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Acetic Anhydride in Aqueous Solution Converts Δ^2-Thiazoline 2-Carboxylate to an Oxalyl Thiolester[1]

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N-Acetyl-*S*-oxalylcysteamine is formed in 70–75% yield from the reaction of acetic anhydride with Δ^2-thiazoline 2-carboxylate at 25°C in near neutral aqueous solution. This reaction is believed to be a good model for how oxalyl thiolesters could be formed *in vivo* from the products of the suspected physiological reactions catalyzed by D-amino acid oxidase and D-aspartate oxidase. The reaction is of special significance because of current evidence that oxalyl thiolesters are important metabolic effectors.

Recent investigations in this laboratory have indicated that Δ^2-thiazoline 2-carboxylate (TC)[3] and its derivatives (Z-TC) are the products of the probable physiological reactions catalyzed by mammalian D-amino acid oxidase and D-aspartate oxidase, respectively (*1–4*). Furthermore, considerable circumstantial evidence has been summarized (*1, 5, 6*) suggesting that such products (or their further metabolites) may participate in controlling animal metabolism, and may function as part of the intracellular messenger system for various hormones, especially insulin and growth factors. Oxalyl thiolesters (OTEs, $RSCOCOO^-$) are potential metabolites of TCs, and very recent findings (*7–10*) have focused attention on the possibility that such compounds may be particularly important metabolic effectors. Thus, it has now been shown (*7*) that OTEs are present in various animal tissues, and, at their known physiological concentrations, they have been found to modify the catalytic activities of at least three important animal enzymes (*8–10*). One known reaction leading to OTEs directly is that catalyzed by L-hydroxy acid oxidase (*1, 11–14*), but, given the apparent importance of OTEs to animal metabolism, the possibility that they may also be formed from TCs needs to be investigated. In the present article, a model reaction for how OTEs might arise *in vivo* from TCs is reported.

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[3] Abbreviations used: OTEs, oxalyl thiolesters; TC, Δ^2-thiazoline 2-carboxylate; Z-TC, Δ^2-thiazoline 2,4-dicarboxylate or its glycine amide; TCs, TC and/or Z-TCs; NAC-S-Ox, *N*-acetyl-*S*-oxalylcysteamine.

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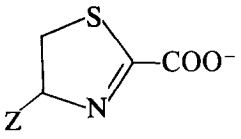
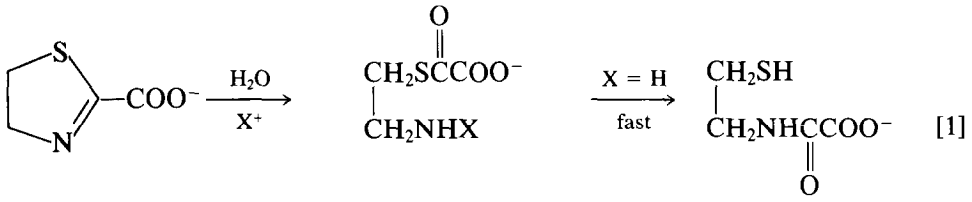


TCs

TC: Z = H (D-amino acid oxidase product)
Z-TC: Z = COO^- or $CONHCH_2COO^-$ (D-aspartate oxidase product)

SCHEME I

OTEs could not be formed physiologically from the direct hydrolysis of TCs because such hydrolyses lead exclusively to *N*-oxalyl products at any pH above 3 (*15*). The *S*-oxalyl derivatives may be fleeting intermediates in these reactions, but the intramolecular oxalyl transfer to give the more stable *N*-oxalyl derivative (Eq. [1], X = H) occurs very rapidly (half-time at pH 7 and 37°C, approximately 0.2 s for *S*-oxalylcysteamine). In contrast to the rapid conversion of *S*-oxalylcysteamine to *N*-oxalylcysteamine at neutral pH, when X is acyl, as in *N*-acetyl-*S*-oxalylcysteamine (NAC-S-Ox), then intramolecular rearrangement does not occur (*16*). Consequently, it seemed reasonable that interaction of TC (or Z-TC) with an electrophilic acyl group (X^+), rather than just the proton, might be a possible physiological reaction to convert TCs to OTEs. *In vivo* the most likely electrophilic acyl reactant would be an anhydride, probably a mixed anhydride of a carboxylic acid and a phosphoric acid. In the present study this possible reaction was modeled using acetic anhydride and TC itself. As reported here, the reaction of acetic anhydride with TC at 25°C and pH near neutral does in fact lead to the formation of NAC-S-Ox as the major product.



[1]

TC X = H: S-oxalylcysteamine
X = acetyl: *N*-acetyl-S-oxalylcysteamine (NAC-S-Ox)

N-oxalylcysteamine

EXPERIMENTAL PROCEDURES

Materials. Unless otherwise noted, commercially available materials, reagent grade or better, were used as received. All water was glass distilled and subsequently passed through a Millipore (Milli Q) reverse-osmosis water purification system.

Some of the Δ^2-thiazoline-2-carboxylate used in this work was prepared as

previously described (3), but a better method for preparing the intermediate ethyl ester by reacting cysteamine with ethyl cyanoformate was developed during the course of the work. This procedure is derived from previous investigations by Shimizu and co-workers (*17*). To a stirred solution of 0.2 mol cysteamine free base in 200 ml absolute ethanol at room temperature was slowly added over 1 h a solution containing 0.2 mol ethyl cyanoformate in 20 ml of the same solvent. After refluxing under N_2 for 24 h, the solvent was evaporated. To the yellowish oil that results was added 20 ml ethanol and 5 g Amberlite IRA 400 (basic). After stirring, the resin was removed by filtration, the solvent evaporated from the filtrate, and the residue distilled under reduced pressure (0.25 mm Hg) to give a 75% yield of a pure colorless oil (bp 80–82°C) that was identical in all respects to the ethyl ester of TC prepared by the earlier method (*3*). TC was obtained from the ester as previously described (*3*).

N,S-Diacetylcysteamine was prepared as described by Gerstein and Jencks (*18*) and *N*-acetyl-*S*-oxalylcysteamine (NAC-S-Ox) as described by Law and Hamilton (*16*). *S*-Acetyl-*N*-oxalylcysteamine was prepared by adding dropwise with stirring 5 mmol acetic anhydride to a cooled (0 to 3°C) solution containing 4 mmol sodium salt of *N*-oxalylcysteamine (*15*) in 5 ml water. During the reaction, the pH of the solution was maintained at 8.0 by the simultaneous addition of 25% sodium hydroxide. Following the reaction, the solution was left at room temperature for 30 min, then acidified to pH 1.5 with 6 N HCl, and extracted with ether. After drying the ether extract with anhydrous sodium sulfate, the ether was evaporated and the solid (70% yield) was recrystallized from ethanol/methylene chloride to give white crystals with mp 102°C. The mass spectrum of this material shows a parent ion at *m/e* 191, and its NMR spectrum ($CDCl_3$) has absorptions at δ 9.0 (s, 1 H), 7.81 (broad s, 1 H), 3.65 (q, 2 H), 3.2 (t, 2 H), and 2.4 (s, 3 H).

Methods. The procedures used for obtaining ultraviolet spectra, rate constants, and thiol concentrations have been given in earlier publications (*3, 8, 14–16*). The HPLC analysis method for *N*-oxalylcysteine has also been described (*7*). Thin-layer chromatographic (TLC) analyses of reaction mixtures that had been treated with hydroxylamine were performed (*11*) using Eastman chromogram plates (cellulose on Mylar), with elution using 1-propanol, 10% aqueous ammonium carbonate, concentrated ammonia (10 : 4 : 8), and visualization with 5% $FeCl_3$ in ethanol containing 0.1 N HCl. Under such conditions, authentic oxalyl monohydroxamate and acetohydroxamic acid have R_f values of 0.51 and 0.78, respectively.

RESULTS

In preliminary experiments, any potential reaction of TC with aqueous acetic anhydride to give NAC-S-Ox was monitored by following the disappearance of the absorption at 270 nm (extinction coefficient, 1.64 mM^{-1} cm^{-1}) due to TC (*3*), and the appearance of absorption at 260 nm (extinction coefficient, 2.9 mM^{-1} cm^{-1}) due to NAC-S-Ox (*8*). These reactions were performed at 25°C and were initiated by adding sufficient neat acetic anhydride to make its initial concentration 10 to 150 mM in 10 to 200 mM phosphate buffer (initial pH 7.4) containing 0.4

mM TC, 1 to 3 mM EDTA, and KCl to give an ionic strength of 1.0 M. At low concentrations of anhydride or very high buffer concentrations, very little increase in absorbance at 260 nm was observed, but at high anhydride and moderate buffer concentrations, a species absorbing at that wavelength is seen. The best conditions found for maximizing the formation of this species are those given in the legend of Fig. 1. Presumably a large excess of the anhydride is required because it hydrolyzes so rapidly under the reaction conditions (*19, 20,* and references therein). The anhydride hydrolysis is catalyzed by bases and nucleophiles so that is probably the reason for the decrease in yield at high buffer concentrations. Some buffer, however, is required or otherwise the pH of the solution drops to approximately 2, under which conditions TC is unstable to hydrolysis (*15*). Even with the 100 mM phosphate buffer used in the experiment of Fig. 1, the pH decreases from an initial value of 7.4 to 4.2 at the end of the reaction. Phosphate itself is not required for the reaction since similar spectral changes are observed if the phosphate buffer is replaced by 100 mM ammonium carbonate (initial pH 7.4).

It can be seen from Fig. 1 that all of the TC reacts under the conditions given. This was confirmed when it was found that the addition of more acetic anhydride after the initial reaction is complete leads to no further changes in the final spectrum. The observation of an absorption at 260 nm at the end of the reaction suggests that an OTE is being formed, but it is clear from the magnitude of the absorption that it is not being formed in quantitative yields; if one assumes that all of the absorption at 260 nm is due to an OTE (*8*), then its yield is 70 to 75%. The

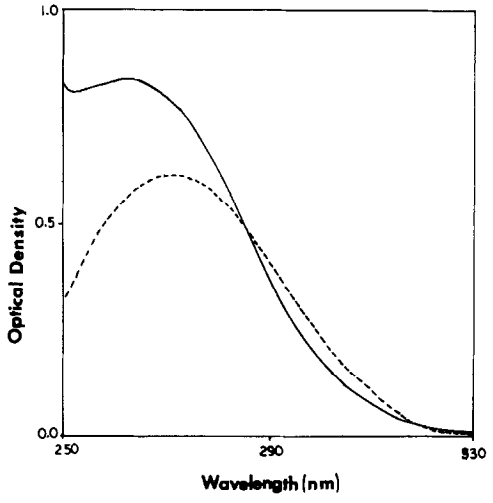


FIG. 1. The ultraviolet spectra of TC (---) and the product (—) that results from the reaction of TC with acetic anhydride. The TC spectrum was recorded at 25°C using 0.4 mM TC in 100 mM potassium phosphate buffer, pH 7.4, containing 1 mM EDTA and sufficient KCl to give an ionic strength of 1.0 mM. At time zero, sufficient acetic anhydride was added to this solution to make its concentration 100 mM, and the mixture was allowed to react at room temperature for 10 to 15 min. During this time the pH of the solution drops to approximately 4.2. After neutralizing to pH 7.4 with concentrated NaOH, the illustrated spectrum of the product was recorded. The final spectrum is not corrected for a volume change of 4% that results from the addition of acetic anhydride and base.

other product formed in the reaction cannot have a free thiol because titration of reaction mixtures with Ellman's reagent (*14*) indicates the absence of any thiol. If, however, reaction mixtures are first reacted with 50 mM hydroxylamine (25°C for 1 hr) and then titrated with Ellman's reagent, the thiol content is 92 ± 3% of the original TC used. Since TC does not react with hydroxylamine under these conditions but thiolesters do, the result indicates that virtually all of the products formed are thiolesters. The nature of the thiolester products was further clarified by subjecting the product of the above hydroxylamine reaction to TLC analysis as outlined under Experimental procedures; spots for both oxalyl monhydroxamate and acetohydroxamic acid were detected, thus indicating that both oxalyl and acetyl thiolester products are formed in the original reaction.

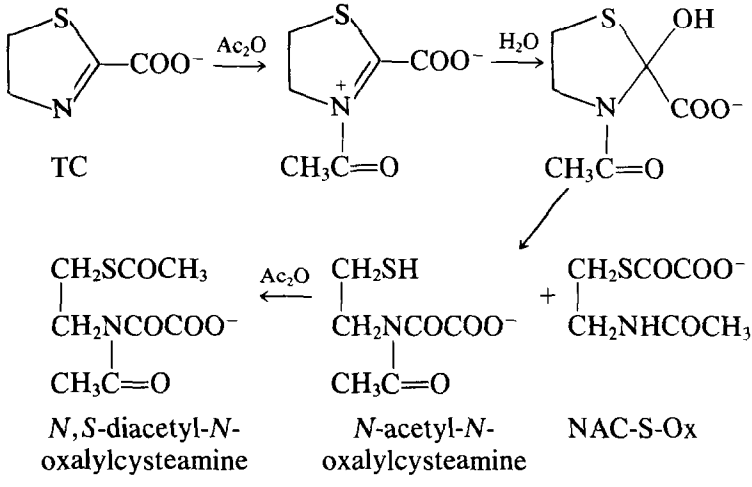
If reaction mixtures from the reaction of TC with acetic anhydride are subjected directly to TLC analysis using cellulose on Mylar, with a developing solvent of 1-butanol, acetic acid, water (6:2:2) and visualization with alkaline nitroprusside spray, two spots are detected, one with an R_f of 0.54 and the other an R_f of 0.66. The former is identical to that given by authentic NAC-S-Ox (*14, 16*) while the latter is of an unknown compound; it is not *S*-acetyl-*N*-oxalylcysteamine which has an R_f of 0.92 nor is it *N,S*-diacetylcysteamine which moves with the solvent front in this system. A further indication that the 260-nm absorbing species, formed in the TC plus acetic anhydride reaction, is NAC-S-Ox, is the observation that, in the presence of nucleophiles (hydroxylamine, cysteamine, and cysteine), the absorption at 260 nm disappears with the same rate constants (within experimental error) as observed when authentic NAC-S-Ox reacts with the same nucleophiles (*16*).

The amount of an oxalyl thiolester in solution can be quantitated as *N*-oxalylcysteine following reaction with cysteine (*7*). When TC plus acetic anhydride reaction mixtures (reaction conditions similar to those in the legend of Fig. 1) were analyzed in this way, the amount of *N*-oxalylcysteine observed indicates that NAC-S-Ox is formed in 70 ± 3% yield, based on the initial amount of TC used. This agrees very well with the amount estimated from the magnitude of the 260 nm absorption (vide ante). In the reaction of cysteine with NAC-S-Ox the other product is *N*-acetylcysteamine, and this compound was also found (by HPLC) following the reaction of TC–acetic anhydride reaction mixtures with cysteine.

DISCUSSION

The results reported here indicate that NAC-S-Ox is a product of the reaction of TC with acetic anhydride in aqueous solution. However, it is also clear from the results that NAC-S-Ox is not the only product; under the best conditions examined (similar to those given in Fig. 1), NAC-S-Ox is formed in a 70 to 75% yield. The identity of the other product was not conclusively established, but the results indicate it is an *S*-acetyl compound which is not *N,S*-diacetylcysteamine or *S*-acetyl-*N*-oxalylcysteamine. The most likely structure for this compound would seem to be *N,S*-diacetyl-*N*-oxalylcysteamine, and that the overall reaction of TC with acetic anhydride (Ac_2O) proceeds as illustrated in Eq. [2]. The hydrolysis of

TC is initiated by protonation on the nitrogen (*15*), so acylation on the same atom (possibly with a mixed anhydride of TC as an intermediate) would give a species which should also add water to give the tetrahedral intermediate. This could break down to give either *N*-acetyl-*N*-oxalylcysteamine or NAC-S-Ox, and apparently the latter is favored by more than 2 to 1 under the conditions utilized. The initially formed *N*-acetyl-*N*-oxalylcysteamine would be expected to react rapidly with acetic anhydride to give the *N*,*S*-diacetyl compound.



[2]

Because acetic anhydride reacts so rapidly with water, a large excess is needed in order to observe its reaction with TC. Unfortunately, the rapid hydrolysis of the excess anhydride causes the acidity of the solution to increase during the reaction, and this could not be prevented by having a concentrated buffer solution, because high buffer concentrations catalyze the anhydride hydrolysis (*20*). Consequently, some change in acidity of the solution was unavoidable during the reaction. Because of these complications, no attempt was made to clarify the details of the conversion by investigating the kinetics of the TC–acetic anhydride reaction. The main focus of the current research was just to demonstrate that an anhydride could convert TC to an OTE, and that has been clearly shown. This demonstration makes it, therefore, very reasonable that OTEs might be formed from TC and Z-TC in vivo. Any such physiological reaction would presumably be enzyme catalyzed and would probably involve some mixed anhydride of a carboxylic acid and a phosphoric acid acting as the electrophile. Given the apparent importance of OTEs to the control of animal metabolism (*7–10*), the possibility that such a reaction may be occurring *in vivo* should clearly be investigated.

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