BIOORGANIC CHEMISTRY 14, 392–404 (1986)

The Nonenzymic Hydrolysis of Δ^2-Thiazoline-2-carboxylate: The Product of the Suspected Physiological Reaction Catalyzed by D-Amino Acid Oxidase[1]

PRASANNA P. VENKATESAN AND GORDON A. HAMILTON[2]

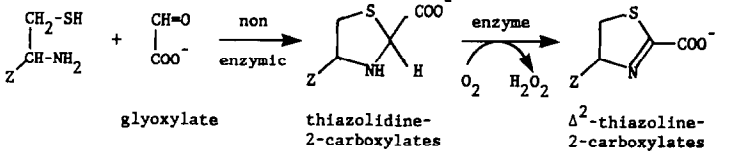
Department of Chemistry, The Pennsylvania State University, University Park, Pennsylvania 16802

Received April 14, 1986

Δ^2-Thiazoline-2-carboxylate, the product of the suspected physiological reaction catalyzed by D-amino acid oxidase, is stable to hydrolysis at 37°C and pH 7 or above, but it hydrolyzes readily at pH 5 or below to give a mixture of *N*- and *S*-oxalylcysteamines; the *N*-oxalyl derivative predominates at pH's above 1 while the *S*-oxalyl compound is the major product at high acidities. The pH-rate profile looks like the superposition of two bell-shaped curves. The initial increase in the rate as the pH is lowered is controlled by a pK_a of 3.95 and from pH 1 to 3 the rate is relatively constant ($k \approx 6.7 \times 10^{-4}$ s^{-1} at 37°C and ionic strength 0.5 M). Below pH 1 the rate increases again to a maximum in 1 M HCl and then decreases in more highly acidic solutions. The rate of conversion of *S*-oxalylcysteamine to *N*-oxalylcysteamine is inversely proportional to the hydrogen ion concentration from pH 3 to 5 but becomes largely independent of pH from pH 1 to 2. In the pH-independent region the rate is comparable with that observed by others for *S*-acetylcysteamine but in the pH-dependent region the rate is 20 to 25 times faster for the oxalyl derivative than for the acetyl compound. At pH 1, *N*-oxalylcysteamine is partially converted to the *S*-oxalyl derivative but the rate of hydrolysis ($k = 1.0 \times 10^{-5}$ s^{-1} at 37°C) to cysteamine and oxalate of this partially equilibrated system occurs at a comparable rate. The results of this investigation are rationalized in terms of what is known about other thiazoline hydrolyses and intramolecular *S* to *N* acyl migrations. The main differences in the present case are presumably due to the fact that thiazoline-2-carboxylate can undergo hydrolysis by two reaction manifolds, one with the carboxyl unprotonated and the other with it protonated. The relevance of these results to possible reactions of thiazoline-2-carboxylate *in vivo* is briefly considered.

INTRODUCTION

Recently we have reported that some thiazolidine-2-carboxylates, formed nonenzymically from certain 2-aminoethanethiols and glyoxylate, are excellent substrates for D-amino acid oxidase (*1–3*) and D-aspartate oxidase (*3, 4*), and that the reactions proceed as shown in Eq. [1] to give Δ^2-thiazoline-2-carboxylates as



[1]

[1] This research was supported by a research grant (AM 13448) from the National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases, Public Health Services.

[2] To whom correspondence should be addressed.

0045-2068/86 $3.00

products. In addition, we have summarized (1-4) considerable evidence that these reactions are almost certainly occurring physiologically. In the case of D-amino acid oxidase, the adduct formed from cysteamine ($Z = H$) and glyoxylate is the likely physiological reactant, whereas for D-aspartate oxidase, the glyoxylate adducts formed from both L-cysteine ($Z = COO^-$) and L-cysteinyl-glycine ($Z = CONHCH_2COO^-$) are potential *in vivo* reactants. Since a large amount of circumstantial evidence (*3, 5, 6*) suggests that either the products of these enzymic reactions or their further metabolites may be involved in controlling metabolism in animals, we have been investigating the reactivities of such compounds in order to: (a) characterize how stable they are under physiological conditions, (b) determine under what conditions they might be converted into other metabolites, and (c) characterize which mechanisms they might be able to use to modify the activities of various enzymes.

Reported here are our investigations of the nonenzymic hydrolysis of Δ^2-thiazoline-2-carboxylate (TC[3]; $Z = H$, Eq. 1), the product of the D-amino acid oxidase reaction. Although the hydrolytic behavior of the carboxylate derivative had not been studied previously, extensive investigations of other thiazolines, especially the 2-methyl derivative, have been performed (*7–12*). As might be expected, several characteristics of the TC hydrolysis are similar to those of the other derivatives, but the presence of the carboxylate group does speed up and modify the reaction considerably, especially at low pH.

EXPERIMENTAL PROCEDURES

Materials. Unless otherwise noted, commercially available materials, reagent grade or better, were used as received. All water used in this work was either doubly distilled, the second time with a Kontes WS-2 glass still following passage through Barnstead organic and ion exchange columns, or glass distilled and then passed through a Millipore (Milli Q) reverse osmosis water purification system. Buffer solutions were prepared by dissolving weighed amounts of the acid and base forms of the buffer material in water, adjusting the pH when necessary, using small amounts of concentrated HCl or 50% potassium hydroxide, and diluting to known volumes. Acetate and chloroacetate buffers were used for reactions run at pH's 4 to 6 and 2 to 4, respectively. Solutions having a pH less than 2 were prepared by dilution of standard hydrochloric acid solutions. For all solutions of pH 0.5 and above, KCl was added to maintain a constant ionic strength of 0.50 M unless otherwise noted. Thiazoline-2-carboxylate was synthesized as previously described (*2*).

The sodium salt of *N*-oxalylcysteamine ($HSCH_2CH_2NHCOCOO^-Na^+$) was prepared by hydrolysis of ethyl *N*-(2-mercaptoethyl)-oxamate (*2*). To 13 g of the ester suspended in 150 ml distilled water maintained at 55°C, carbonate free sodium hydroxide solution (1 M) was added dropwise until the pH stabilized at 7. The water was evaporated and the white solid washed with ether a few times to

[3] Abbreviation used: TC, Δ^2-thiazoline-2-carboxylate.

remove traces of unreacted ester. After recrystallization from methanol and ether, 7.3 g (58%) of a white solid with the following characteristics was obtained:

Elemental anal. Found (theory in parentheses): C 28.20 (28.07), H 3.52 (3.53), N 7.94 (8.18), and S 17.83 (18.73); IR (KBr) 3341, 2930, 2550, 1657, 1626, 1520, 1470, 1420, 1407, 1350, 1286, 1254, 1215, 1190, 1065, 915, 860, 786, 742 and 720 cm^{-1}; ^{13}C NMR (d_6-DMSO) δ 165.7, 163.4, 42.3, 23.2; equivalent weight of 171 determined by titration with 5,5′-dithiobis(2-nitrobenzoate).

Despite several attempts (*13*), a pure crystalline preparation of S-oxalylcysteamine ($H_3N^+CH_2CH_2SCOCOO^-$) could not be obtained, mainly because it rearranges so readily to the *N*-oxalyl derivative. However, solutions containing the *S*-oxalyl compound could be prepared as follows. Oxalyl chloride (0.3 mol) in 100 ml dichloromethane was mixed with a suspension of 0.1 mol of cysteamine hydrochloride in 100 ml of the same solvent and allowed to react under N_2. Following evaporation of the solvent and excess oxalyl chloride, a mixture of solids, which probably contained unreacted cysteamine hydrochloride as well as some oxalylated derivative, was obtained. Solutions of this material in aqueous acid (pH 1) initially have a uv absorption maximum at 276 nm, which is probably due to an acid chloride, anhydride, or dithio ester derivative of the desired compound. In any event, within minutes at room temperature the absorption at 276 nm disappears and is replaced by an absorption at 259–260 nm which is characteristic of the $RSCOCOO^-$ structure (*14–16*). As expected, if this compound is *S*-oxalylcysteamine, then the absorption disappears rapidly at pH 3 or above, due to its rearrangement to *N*-oxalylcysteamine (identified by TLC). For experiments requiring *S*-oxalylcysteamine, the pH 1 solution containing the 260-nm absorbing species was prepared from the solid material just prior to use.

Methods. Routine ultraviolet spectra were obtained using a Hitachi Model 100-80 A computerized spectrophotometer. Thiol concentrations were determined by titration with 5,5′-dithiobis(2-nitrobenzoate) as described by Ellman (*17*); the extinction coefficient for the product was assumed to be 14.15 mM^{-1} (cm^{-1} at 412 nm (*18*). Acid ionization constants for TC and *N*-oxalylcysteamine were determined by the general method described by Albert and Serjeant (*19*). At neutral pH TC has an absorption maximum at 270 nm (extinction coefficient, 1.64 mM^{-1} cm^{-1}), while in acid (pH 1 to 3) its maximum is at 286 nm (extinction coefficient approximately 2.4 mM^{-1} cm^{-1}). Since the maximum changes occur around 290 nm, data collected at 280, 290, and 300 nm were used to obtain the pK_a at 37°C with 20 mM buffers present and a total ionic strength of 0.5 M (KCl). For *N*-oxalylcysteamine, spectral changes at 240 to 260 nm were used, again at 37°C and ionic strength 0.5 M.

All reported rate constants were obtained by standard spectrophotometric techniques using a Gilford 240 spectrophotometer, equipped with an automatic sample changer, a strip chart recorder, and a constant-temperature circulating water bath. The hydrolysis of TC was followed at 270 to 286 nm (depending on pH) and the rates of various reactions involving *S*-oxalylcysteamine were monitored at 260 to 263 nm. All reactions of TC were run in quadruplicate and good first-order kinetics were observed for at least three half-lives. The observed first-order rate constants (k_{obs}) were calculated by standard methods, although in some cases a small cor-

rection was made (*13*) to the optical density at infinite time due to a linear drift in the baseline. Prior to initiating the reaction by adding a small aliquot of a solution containing TC, the other components (total volume, 3 ml) were temperature-equilibrated at 37°C for 1 h. Usually the concentration of TC in the reaction solution was 0.4 mM. The pH and temperature were checked both before and after each reaction.

The amounts of *S*-oxalylcysteamine and *N*-oxalylcysteamine, formed from TC under a given set of conditions, were estimated from the absorption at 260 nm when it reaches its maximum point (before further reactions of the thiolester start becoming significant). At 260 nm both oxalyl derivatives absorb but the absorption due to the *S*-oxalyl compound is much greater. Thus, the extinction coefficient for *N*-oxalylcysteamine is very low at high pH, being 0.1 mM^{-1} cm^{-1} at pH 3 and above and increases to only 0.3 mM^{-1} cm^{-1} in more acidic solutions (pK_a = 1.8). Although it was not possible to obtain a pure sample of *S*-oxalylcysteamine and thus measure its extinction coefficient, a reasonable estimate for it at various pH's can be made from what is known about analogous compounds. In other investigations, it has been found (*16*) that essentially all oxalyl thiolesters, at pH's where they exist as $RSCOCOO^-$ (*R* = alkyl), have an absorption maximum at 260 nm with an extinction coefficient of 2.9 mM^{-1} cm^{-1}. Consequently, this value was assumed to hold for *S*-oxalylcysteamine as well at pH's of 1 or above (from the spectral changes that *S*-oxalylcysteamine undergoes in HCl solutions, it is estimated that its pK_a is approximately 0.5). The protonated form (on the carboxylate group) of *S*-oxalylcysteamine probably has an extinction coefficient at 260 nm very close to the same value. In other work (*20*) it has been found that EtSCOCOOEt has an absorption maximum in ethanol at 269 nm with an extinction coefficient of 3.35 mM^{-1} cm^{-1}. At 260 nm its extinction coefficient is 3.0 mM^{-1} cm^{-1}. This value was assumed to hold for *S*-oxalylcysteamine at pH 0.0 and below.

Using the above extinction coefficients it is thus possible to calculate the amounts of the two oxalyl derivatives formed in the hydrolysis reactions assuming that all the initial TC is converted into these products. To the extent that some further reaction of the *S*-oxalylcysteamine may have occurred, the method will tend to underestimate the amount of the *S*-oxalyl derivative and overestimate that of the *N*-oxalyl derivative. However, because the rate of these further reactions of the *S*-oxalyl compound is considerably less than the rate of hydrolysis of TC, the values will not be very much in error.

RESULTS

General observations. In solution at room temperature and neutral to basic pH's, TC is stable for hours to days (approximately a 5% decrease in the absorption at 270 nm is seen after 60 h at pH 7). However, in acid solution the absorption due to TC rapidly disappears. Shown in Fig. 1 are some spectral changes observed with time at pH's 1 (Fig. 1a) and 3 (Fig. 1b). These results indicate not only that

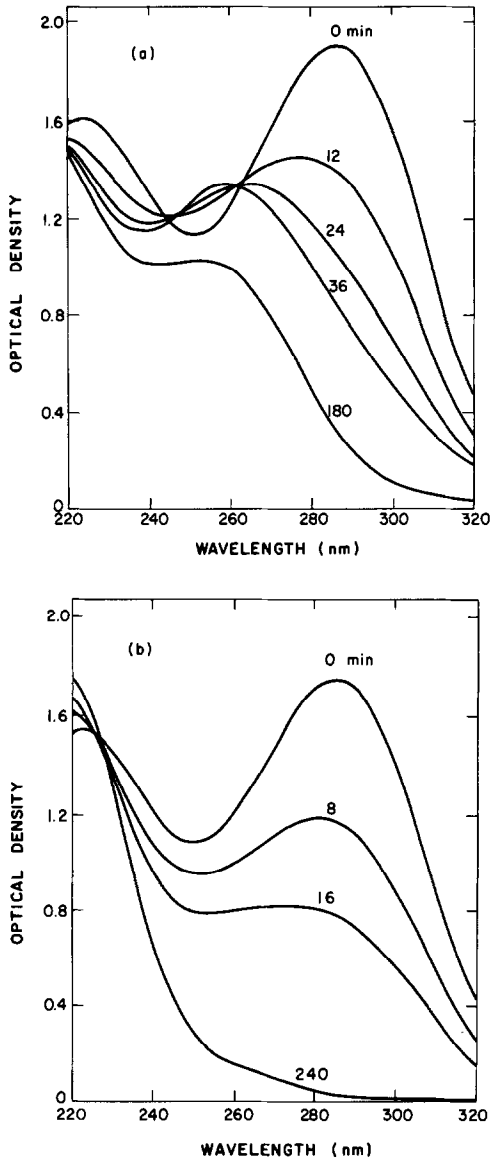


FIG. 1. The uv spectral changes occurring during the hydrolysis of TC at pH 1 (a) and pH 3 (b). The numbers on the figures are the times when the spectral scans were initiated at 320 nm; the scanning rate was 100 nm per min. The time-zero scan was initiated immediately after mixing a small aliquot of a TC solution with the temperature-equilibrated acid or buffer solution. General reaction conditions: 37°C; ionic strength, 0.50 M (KCl); initial TC concentration, 0.8 mM; 0.1 M HCl (a) and 20 mM chloroacetate buffer (b).

the reaction occurs readily, but also that different products are obtained at the two pH's. Such results are not unexpected because thiazolines are known to hydrolyze in acid to give a mixture of *N*- and *S*-acyl derivatives, the ratio of which depends on pH (*7–12*). In the present case, oxalylcysteamine derivatives would

be formed. Since *S*-oxalylcysteamine absorbs strongly at 260 nm, whereas *N*-oxalylcysteamine shows end aborption only down to 220 nm, the results in Fig. 1 indicate that at pH 3 the *N*-oxalyl derivative is by far the major product, but a considerable amount of the *S*-oxalyl product is formed at pH 1. At pH 3 a reasonably tight isosbestic point is observed, suggesting that the reaction is the simple conversion of the reactant to *N*-oxalylcysteamine. However, at pH 1 it is evident that the initially formed product undergoes a further reaction. Most of the subsequent reaction is probably the conversion of the *S*-oxalyl derivative to the *N*-oxalyl compound, but hydrolysis to oxalate and cysteamine is also occurring. Evidence for this was obtained when the products from the pH-1 reaction were analyzed by thin-layer chromatographic techniques using silica gel G and butanol, methanol, acetic acid, and water in the proportion of 4 : 4 : 1 : 1 as the eluting agent. With this technique, it was found that both the *N*- and *S*-oxalylcysteamines are formed in the early stages of the reaction and that these are the only products detectable initially. However, after approximately 70 min reaction time, cysteamine can also be detected. A further indication that the 260-nm absorbing product of the reaction at pH 1 is *S*-oxalylcysteamine is the finding that it disappears at approximately the same rate as the chemically synthesized compound under the same conditions ($k = 0.85 \times 10^{-4}$ s^{-1} at pH 1, ionic strength 0.5 M, 37°C).

The effect of buffer concentration on the rate of hydrolysis of TC was investigated from pH 2.0 to 4.5 and very little buffer catalysis was observed; when the buffer concentration is varied from 25 to 200 mM the observed first-order rate constant increases by about 20 to 30% at pH 3.5 to 4.5 (acetate buffers at pH 4.0 and 4.5 and chloroacetate buffer at pH 3.5) but does not change detectably at pH 2.0 to 3.0 (chloroacetate buffers). There is also only a very small ionic strength effect on the rate of the hydrolysis of TC. For example, for reactions run at pH 1.1 and 37°C, changing the ionic strength from 0.1 to 2.9 M (KCl) causes a decrease in rate of only 32%. Despite the small effects of buffer concentration and ionic strength, however, in all subsequent kinetic experiments low buffer concentrations were used and the ionic strength was kept constant at 0.50 M (except in strong acid where it could not be controlled).

Effects of pH on the hydrolysis of thiazoline-2-carboxylate. In Fig. 2 the pH-rate profile for the hydrolysis of this compound from pH 5.5 to an H_0 of -2 is illustrated. The pH-rate profile for 2-methylthiazoline (*9*), which others have investigated, is also shown for comparison. It is seen that at pH's above 1 the curves for the two compounds are very similar, but that in more acidic solutions there is an additional mechanism for hydrolysis of TC that is not available to the methyl derivative. The pH-rate profile for the methyl compound is a typical bell-shaped curve, but it appears that two such bell-shaped curves are superimposed in the pH-rate profile for TC. Such results can be readily rationalized, at least qualitatively, because of the extra protonizable group on the carboxylate compound.

The effects of pH above pH 2 are presumably due in both cases to the requirement that the nitrogen be protonated in order for the reaction to occur. There is some shift in the curves for the two compounds in this pH region because of their different pK_a's; the pK_a for the methyl compound is 5.25 (*9*), whereas that for TC,

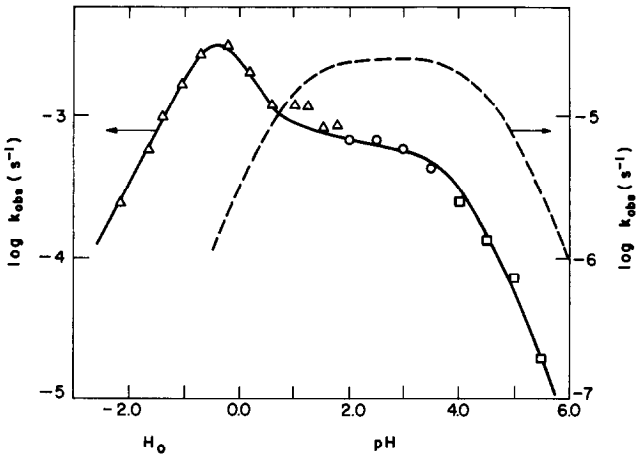


FIG. 2. The pH-rate profile for the hydrolysis of TC at 37°C (solid line, left scale) and, for comparison, that for 2-methylthiazoline at 30°C (dashed line, right scale) taken from reference 9. For TC hydrolyses, the acidity of the solutions was controlled by (Δ) HCl, (○) 20 mM chloroacetate buffers, or (□) 20 mM acetate buffers, and the ionic strength was kept constant at 0.50 M (KCl) for all pH's of 0.5 and above. The H_0 values for the HCl solutions were taken from those given by Paul and Long (*21*).

determined by the spectrophotometric method given in the experimental section, is 3.95. One can also obtain a pK_a for TC from the kinetic data obtained at pH's 2 to 5. In this region the reaction is presumably occurring as given in Eq. [2]. If so, then k_{obs} is given by Eq. [3] and k and K_a

$$TC + H^+ \overset{K_a}{\rightleftharpoons} TCH^+ \overset{k}{\rightarrow} \text{products} \qquad [2]$$

$$k_{obs} = \frac{k[H^+]}{[H^+] + K_a} \qquad [3]$$

can be obtained from the slope and intercept of a $1/k_{obs}$ vs $1/[H^+]$ plot. Such a plot of the data is linear and gives $k = 6.7 \times 10^{-4}$ s^{-1} and p$K_a = 3.84$. Since the spectrophotometric value is considered to be the more accurate, it was used, in conjunction with the value for k, to calculate a theoretical pH-rate curve, and the line from pH 2.5 to 5.5 of Fig. 2 is such a curve.

At greater acidities the carboxylate group of TC will protonate and this compound would be expected to have a reactivity different from that of the zwitterion. Presumably this is the reason for the second bell-shaped curve in the acid region. Although an accurate pK_a for the carboxylic acid group of TC was not determined, from the spectral changes that occur it can be estimated to be approximately −1 (in the pH 1 to 2 region TC has an absorption maximum at 286 nm but in 6 M HCl it has shifted to 300 nm). Because the ionic strength is not constant in this region and the solvent composition is changing, no attempt was made to quantitatively fit the data obtained at high acidities. Thus, in Fig. 2, the line through the experimental points from H_0 of −2 to pH 2 is merely a qualitative fit to the data.

As indicated in Fig. 1, the products of the hydrolysis of TC change with pH. Using the procedure described in the experimental section, the percentage yields

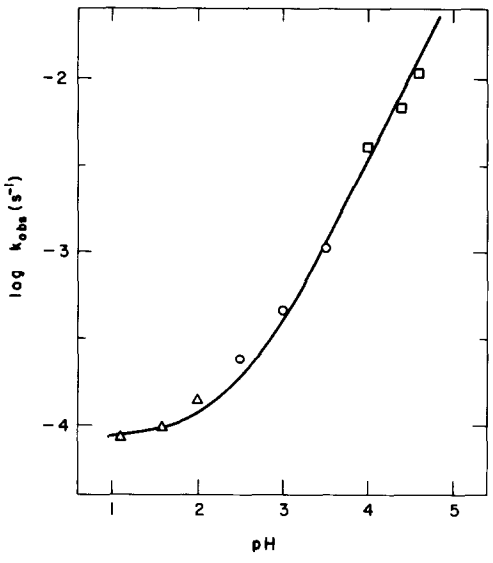


FIG. 3. The effects of pH on the observed first-order rate constant for the conversion of *S*-oxalylcysteamine to *N*-oxalylcysteamine at 37°C and ionic strength of 0.50 M (KCl). The acidities of the solutions were controlled by (△) HCl, (○) 20 mM chloroacetate buffers, or (□) 20 mM acetate buffers. The reactions were followed by the decrease in absorbance at 260 nm.

of *S*-oxalylcysteamine at pH's 1.0, 2.0, 2.5, 3.0, and 3.5 were estimated to be 56, 19, 6, 3, and 1, respectively. For reactions in the more strongly acid solutions, the yields of thiolester as a function of HCl concentration were estimated to be as follows (HCl concentration in molarity, time in minutes to reach the maximum absorption at 260 nm, percentage yield of thioester): 1 M, 40 min, 75%; 2 M, 35 min, 96%; 3 M, 30 min, 96%; 4 M, 60 min, 68%; 5 M, 70 min, 74%; 6 M, 80 min, 49%.

Reactions of the S- and N-oxalylcysteamines. A characteristic feature of the thiazoline-acylcysteamine systems is that the acylcysteamine products are readily interconverted, with the direction of the reaction being dependent on the pH (*7–12*). To compare the present system with those studied earlier, a few of the characteristics of these interconversions were studied. Shown in Fig. 3 are some results illustrating the effect of pH on the conversion of *S*-oxalylcysteamine to the *N*-oxalyl derivative. The line through the points is a theoretical one based on a specific mechanism and derived rate constants (see Discussion section).

If *N*-oxalylcysteamine is incubated at pH 1.0 (37°C and ionic strength 0.50) and the absorption at 260 nm monitored, an initial increase with time is noted followed by a first-order decay. The increase is due to the formation of *S*-oxalylcysteamine and the decay results from its hydrolysis to oxalate and cysteamine. The maximum at 260 nm occurs after 7.8 h and is more than twice the original absorbance due to the *N*-oxalylcysteamine. From the absorbance one can calculate that at this point approximately 14% of the original compound is present as *S*-oxalylcysteamine, but that is just a steady state rather than an equilibrium concentration

because the hydrolysis to oxalate and cysteamine proceeds at a comparable rate. From the eventual first-order decay of the 260-nm absorption, the rate constant for hydrolysis of the oxalylcysteamine system to oxalate and cysteamine was calculated to be 1.0×10^{-5} s^{-1}.

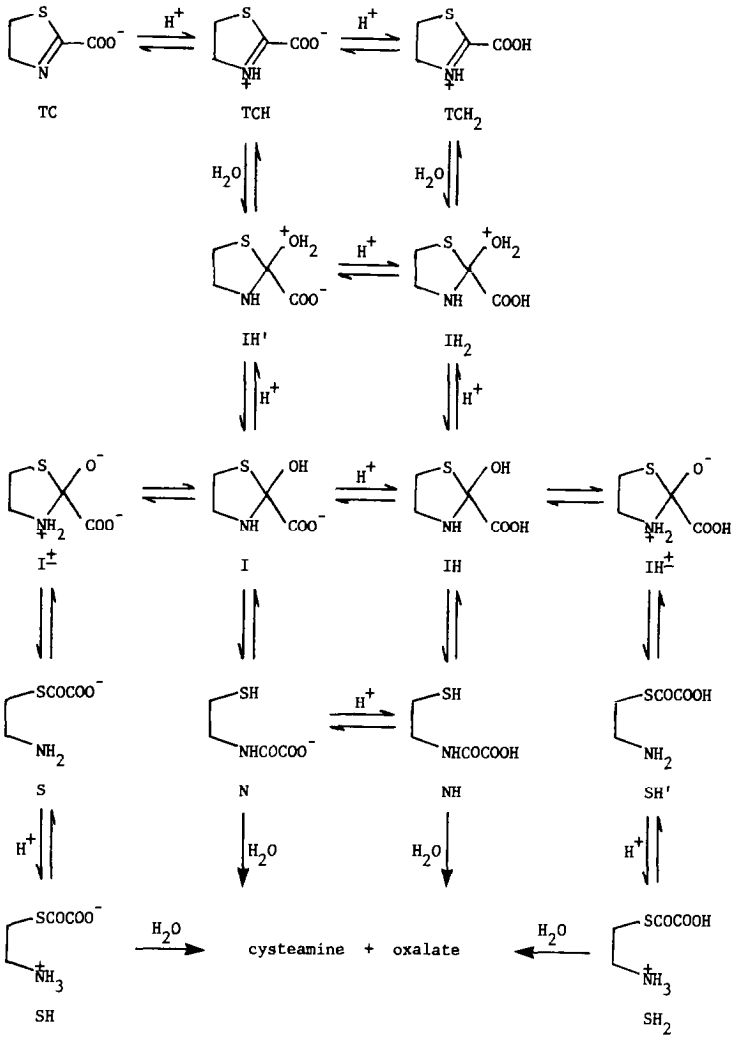
Reactivity of thiazoline-2-carboxylate with nucleophiles. Some preliminary experiments were performed to determine whether TC reacts with various nucleophiles under physiological conditions. The results indicate that TC can be forced to react with nucleophiles, but that the reactions are so slow that they would not be significant physiologically unless they were enzyme catalyzed. Thus, at 37°C and pH 7 in the presence of 10 mM cysteamine or hydroxylamine, no detectable decrease in TC absorbance at 270 nm is observed. At higher temperatures or with greater concentrations of nucleophiles, some reaction does occur, as indicated by a decrease in the thiazoline absorbance at 270 nm. However, because these reactions occur too slowly to be of metabolic importance, they have not been studied further.

DISCUSSION

The present results, when considered in conjunction with those obtained by others (*7–12*) using different thiazoline derivatives, suggest that the hydrolysis of TC occurs as outlined in Scheme 1. Although this seems like a complex mechanism, the scheme actually has the minimum number of steps that need to be considered for the reaction (not all kinetically equivalent structures that differ only by a proton transfer are illustrated). Because the mechanism is so complex, however, and because the current experiments were not extensive enough to be able to analyze them quantitatively in terms of this mechanism, most of the discussion is qualitative in nature. Nevertheless, some general conclusions concerning how the reaction probably proceeds can be made.

At pH's of 2 and above, the pH-rate profile for TC hydrolysis (Fig. 2) is very similar to that for 2-methylthiazoline except that it is shifted somewhat because the pK_a for TCH (3.95) is lower than that for the methyl derivative (5.25), and the rate constant for the conversion of TCH to hydrolysis products is about 15 times greater than the corresponding rate constant for the conversion of protonated 2-methylthiazoline to hydrolysis products ($k = 6.7 \times 10^{-4}$ s^{-1} for TCH at 37°C and $k = 2.6 \times 10^{-5}$ s^{-1} for the protonated 2-methylthiazoline at 30°C). As with the other thiazolines, the product ratio changes in a pH region where the rate is constant, so the steps that determine which product will be formed must occur subsequent to the rate-determining step. Consequently, the rate-determining step in this pH region is almost certainly the conversion of TCH to IH′, i.e., the step analogous to the one that is the rate-determining step with other thiazolines.

For all previously studied thiazolines (*7–12*), the pH-rate profile for the hydrolysis is a simple bell-shaped curve, with the decrease in rate at high acid concentrations being due to protonation of the intermediate and the fact that there is no mechanism for such a species to proceed to hydrolysis products. The rate thus decreases because the only reaction available to the protonated intermediate is



SCHEME 1.

reversal to starting thiazoline, presumably through a species analogous to IH'. As noted in the previous section, the pH-rate profile for TC (Fig. 2) appears to be the superposition of two bell-shaped curves. However, the rate decrease expected in acid for one of them (presumably the one in the more basic pH region) is not seen. Almost certainly the reason for these characteristics is because TC has two hydrolytic reaction manifolds, both of which are probably the same as observed with other thiazolines. In one manifold the carboxyl group of TC is ionized (left side of Scheme 1) and in the other it is in the protonated form (right side of Scheme 1). With such a mechanism it is readily apparent why the rate decrease in acid of the putative first bell-shaped curve is not observed; the protonated intermediate can

now not only exist as IH′ but also as IH, which is a part of the second reaction manifold and can thus proceed to give hydrolysis products. As the acidity is increased below pH 2, the rate of the hydrolysis begins to increase, presumably because the TCH_2 to IH_2 step starts becoming significant. However, at still higher acidities, eventually all of the intermediate will be converted into IH_2 (or its kinetic equivalent) and the rate will slow down, as observed, because again the only reaction available to the protonated intermediate is reversal to the starting thiazoline.

In the hydrolysis of other thiazolines, it is known that the proton transfer step, analogous to the interconversion of I and $I^{\pm}$, can be slow relative to the cleavage of I to the *N*-acylcysteamine, and that this is the reason for the change in product ratio with pH. Presumably the same explanation holds for the present case as well in the pH region down to 1 or 2. The observation that at higher acidities (2 to 3 M HCl) the yield of *S*-oxalylcysteamine approaches 100% suggests that the ring cleavage through $IH^{\pm}$ occurs considerably faster than that through IH. The reason for the dropoff in thiolester yield at very high acidities (6 M HCl) is not known; however, it may be more apparent than real. The yield of thiolester would be underestimated if further hydrolysis to cysteamine and oxalate is occurring at a rate comparable with the rate of hydrolysis of TC to the oxalyl derivatives, and such may be the case at the high acidities where the TC reaction is relatively slow.

The effect of pH (Fig. 3) on the conversion of *S*-oxalylcysteamine to the *N*-oxalyl derivative can be explained in the same way as others (*10, 22*) have rationalized similar effects for the reaction of the *S*-acetyl derivative. In the pH range studied here, apparently the $I^{\pm}$ to I conversion is always rate limiting, with this step being acid-catalyzed at the low pH's and with a water-catalyzed reaction taking over at higher pH's. Since the *S*-oxalylcysteamine exists in this pH region virtually completely as the protonated species (SH), the acid-catalyzed reaction of $I^{\pm}$ is thus independent of pH whereas the water-catalyzed reaction depends inversely on the hydrogen ion concentration. Although data were not collected at a low enough pH to have the reaction completely independent of pH, the curvature in the plot of the data in Fig. 3 indicates that such a situation is being approached.

From the data in Fig. 3 one can readily extract combinations of rate and equilibrium constants. The steps involved in the reaction, as well as the appropriate constants for each step, are given in Eq. [4], and the derived expression for k_{obs} if this mechanism holds is given in Eq. [5] (this expression holds only in the pH

$$\mathrm{SH} \underset{\mathrm{H^+}}{\overset{K_a}{\rightleftharpoons}} S \overset{K}{\rightleftharpoons} \mathrm{I^{\pm}} \xrightarrow{k_{\mathrm{H}}[\mathrm{H^+}] + k_0} \text{products} \qquad [4]$$

$$k_{obs} = \frac{KK_ak_0}{[\mathrm{H^+}]} + KK_ak_{\mathrm{H}}. \qquad [5]$$

region where the amino group of *S*-oxalylcysteamine is protonated). The best fit of the data gives a value of 3.2×10^{-7} M s^{-1} for KK_ak_0 and 0.85×10^{-4} s^{-1} for KK_ak_{H}. The line in Fig. 3 is the calculated line with these values for the constants and it can be seen that the fit is reasonable. Some of the deviation from the line may be due to a small amount of buffer catalysis, especially when the acid form of the

buffer is in excess, but, since the buffer concentration is low (20 mM), this should not cause the KK_ak_0 value to be much in error.

Related constants for the reaction of *S*-acetylcysteamine can be obtained from published data. The value for KK_ak_0, calculated from the data of Martin and Hedrick (*22*) obtained at 35°C, is 1.02×10^{-8} M s^{-1}, and that calculated from data of Barnett and Jencks (*10*) obtained at 50°C is 4.3×10^{-8} M s^{-1}. Since the present experiments were done at 37°C, the comparison of the rate constants indicates that this sequence of steps proceeds 20 to 25 times more rapidly with *S*-oxalylcysteamine as reactant than it does with the *S*-acetyl derivative. The value for KK_ak_H, calculated from Martin and Hedrick's data at 35°C, is 1.1×10^{-4} s^{-1}, and that from Barnett and Jencks' data at 50°C is 3.2×10^{-4} s^{-1}. Therefore, for the acid-catalyzed reaction, *S*-oxalylcysteamine reacts at about the same rate, or even slightly slower, than *S*-acetylcysteamine. Since k_H is probably diffusion controlled and the same for both compounds, this means that KK_a must be approximately the same for both as well. Consequently, the difference in KK_ak_0 between the two compounds must be due to a difference in k_0, the water catalysis constant. Possibly the reason that the oxalyl compound reacts more rapidly is because the carboxylate acts as an intramolecular general base (perhaps with one or more bridging water molecules) to trap the intermediate zwitterion ($I^{\pm}$) so that it can go on to products.

With regard to what noncatalyzed reactions TC might undergo *in vivo*, the results reported here indicate that, at neutral pH and 37°C, TC would be stable for hours to days. Therefore, if TC is formed *in vivo* it should be detectable, unless it is subject to some rapid enzyme-catalyzed reaction. Because of the correlations suggesting that TC may be involved in controlling metabolism (*3, 5, 6*), it had been proposed (*1*) that TC might react with various nucleophilic groups on enzymes to give covalently modified derivatives with altered catalytic activity. The finding that TC reacts very slowly with amine and thiol nucleophiles under physiological conditions makes this unlikely unless the reactions of TC with the proteins were enzyme-catalyzed.

Very recently it has become evident that oxalyl thiolesters ($RSCOCOO^-$) are present in animal cells (*23*) and are probably acting as metabolic effectors; they have been shown to alter the activities of at least two metabolically important enzymes (*16, 24, 25*) when the oxalyl thiolesters are present at physiological concentrations. This raises the question of whether TC could be a source of oxalyl thiolesters *in vivo*. The results reported here indicate that the nonenzymic hydrolysis of TC at neutral pH is certainly too slow to be metabolically significant. If an enzyme were present to catalyze the simple hydrolysis of TC, then one could conclude from the current results that only the *N*-oxalyl derivative would be formed in reasonable amounts at neutral pH. Even if the enzyme directed the hydrolysis to give the *S*-oxalyl derivative, one could calculate from KK_ak_0 that, at pH 7.4 and 37°C, the half-time for the nonenzymic *S* to *N* transfer would be only 0.09 s. Consequently, oxalyl thiolesters could not result from the enzymic hydrolysis of TC unless either the enzyme directed a modification of the amino group of the product (possibly an acylation; *N*-acyl-*S*-oxalylcysteamines do not rearrange), or the initially formed *S*-oxalylcysteamine is very efficiently trapped by another

thiol while it is enzyme-bound. Both of the above are real possibilities that require further investigation.

REFERENCES

1. HAMILTON, G. A., BUCKTHAL, D. J., MORTENSEN, R. M., AND ZERBY, K. W. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 2625–2629.
2. NABER, N., VENKATESAN, P. P., AND HAMILTON, G. A. (1982) *Biochem. Biophys. Res. Commun.* **107,** 374–380.
3. HAMILTON, G. A. (1985) *Adv. Enzymol.* **57,** 85–178.
4. BURNS, C. L., MAIN, D. E., BUCKTHAL, D. J., AND HAMILTON, G. A. (1984) *Biochem. Biophys. Res. Commun.* **125,** 1039–1045.
5. HAMILTON, G. A., AND BUCKTHAL, D. J. (1982) *Bioorg. Chem.* **11,** 350–370.
6. HAMILTON, G. A., BUCKTHAL, D. J., AND KALINYAK, J. (1982) *in* Oxidases and Related Redox Systems (King, T. E., Mason, H. S., and Morrison, M., eds.), pp. 447–459, Pergamon, New York.
7. MARTIN, R. B., LOWEY, S., ELSON, E. L., AND EDSALL, J. T. (1959) *J. Amer. Chem. Soc.* **81,** 5089–5095.
8. MARTIN, R. B., AND PARCEL, A. (1961) *J. Amer. Chem. Soc.* **83,** 4830–4835.
9. SCHMIR, G. L. (1965) *J. Amer. Chem. Soc.* **87,** 2743–2751.
10. BARNETT, R. E., AND JENCKS, W. P. (1969) *J. Amer. Chem. Soc.* **91,** 2358–2369.
11. CERJAN, C., AND BARNETT, R. E. (1972) *J. Phys. Chem.* **76,** 1192–1195.
12. JENCKS, W. P. (1976) *Acc. Chem. Res.* **9,** 425–432.
13. VENKATESAN, P. P. (1984) Ph.D. thesis, The Pennsylvania State University.
14. KOCH, VON J., AND JAENICKE, L. (1962) *Liebigs Ann. Chem.* **652,** 129–139.
15. BRUSH, E. J., AND HAMILTON, G. A. (1981) *Biochem. Biophys. Res. Commun.* **103,** 1194–1200.
16. GUNSHORE, S., AND HAMILTON, G. A. (1986) *Biochem. Biophys. Res. Commun.* **134,** 93–99.
17. ELLMAN, G. L. (1959) *Arch. Biochem. Biophys.* **83,** 70–77.
18. RIDDLES, P. W., BLAKELEY, R. L., AND ZERNER, B. (1979) *Anal. Biochem.* **94,** 75–81.
19. ALBERT, A., AND SERJEANT, E. P. (1962) Ionization Constants of Acids and Bases, pp. 69–91, Wiley, New York.
20. AL-ARAB, M. M., AND HAMILTON, G. A. (1987) *Bioorg. Chem.*, in press.
21. PAUL, M. A., AND LONG, F. A. (1957) *Chem. Rev.* **57,** 1–45.
22. MARTIN, R. B., AND HEDRICK, R. I. (1962) *J. Amer. Chem. Soc.* **84,** 106–110.
23. SKORCZYNSKI, S. S., AND HAMILTON, G. A. (1986) *Fed. Proc.* **45,** 1689 (abstract 1217).
24. KISELICA, S. G., AND HAMILTON, G. A. (1986) *Fed. Proc.* **45,** 1689 (abstract 1219).
25. HARRIS, R. K., AND HAMILTON, G. A. (1986) *Fed. Proc.* **45,** 1689 (abstract 1218).