HCl and hydrolyzing very slowly in neutral solutions. A reaction scheme is proposed, assuming a hydroxythiazolidine as an intermediate, to explain the observed results, including the appearance of both N- and S-acetyl-β-mercaptoethylamines. Studies on the reactions of the two latter compounds in solution at various pH values gave results concordant with the scheme. Velocity constants for most of the processes involved in the scheme are recorded. The pK_a value of the conjugate acid of 2-methylthiazoline was found to be 5.22 ± 0.02 at ionic strength 0.10. The reactions of N-acetyl-β-mercaptoethylamine and of glutathione, to yield thiazoline rings or thiol esters, are compared. It is concluded that the open chain N-acetyl form is thermodynamically stable in neutral solution relative to the thiazoline, although the hydrolysis of the latter is very slow near pH 7 in solutions of 2-methyl-Δ^2-thiazoline. The implications of the observations for the hypothesis of thiazoline formation from cysteinyl residues in proteins are discussed.

In 1940 Linderstrøm-Lang and Jacobsen[2] investigated the opening of the ring of 2-methyl-Δ^2-thiazoline to yield the sulfhydryl compound N-acetyl-β-mercaptoethylamine and its S-acetyl isomer. They pointed out the possible importance of this type of structure, which might explain the unreactivity of many sulfhydryl groups in native proteins, and their reactivity after the protein was denatured. Since then a thiazoline ring has been indicated in Bacitracin-A[3] and the proposal of Calvin[4] that thiazoline ring formation occurs in strongly acid solutions of glutathione has been supported by further work.[5,6]

The formation of an apparently stable thiazoline structure in strongly acid solutions of glutathione[6] stood in apparent contrast to the results of Linderstrøm-Lang and Jacobsen[2] which suggested that the acid form of 2-methyl-Δ^2-thiazoline is unstable. These considerations led us to undertake a more extensive kinetic study of the hydrolysis of this compound, over a range of pH from concentrated HCl solutions to pH 9. In addition its formation from N-acetyl-β-mercaptoethylamine has been studied as an alternative reaction to an N→S acyl transfer. Some further observations on molecular rearrangements in acid solution of glutathione are reported and certain implications discussed.

Experimental

Preparations of 2-methyl-Δ^2-thiazoline were obtained from the Aldrich Chemical Co. of Milwaukee and from Light and Co., England. The middle cut of the vacuum distilled product was used in the experiments. Both products gave identical absorption spectra which are recorded in Fig. 1 under various conditions. The spectrum in neutral solution in Fig. 1, curve B, is in agreement with that obtained in Linderstrøm-Lang's laboratory and recorded in ref. 3. However, the extinction coefficients shown in curve B of Fig. 1 are about 30 times greater than those reported by Basford and Huennekens,[7] though the shape of the curve is nearly identical. The spectrum in methanol differs considerably from that previously reported in this solvent.[8] The ultraviolet spectrum in acid solution has been reported.[4] This spectrum was uniform from pH 2 to concentrated HCl as was the Raman spectrum (see Fig. 2).

The pKa of 2-methylthiazoline at 25° and 0.10 μ was found to be 5.22 ± 0.02 as determined by titration with the glass electrode and by spectrophotometric titration.[9] Linderstrøm-Lang and Jacobsen[2] reported a value of 5.37 under the same conditions; their measurements, however, were made with a quinhydrone rather than a glass electrode. We are unable to offer an explanation of the discrepancy between their results and ours. We noted, as they did, that the hydrolysis of thiazoline during the course of the measurements of pH and of absorbancy caused the readings to vary with time in the more acid solutions. It was therefore necessary to plot such measurements as a function of time and extrapolate back to zero time. When this was done, however, consistent values of pK_a were obtained over the pH range from 4.5 to 6.0.

The Raman spectrum of 2-methylthiazoline was determined, with a Cary Raman spectrophotometer, model 81, on the pure liquid, on a solution of the free base in water, and on the solution of its conjugate acid in HCl, and also in DCl in D_2O solution. The data are recorded in Table I and the recorded spectrum for the acid solution in HCl is shown in Fig. 2. It will be seen that the spectrum of the free base in water is very close to that of the pure liquid, whereas marked changes occur on the addition of a proton to the nitrogen of the thiazoline ring. Addition of a deuteron to the nitrogen (right hand column of Table I) gives a somewhat different spectrum from that due to proton addition. The lines which are unchanged by proton addition are in general also unaffected by deuteron addition to the nitrogen. Further discussion of the uses of the Raman spectra in studying the reactions of 2-methyl-Δ^2-thiazoline is given later.

N-Acetyl-β-mercaptoethylamine was synthesized by two methods; that of Kuhn and Quadbeck[10] gave a product contaminated with about 8% thiazoline as determined spectroscopically. For the experiments reported here material was prepared by slowly adding to one mole of acetyl chloride a cold chloroform solution containing two moles of β-mercaptoethylamine (cysteamine) obtained from the Mann Research Laboratories. The chloride salt of the latter was filtered off

(1) The work was supported by grants from the United States Public Health Service (H-3169) and the National Science Foundation (G-3230). A preliminary account was reported at the meeting of the American Society of Biological Chemists at Atlantic City, April, 1959 (see *Federation Proc.*, **18**, 282 (1959)).

(2) (a) K. Linderstrøm-Lang and C. F. Jacobsen, *Compt. rend. trav. lab. Carlsberg, Ser. chim.*, **23**, 289 (1940); (b) K. Linderstrøm-Lang and C. F. Jacobsen, *J. Biol. Chem.*, **137**, 443 (1941).

(3) J. R. Weisiger, W. Hausmann and L. C. Craig, THIS JOURNAL, **77**, 3123 (1955).

(4) M. Calvin in "Glutathione," edited by S. Colowick, *et al.*, Academic Press, Inc., New York, N. Y., 1954, p. 3.

(5) (a) D. Garfinkel, THIS JOURNAL, **80**, 4833 (1958); (b) G. Préaux and R. Lontie, "Protides of the Biological Fluids," Elsevier Pub. Co., Amsterdam, 1958, p. 217.

(6) R. B. Martin and J. T. Edsall, *Bull. soc. chim. biol.*, **40**, 1763 (1958).

(7) R. E. Basford and F. M. Huennekens, THIS JOURNAL, **77**, 3878 (1955). In a private communication Dr. F. M. Huennekens has informed us that he has rechecked and repeated his measurements and finds that his results are now in general agreement with ours.

(8) R. Kuhn and F. Drawert, *Liebigs. Ann. Chem.*, **590**, 55 (1954).

(9) For the methods used, see R. B. Martin, J. T. Edsall, D. B. Wetlaufer and B. R. Hollingworth, *J. Biol. Chem.*, **233**, 1429 (1958).

(10) R. Kuhn and G. Quadbeck, *Ber.*, **84**, 844 (1951).

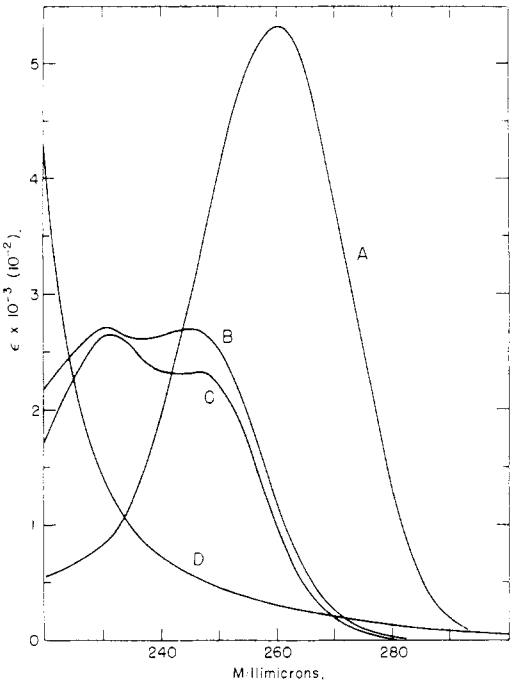

Fig. 1.—Curves A, B, C are spectra of 2-methylthiazoline in 0.1 M HCl, phosphate buffer, pH 8.2, and methanol, respectively, ordinate scale $\epsilon \times 10^{-3}$. Curve D represents N-acetyl-β-mercaptoethylamine in water, scale $\epsilon \times 10^{-2}$.

and evaporation of chloroform from the filtrate yielded an oil similar to that previously obtained.[7,10] The difference between the spectrum recorded in Fig. 1 and that previously reported by Basford and Huennekens[7] is probably to be explained by the presence of thiazoline as an impurity in their sample. Freshly prepared product was used in all experiments reported here, since thiazoline is formed on standing. S-Acetyl-β-mercaptoethylamine was synthesized from acetyl chloride and β-mercaptoethylamine hydrochloride as described by Wieland and Bokelmann.[11] The ultraviolet spectrum exhibits a maximum at 229 mμ with a molar extinction coefficient of 4350. The glutathione and equipment used have been described previously.[6]

Results

It has been shown previously that the initial products of hydrolysis of 2-methyl-Δ^2-thiazoline are N- and S-acetyl-β-mercaptoethylamine.[2] These products may decompose to form acetic acid and β-mercaptoethylamine. The latter process is irreversible under the conditions of the experiments reported here.

The rate of disappearance of thiazoline[12] was followed by observing the change in absorbancy at 260 mμ and was found to be first order. By observing the rate of change of the absorbancy at 230 mμ, the rate of formation of S-acetyl-β-mercaptoethylamine was deduced.

To analyze the course of the reaction the extinction coefficient of each species contributing to the absorbancy at a given wave length must be known. These expressions were used at 260 and 230 mμ, respectively

$$A_{260} = 5300(TH^+) + 1250(T) + 32(N\text{–}SH) + 80(S\text{–}NH_3^+) + 14(MH^+)$$

(11) T. Wieland and E. Bokelmann, *Ann. Chem.*, **576**, 20 (1952).

(12) For brevity we shall denote 2-methyl-Δ^2-thiazoline simply as thiazoline in the following discussion. For the abbreviations used to denote this and other compounds, see Fig. 4.

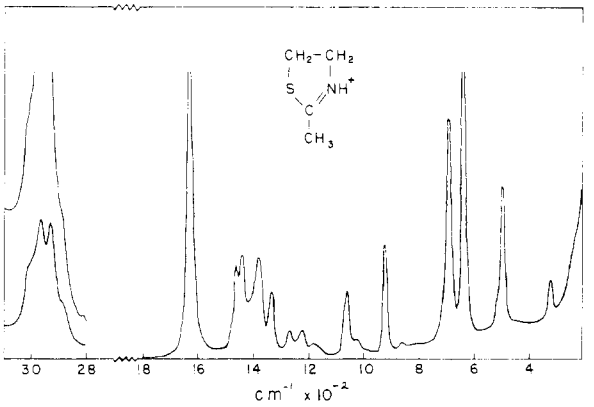

Fig. 2.—Raman spectra of 2 ml. of 2-methyl-Δ^2-thiazoline in 5 ml. of concentrated HCl. This spectrum is invariant with time in strong acid. Initial spectrum at pH 1, as described in the text, is identical with that shown here, but it changes with time as described in the discussion of kinetics. The peaks in the region 2800–3000 cm.$^{-1}$ were off the scale when recorded at the same setting as the other Raman bands. The lower tracing in this region shows a reading recorded at 0.40 sensitivity of the instrument.

$$A_{230} = 830(TH^+) + 2700(T) + 146(N\text{–}SH) + 4350(S\text{–}NH_3^+) + 80(MH^+)$$

where (TH^+) and (T) represent the molar concentrations of the acidic and basic forms of thiazoline, respectively, (N–SH) and $(S\text{–}NH_3^+)$ the concentrations of N- and S-acetyl-β-mercaptoethylamine, respectively, and (MH^+) that of the conjugate acid of β-mercaptoethylamine. Acetic acid does not absorb appreciably at these wave lengths. All the values were determined by direct measurement.

For a solution of thiazoline (T) and its conjugate acid (TH^+) the initial rate of transformation into both the N- and S-acetyl-β-mercaptoethylamines (N–SH and $S\text{–}NH_3^+$, respectively) may be simply represented as

$$N\text{–}SH \xleftarrow{k_3'} T \xrightarrow{k_5'} S\text{–}NH_3^+$$

Then

$$-\frac{dC_T}{dt} = (k_3' + k_5')C_T = k_1'C_T \quad (1)$$

where

$$C_T = (T) + (TH^+) \quad (2)$$

For this case

$$A_{260} = \epsilon_{260}(C_T) + \epsilon_p(P) \quad (3)$$

Here ϵ_{260} is the observed extinction of the thiazoline at zero time (5300 for solutions of pH < 3) and ϵ_p is the extinction of the products which is nearly negligible, but may be taken as 100. From the above two equations an expression for k_1' is obtained

$$k_1' = \frac{-1}{(\epsilon_{260} - \epsilon_p)C_T}\frac{dA_{260}}{dt} \quad (4)$$

This is the rate constant plotted in Fig. 3 (open circles) for the disappearance of thiazoline.

Similarly at 230 mμ for a solution containing only thiazoline initially, we may write $A_{230} = \epsilon_{230}(C_T) + 146(N\text{–}SH) + 4350(S\text{–}NH_3^+)$. Since k_1' is known from the observations at 260 mμ, k_5' may

TABLE I

RAMAN SPECTRA OF 2-METHYL-Δ^2-THIAZOLINE AND ITS CONJUGATE ACID

Raman frequencies are recorded to nearest 5 cm.$^{-1}$ in units of cm.$^{-1}$. Numbers in parentheses are intensities relative to 495 cm.$^{-1}$ line arbitrarily taken as 10. An S signifies a shoulder on a neighboring peak. The intensities recorded here and in reference 6, on the Cary Raman Spectrophotometer, which gives photoelectric recording, are of much higher precision than those reported in earlier work on Raman spectra from this laboratory, which were based on visual estimates of photographs of the spectra.

A spectrum identical to that recorded below for thiazoline in concentrated DCl was obtained after standing for five months at room temperature. This indicates both the stability of thiazoline and the non-exchangeability of the carbon bound hydrogens in concentrated acid.

	30 volume % soln. in		
Pure liquid	Free base in water	Thiazolinonium ion in concd. HCl (H_2O)	Concd. DCl (D_2O)
320 (4)	320 (3)	320 (3)	320 (3)
495 (10)	495 (10)	495 (10)	495 (10)
615 (17)	630 (13)	635 (19)	635 (15)
650 (16)	655 (12)	690 (15)	680 (15)
710 (6)	710 (4)	S 710	S 700
845 (1)		865 (1)	865 (3)
900 (3)	910 (5)	920 (8)	
955 (3)	965 (2)		975 (3)
995 (1)	1000 (1)	1025 (1)	1010 (5)
1055 (1)	1055 (1)	1060 (5)	
1145 (3)	1160 (2)	1185 (1)	1175 (1)
1200 (1)		1225 (2)	1225 (2)
1265 (1)	1265 (1)	1265 (2)	1280 (1)
1310 (2)	1320 (2)	1335 (5)	1335 (2)
1370 (2)	1375 (3)	1380 (7)	1380 (4)
		S 1400	
1435 (12)	1440 (11)	1440 (7)	1440 (6)
S 1455	S 1455	1465 (6)	1465 (7)
1635 (12)	1635 (13)	1630 (21)	1610 (28)
2730 (1)			
2860 (13)	2870 (10)	S 2890 (7)	S 2890 (7)
S 2890	S 2900		
2925 (36)	2935 (30)	2935 (20)	2935 (20)
S 2950 (23)	2965 (19)	2970 (20)	2970 (23)
2990 (15)	3000 (10)	S 3015 (12)	S 3015 (13)

be computed by the expression

$$k_5' = \frac{k_1'(\epsilon_{230} - 146) + (1/C_T)(dA_{230}/dt)}{4200} \quad (5)$$

This rate constant is plotted in Fig. 3 (full circles) as a function of pH for the appearance of S-acetyl-β-mercaptoethylamine. It is not possible to determine directly the rate of formation of the N-acetyl derivative because of its lack of a characteristic spectrum (Fig. 1).

The main feature of the curve for the disappearance of thiazoline is that a maximum is obtained at about pH 3. In order to explain the maxima in the curve the scheme pictured in Fig. 4 is proposed as a kinetic expression of the data. A hydroxythiazolidine (HOTH) is postulated as an intermediate between thiazoline and both N- and S-acetyl-β-mercaptoethylamine. Assuming a steady-state condition for (HOTH), equations (6) to (9) are indicated by the mechanism

$$-dC_T/dt = k_1(TH^+) - k_2(H^+)(HOTH) \quad (6)$$

$$d(S\text{-}NH_3^+)/dt = k_5(HOTH) - k_6(S\text{-}NH_2) \quad (7)$$

$$d(N\text{-}SH)/dt = k_3(HOTH) - k_4(N\text{-}SH) \quad (8)$$

$$(HOTH) = [k_1(TH^+) + k_4(N\text{-}SH) + k_6(S\text{-}NH_2)]/[k_2(H^+) + k_3 + k_5] \quad (9)$$

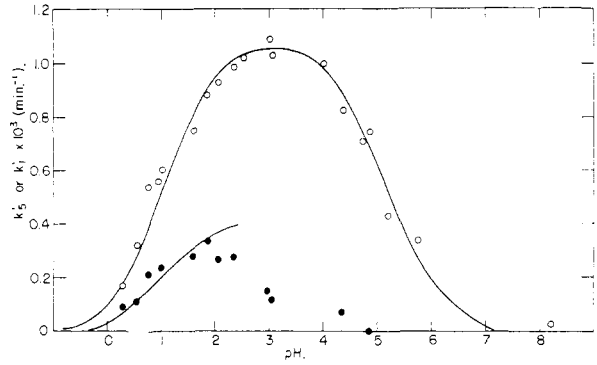

Fig. 3.—Initial rate of hydrolysis of 2-methylthiazoline as a function of pH (open circles). Rate of appearance of S-acetyl-β-mercaptoethylamine (full circles). Curves are theoretical. Temperature 25° and 0.10 ionic strength. Buffers: pH 0.28–2.35, HCl; pH 2.52–3.05, chloroacetic acid; pH 4.01–5.20, acetic acid; pH 5.72–8.21, phosphate. Except for the acid solutions the buffer concentration is about 0.03 M.

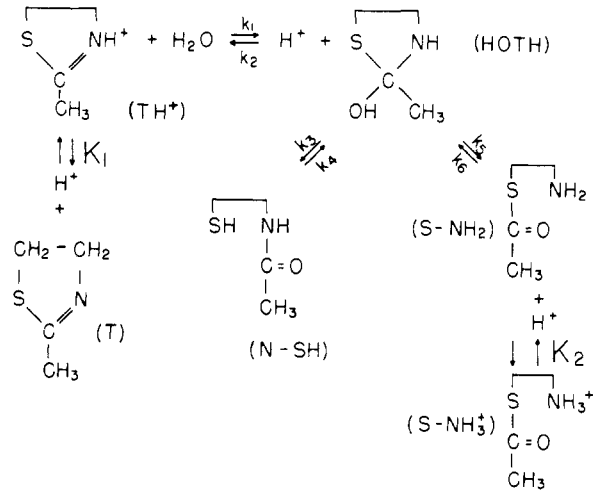

Fig. 4.—Scheme for the interrelationships between thiazoline and N- and S-acetyl-β-mercaptoethylamines. Small k's denote rate constants and large K's equilibrium constants. The numerical values for the various constants, as deduced from the experimental data by methods reported in the text are as follows: $k_1 = 1.05 \times 10^{-3}$ min.$^{-1}$; $k_3/k_2 = 0.062$ molar; $k_5/k_2 = 0.038$ molar; $k_4 = 1.5 \times 10^{-4}$ min.$^{-1}$; $k_6K_2 = 3.2 \times 10^{-6}$ min.$^{-1}$ molar^{-1}; $pK_1 = 5.22$; $pK_2 \cong 10$ (assumed); therefore $k_6 \cong 3 \times 10^4$ min.$^{-1}$.

Also define $K_1 = (T)(H^+)/(TH^+)$ and $K_2 = (S\text{-}NH_2)(H^+)/(S\text{-}NH_3^+)$. If thiazoline alone is present at the beginning of the experiment, substitution of the expression for (HOTH) into $-dC_T/dt$ gives for the initial rate

$$\frac{-dC_T}{dt} = \frac{k_1C_T(H^+)[(k_3 + k_5)/k_2]}{[K_1 + (H^+)][(H^+) + (k_3 + k_5)/k_2]} = k_1'C_T \quad (10)$$

This equation is of the form required to fit the curve of Fig. 3. The rate constant k_1' approaches zero at very high or very low values of (H^+), and rises to a maximum at $(H^+) = \sqrt{K_1(k_3 + k_5)/k_2}$. The curve for the rate of disappearance of thiazoline in Fig. 3, as a function of pH, is drawn for the pK_1 value of 5.22 as determined by titration, a

$(k_3 + k_5)/k_2$ value of 0.10 molar and $k_1 = 1.05 \times 10^{-3}$ min.$^{-1}$.

For the appearance of S-acetyl-β-mercaptoethylamine from an initial solution of thiazoline

$$\frac{d(S\text{-}NH_3^+)}{dt} = \frac{k_5k_1(TH^+)/k_2 - k_6(S\text{-}NH_3^+)K_2[1 + k_3/(H^+)k_2]}{[(H^+) + (k_3 + k_5)/k_2]} = k_5'C_T \quad (11)$$

In this case the terms for the back reaction are retained, since they become important at pH values greater than 2. For this reason the lower curve drawn in Fig. 3 is not extended beyond pH 2. For the 5 determinations of rate from pH 0.55 to 1.86 the ratio of $k_5'/k_1' = 0.38 \pm 0.03$. This gives a value of $k_5/k_2 = 0.038$ molar, thus $k_3/k_2 = 0.062$ molar. At greater values of pH the ratio k_5'/k_1' is less than 0.38, presumably due to the back reaction of velocity constant k_6. The S-acetyl-β-mercaptoethylamine is known to be unstable in neutral solution.[11] At pH 0.28 the ratio is 0.53 which may be attributed to the back reaction of velocity constant k_4. Of course, alternative modes of formation of products in addition to those represented in Fig. 4 may also explain the lack of constancy of k_5'/k_1' outside the pH range of 0.5 to 2.

Kinetic experiments also were performed with initial solutions of N-acetyl-β-mercaptoethylamine. The rate of thiazoline formation may be followed at 260 mμ by an analysis similar to that previously described for thiazoline. The scheme of Fig. 4 yields

$$dC_T/dt = k_4(H^+)(N\text{-}SH)/[(H^+) + (k_3 + k_5)/k_2] \quad (12)$$

for the appearance of thiazoline. In solutions from 2 to 10 M in HCl, for which $(H^+) >> [(k_3 + k_5)/k_2]$ the rate is approximately constant as predicted and k_4 is found to be about 1.5×10^{-4} min.$^{-1}$.

Similarly, the rate of appearance of S-acetyl-β-mercaptoethylamine may be deduced from Fig. 4 to be

$$d(S\text{-}NH_3^+)/dt = [k_4(N\text{-}SH)k_6/k_2]/[(H^+) + (k_3 + k_5)/k_2] \quad (13)$$

As is indicated, the rate decreases to zero as the acidity increases, but the mechanism is scarcely valid at high acidities where the hydrolysis of the thiol ester is appreciable. Utilizing the value of k_4 obtained above, a value of k_5/k_2 of about 0.04 molar is obtained from data in the pH range 0.3 to 1.6. This is in good agreement with the same ratio as obtained from experiments starting with solutions of thiazoline.

The rate of hydrolysis of S-acetyl-β-mercaptoethylamine also was studied. The rate of decomposition into β-mercaptoethylamine and acetic acid at 25° in 6 M HCl, calculated from the disappearance of the absorption at 230 mμ, gave a first-order rate constant of 7×10^{-4} min.$^{-1}$. No increase in absorption occurred at 260 mμ, indicating that thiazoline formation did not take place and the formation of N-acetyl-β-mercaptoethylamine is not expected. Since this rate constant for decomposition is considerably less than what would be inferred from results on other thioesters,[13] the rate of decomposition will be assumed negligible in the much less acid solutions to be discussed below. It might be expected that the presence of a positive charge on the S-acetyl-β-mercaptoethylamine would greatly hinder the acid-catalyzed decomposition.

In these less acid solutions the S-acetyl-β-mercaptoethylammonium ion may disappear in two ways

$$TH^+ \xleftarrow{k_2'} S\text{-}NH_3^+ \xrightarrow{k_3'} N\text{-}SH$$

Since $A_{230} = 830(TH^+) + 146(N\text{-}SH) + 4350(S\text{-}NH_3^+)$ the change in absorbancy may be expressed as a function of the rate constants

$$[-1/(S\text{-}NH_3^+)][dA_{230}/dt] = 3500k_2' + 4200k_3' \cong 4200k_6' \quad (14)$$

The coefficients of the primed rate constants are nearly the same and the reaction of velocity constant k_3' is predominant, especially at the higher end of the pH range studied. Hence the right-hand side of the equation may be well represented by $4200k_6'$.

For the disappearance of S-acetyl-β-mercaptoethylammonium ion the scheme of Fig. 4 yields

$$-d(S\text{-}NH_3^+)/dt = k_6K_2(S\text{-}NH_3^+)[1 + k_3/k_2(H^+)]/[(H^+) + (k_3 + k_5)/k_2] = (k_2' + k_3')(S\text{-}NH_3^+) = k_6'(S\text{-}NH_3^+) \quad (15)$$

The first term in square brackets in the numerator represents thiazoline formation and the second N-acetyl-β-mercaptoethylamine formation.

If the ratios k_3/k_2 and $(k_3 + k_5)/k_2$ are taken from the results on thiazoline the product k_6K_2 may be evaluated. For the pH range 1.8–3.1, $k_6K_2 = 3.2 \pm 0.1 \times 10^{-6}$ min.$^{-1}$ molar^{-1} at 25° and 0.10 ionic strength. If K_2 is taken as about 10^{-10} molar, then $k_6 \cong 3 \times 10^4$ min.$^{-1}$ approximately.

In terms of the formulation of Fig. 4 the following equilibrium constants may be defined and their values obtained from the rate constants.

$$K_{ST} = (S\text{-}NH_3^+)/(TH^+) = k_5k_1/k_2k_6K_2 = 12 \quad (16)$$

$$K_{NS} = (H^+)(N\text{-}SH)/(S\text{-}NH_3^+) = k_3k_6K_2/k_4k_5 = 0.035 \text{ molar} \quad (17)$$

$$K_{NT} = (N\text{-}SH)/(T) = k_1k_3/K_1k_2k_4 = 7 \times 10^4 \quad (18)$$

We note, from (18), that thiazoline in neutral solution should be thermodynamically unstable, relative to N-acetyl-β-mercaptoethylamine, even though its *rate* of hydrolysis at pH near 7 is very small compared to that at pH 3.

Solutions which were in the pH range 0.95 to 1.59, containing initially either thiazoline or N-acetyl-β-mercaptoethylamine, were followed for several weeks until the relative absorbancies at 230 and 260 mμ were constant. An estimate of the thiazoline concentration then was made from the absorption at 260 mμ, allowing for some product absorption. From the known extinction of thiazoline at 230 mμ the absorbancy due to thiazoline may be subtracted from the total absorbancy at 230 mμ and the concentration of S-acetyl-β-mercaptoethylammonium ion estimated. The ratio $(S\text{-}NH_3^+)/(TH^+)$ is found to be 11 ± 1, which is equal within experimental error to that calculated from the rate constants in equation 16.

Additional rate experiments were followed by observing the change in the Raman spectra of thiazoline (approximately 3 M) with time at room

(13) I. H. Noda, S. A. Kuby and H. A. Lardy, THIS JOURNAL, **75**, 913 (1953).

temperature and about *p*H 1 (Fig. 2). The rate of disappearance of the 1628 cm.$^{-1}$ line due to the C=N vibration is almost twice as fast as the rate of appearance of the 2575 line due to S–H stretching. This result supports the conclusions obtained by ultraviolet spectrophotometry. The 1628 line had fallen to half its initial intensity in approximately 96 hr. at 25°. From a similar run at 69°, which was followed in the Raman spectrophotometer, the half-life of thiazoline, from the disappearance of the 1628 line, was approximately 1.7 hr. Hence the activation energy for the disappearance of thiazoline is very roughly estimated as 19 kcal./mole.

A solution of 2 g. of N-acetyl-β-mercaptoethylamine in 5 ml. of concentrated HCl gave a Raman spectrum which slowly changed over a period of about a month until the final spectrum was indistinguishable from that of thiazoline in HCl. This indicates that the irreversible breakdown to acetic acid and β-mercaptoethylamine is practically negligible under these conditions. The conversion of N–SH to the conjugate acid of thiazoline, rather than to the S–NH_3^+ ion, might at first sight appear paradoxical, since the latter ion is favored thermodynamically according to equation 16. However, the kinetic analysis shown in Fig. 4 indicates that the conversion of N–SH to TH^+ goes very rapidly in comparison with the conversion to S–NH_3^+, which is exceedingly slow in strongly acid solutions.

Reaction with Ammonia.—Linderstrøm-Lang and Jacobsen[2] observed that the addition of ammonium ion to a solution of thiazoline yields a product which gives a thiol reaction with porphyrindin. Since the equilibrium constant for the reaction was independent of *p*H near the neutral point, they formulated the reaction as $TH^+ + NH_3$ or $T + NH_4^+$. They favored the latter formulation of the kinetically indistinguishable pair. However, in analogy with the hydrolysis reaction the former will be considered here.

$$TH^+ + NH_3 \underset{k_8}{\overset{k_7}{\rightleftharpoons}} \text{product}$$

The initial reaction rate may be formulated as

$$-dC_T/dt = k_7(TH^+)(NH_3) = k_7C_TC_NK_3(H^+)/K_1[K_3 + (H^+)] = k_7'C_T \quad (19)$$

where C_N is the total concentration of ammonia and ammonium ion and K_3 is the ionization constant of ammonium ion. The reaction was studied at 25° and $\mu = 0.10$ by following the rate of disappearance of the absorption of thiazoline at 245 mμ. At these conditions pK_3 for the ammonium ion was taken as 9.37. The experiments performed are listed in Table II. The hydrolysis due to water will be less than 5% under the conditions described and is therefore not considered.

TABLE II

THE REACTION OF THIAZOLINE AND AMMONIA AT 25° AND $\mu = 0.10$

$C_T \times 10^4$, M	C_N	*p*H	$k_7' \times 10^4$, min.$^{-1}$	k_7, min.$^{-1}$ M^{-1}
2.96	0.100	8.69	10.0	171
2.96	.100	9.84	3.20	174
1.48	.100	8.79	10.0	176
2.96	.040	8.78	3.88	172

The absorbancy after several days was about 10% of the initial value at 245 mμ. It is not certain whether this is due to thiazoline remaining at equilibrium or to the product. The complete spectrum of the final mixture exhibits absorption which increases gradually with decreasing wave length until a maximum is reached at about 208 mμ with a molar extinction coefficient of about 7×10^3.

The rate constants of Table II were also evaluated from the standard logarithmic plots of a reversible reaction utilizing the final absorption discussed above. Substantially the same results are obtained for the rate constant from the linear plots supporting the inference that the reaction proceeds nearly to completion under the experimental conditions.

Glutathione.—Thiazoline formation has been inferred to occur in strongly acid solutions of glutathione. Some of the evidence from Raman spectra for the appearance of a thiazoline structure in glutathione has been previously summarized.[6] Figures 5 and 6 show the Raman spectrum of glutathione in concentrated HCl after mixing and 64 hours later, respectively. These may be compared with the Raman spectrum of 2-methylthiazoline in concentrated HCl, as shown in Fig. 2.

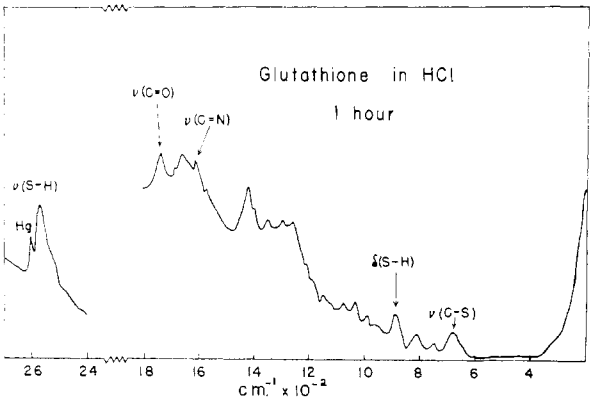

Fig. 5.—Raman spectrum of 2 g. of glutathione in 5 ml. of concentrated HCl, 1 hr. after mixing. The rise in the base line of the spectrum is due to fluorescence. The peak marked Hg is the line at 20336 cm.$^{-1}$ in the spectrum of the mercury arc, 2602 cm.$^{-1}$ away from the exciting line (22938 cm.$^{-1}$). Compare the relative intensity of this line and of the Raman S–H stretching frequency at 2575 cm.$^{-1}$, in Figs. 5 and 6.

It has been demonstrated that N-acetyl-β-mercaptoethylamine yields both the S-acetyl derivative and thiazoline in acid solutions. An attempt was made to demonstrate an N to S acyl transfer in glutathione. The spectra of a solution 1.65×10^{-3} *M* in glutathione and 2.66 *M* in HCl were taken after mixing and five days later. The spectra, as well as the difference between them, are shown in Fig. 7. The difference spectrum has a peak at 232 mμ, characteristic of thiol esters. Assuming an extinction coefficient of 4400, it may be inferred that about 10% of the original glutathione is in the thiol ester form.

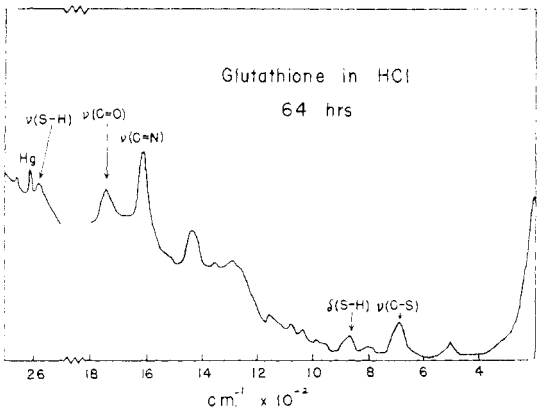

Fig. 6.—Raman spectrum of solution shown in Fig. 5 64 hr. later.

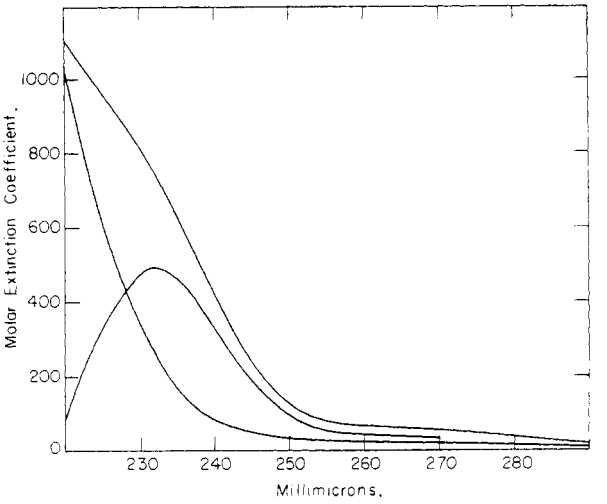

Fig. 7.—Ultraviolet spectra of 1.65×10^{-3} M glutathione in 2.66 M HCl. Lower curve taken directly after mixing is subtracted from readings taken five days later (upper curve) to yield curve with maximum at 232 mμ.

Some cyclic compounds containing an asymmetric center often show strong optical activity. The optical rotation of a solution of 2 g. of glutathione in 12 ml. of concentrated HCl was followed with time. Starting from an initial specific rotation $[\alpha]^{20}D$ of about +3° a final value of about +70° was observed after 3 days. Since only about 4/5 of the glutathione is in the thiazoline form under these conditions,[6] the specific rotation of glutathione in the thiazoline form may be estimated as approximately +90°. The rate of change of the optical rotation with time is consistent with the ultraviolet and Raman studies for the appearance of the thiazoline derivative in strongly acid solutions of glutathione.

Discussion

Linderstrøm-Lang and Jacobsen[2] measured the rate of formation of free thiol groups by titration with porphyrindin during thiazoline hydrolysis in the *p*H range 3 to 7, at 60°. Some of their work was performed at 20° with varying ionic strength. A weighted average of their values from *p*H 6.3 to 7.4 (the only range over which the values at different temperatures may be compared), calculated by assuming a heat of ionization of the thiazolinonium ion of 8 kcal./mole, yields 0.65×10^{-3} min.$^{-1}$ at 20° for k_1 as defined in this paper.[14] Assuming an activation energy of 19 kcal./mole as estimated from the Raman data this value becomes 1.05×10^{-3} min.$^{-1}$ at 25°, which is in good agreement with the result obtained here by measuring the rate of disappearance of absorption due to thiazoline at 260 mμ. This indicated identity of the rate of disappearance of thiazoline with the rate of appearance of a free sulfhydryl group is in agreement with the conclusion from the results of this paper that only N- and not S-acetyl-β-mercaptoethylamine is formed from thiazoline at values of *p*H greater than 5.

The reaction scheme of Fig. 4 postulates that the addition of water is a step separate from breakdown into products. There is substantial evidence for this in various thiazoline derivatives.[15] A similar intermediate has been postulated for oxime and semicarbazone formation.[16] The scheme represented in Fig. 4 is almost certainly not complete, but is adequate to explain our data. In particular, it is quite possible that it is the conjugate acid form of the hydroxythiazolidine (which we would abbreviate as $HOTH_2^+$) that decomposes into products. However, to yield a curve with a maximum it is necessary to assume that $HOTH_2^+$ reacts with OH^-. No advantage is to be derived in the analysis of the data presented here, by writing the decomposition of the hydroxythiazolidine intermediate as a second order reaction, involving the OH^- ion, although such an alternative formulation is of course possible. As a consequence of the steady-state approximation the equilibrium involving the conjugate acid of hydroxythiazolidine need not be explicitly considered in the scheme of Fig. 3.

Several experiments performed at 0.10 ionic strength, but at appreciably greater buffer concentrations than those of Fig. 2, indicated some general catalysis. However, since the effect is relatively small, an extended study of general acid or base catalysis has not been made.

For the reaction of thiazoline with ammonia the rate constant of 170 min.$^{-1}$ M^{-1} may be compared with a value of about 300 min.$^{-1}$ M^{-1} obtained by measuring the appearance of a thiol group with porphyrindin.[2] The rate constants are comparable considering that the latter value was obtained in 5.8 guanidinium bromide at 20°.

As mentioned above the reaction between thiazoline and ammonia proceeds practically to completion. However, Linderstrøm-Lang and Jacobsen observed a definite equilibrium mixture, though at lower *p*H values than those in Table I. The discrepancy may be at least partially resolved if the product has a pK_a of about 8. This is not unreasonable for the sulfhydryl group of the suggested amidine product.[2]

(14) In Table IX, page 313 of reference 2a, the values of the rate constant of column 4 (apparently given in min.$^{-1}$) are a factor of ln 10 smaller than the rate constant derived from the "per cent. thiazoline split in 100 min." of column 5. The value quoted above is derived from their column 4 as the result from the data of column 5 would be 2.3 times as great and would be wholly inconsistent with the results of this paper.

(15) J. C. Crawhall and D. F. Elliott, *J. Chem. Soc.*, 3094 (1952).

(16) W. P. Jencks, THIS JOURNAL, **81**, 475 (1959).

The experiment with glutathione in 2.88 *M* HCl which indicates the formation of a thiol ester, coupled with the previous evidence for thiazoline formation, strongly suggests that glutathione may participate in a scheme similar to that of Fig. 4. The formation of an S-acyl derivative would at least partially explain the lack of reversibility when the once formed thiazoline derivative is taken to neutral solution and then returned to strong acid.[17] As in the case of N-acetyl-β-mercaptoethylamine the rate of thiazoline formation in glutathione is constant from 2 to 6 *M* acid. The rate constant is about half the value of k_4 observed for N-acetyl-β-mercaptoethylamine. However, both rates are subject to some uncertainty, due to amide hydrolysis.

Although the rate constants for thiazoline formation in the two compounds are of the same order of magnitude, this is not so for the equilibrium situation. For N-acetyl-β-mercaptoethylamine the equilibrium ratio $(TH^+)/(N\text{–}SH)(H^+)$ has a value of about 2.3 molar^{-1}. The comparable ratio for glutathione[6] is of the order of 3×10^{-3} molar^{-1}. The formation of thiazoline-4-carboxylic acid from N-formylcysteine[18] appears to proceed rapidly toward an equilibrium condition intermediate between the values characteristic for the formation of the thiazoline derivatives of glutathione and N-acetyl-β-mercaptoethylamine.

(17) Observed by several authors, cited in reference 6. See also the discussion of Préaux and Lontie, 5b.

(18) D. Cavallini, B. Mondovi and C. De Marco, *Experientia*, **13**, 436 (1957).

The occurrence of thiazoline rings in proteins has been advanced as one possible explanation for the "masking" of –SH groups, *e.g.*, in serum albumin.[19] Insofar as the properties of 2-methylthiazoline described here may provide information concerning proteins, little support is given to such a view except in solutions more acid than *p*H 1. Simpson and Saroff[19] obtained a maximum rate of decrease of sulfhydryl titer at *p*H 3; however, this is the same *p*H at which 2-methylthiazoline has a maximum rate of decomposition. For the physiological *p*H range the inferred equilibrium constant $K_{NT} = (N\text{–}SH)/(T) = 7 \times 10^4$ strongly favors the amide over the thiazoline form. The situation may be even more unfavorable for thiazoline formation if glutathione is typical of the proteins. However, the basic form of the thiazoline structure once formed may well be stable kinetically speaking. Although the thiazoline derivative may not hydrolyze, its rate of reaction with the ever-present amines would be quite significant in the *p*H 7–8 range. It is of course possible that particular proteins may contain regions in which a special type of configuration favors the formation of a thiazoline ring. The results of the study presented here, however, appear unfavorable to the thiazoline ring hypothesis as a general proposal to explain the "masked" sulfhydryl groups of proteins.

(19) R. B. Simpson and H. A. Saroff, THIS JOURNAL, **80**, 2129 (1958).

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Dye Sensitized Photoöxidation[1]

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RECEIVED APRIL 3, 1959

Only those dyes which are capable of being photoreduced can act as sensitizers for photoöxidation. Both reactions proceed through a metastable long-lived excited state of the dye. A kinetic study using proflavine as the sensitizer and *p*-toluenediamine as the substrate shows that the dye molecule in the long-lived state reacts with oxygen or forms a labile peroxide which in turn oxidizes the substrate. The quantum yield of the reaction decreases markedly with increasing dye concentration due to concentration quenching of the long-lived state and of the peroxide. The large limiting quantum yield achieved, namely, 3.0, is attributed to the formation of a polymer of the oxidized aromatic amine.

Introduction

Nearly sixty years ago, Raab[3] discovered that microörganisms, if stained with certain dyes, are inactivated by visible light. This phenomenon, sometimes referred to as "photodynamic action," requires the presence of oxygen and consists of a dye-sensitized photoöxidation of the substrate.[4] The dye itself is not consumed in the over-all process and may be used over and over again, as long as some autoxidizable substrate remains.

It is the purpose of the present paper to describe certain experiments which help to elucidate the mechanism of photodynamic action. For most of the quantitative studies we have employed proflavin (3,6-diaminoacridine) as the sensitizer. The photoreductive properties[5] and the photodynamic action on biological substrates[6–8] of this dye and its analogs have been studied. As will be shown, there is a correlation between these two apparently diverse phenomena which also holds for dyes of other classes. As a model substrate, we have employed mainly *p*-toluenediamine. This substance is readily susceptible to photoöxidation,

(1) (a) Presented before the 135th National Meeting of the American Chemical Society, Boston, April 9, 1959. (b) This paper represents a part of the dissertation submitted in June, 1956, by Malcolm E. Schrader to the faculty of the Graduate School of the Polytechnic Institute of Brooklyn in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

(2) Public Health Service Research Fellow of the National Cancer Institute.

(3) O. Raab, *Z. Biol.*, **39**, 524 (1900).

(4) H. Blum, "Photodynamic Action and Diseases Caused by Light," Reinhold Publ. Corp., New York, N. Y., 1941.

(5) F. Millich and G. Oster, THIS JOURNAL, **81**, 1357 (1959).

(6) G. Oster and A. D. McLaren, *J. Gen. Physiol.*, **33**, 215 (1950).

(7) G. Oster, *Trans. Faraday Soc.*, **47**, 660 (1951).

(8) J. S. Bellin, R. W. Kimball and G. Oster, to be published.