BIOORGANIC CHEMISTRY 14, 378–391 (1986)

The Reaction of Oxalyl Thiolesters with Nucleophiles, Especially Thiols[1]

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Received May 12, 1986

Since there is evidence that oxalyl thiolesters ($RSCOCOO^-$) are present in animal cells, and possibly may participate in the control of metabolism, the present study was undertaken to characterize their reactivity with nucleophiles so that one could gain a better understanding of how they might be affecting the activities of enzymes. At 25°C and neutral pH, *N*-acetyl-*S*-oxalyl-2-aminoethanethiol (NAC-S-Ox) reacts rapidly with cysteamine (2-aminoethanethiol) to give *N*-acetylcysteamine and *N*-oxalylcysteamine. Under similar conditions, other aminothiols, such as cysteine, homocysteine, penicillamine, and cysteine ethyl ester, also react rapidly with NAC-S-Ox, but non-thiol-containing amines, such as alanine, alanine ethyl ester, glycine, and *S*-methylcysteine, react more than four orders of magnitude less rapidly. The aminothiol reactions apparently proceed by rate-determining oxalyl transfer to the thiol group followed by a rapid intramolecular *S*- to *N*-oxalyl migration. The reactions follow second-order kinetics with the thiolate anion being the reactive nucleophile. At 25°C and ionic strength 1.0 M, k_N, defined in the equation, rate = $k_N[RS^-][NAC\text{-}S\text{-}Ox]$, has the following values ($M^{-1}\ s^{-1}$) for the anion of the reacting thiol: cysteamine, 170; cysteine, 260; cysteine ethyl ester, 76; homocysteine, 380. Rate data for the reaction of NAC-S-Ox with hydroxylamine, imidazole, hydroperoxide, and hydroxide were also obtained. The reaction of *S*-oxalyl-*p*-thiocresol with thiol anions under the same conditions gives the following values for k_N ($M^{-1}\ s^{-1} \times 10^{-3}$): glutathione, 5.6; *N*-acetylcysteamine, 3.7; pantetheine, 4.8; 8-mercaptooctanoic acid, 4.5; 6-mercaptooctanoic acid, 1.0; dihydrolipoic acid, 8.2. These results indicate that oxalyl transfers from oxalyl thiolesters to thiol anions occur more than two orders of magnitude more rapidly than corresponding acetyl transfers, and that under physiological conditions any *in vivo* oxalyl thiolester would equilibrate within minutes with virtually every thiol in the cell, including those attached to enzymes. Consequently, it is proposed that one mechanism by which oxalyl thiolesters may function *in vivo* to alter the catalytic activities of enzymes is to covalently modify enzymic thiols by acylation with an oxalyl group.

INTRODUCTION

It is becoming increasingly probable that oxalyl thiolesters ($RSCOCOO^-$) are important mammalian metabolites. The first indications that this might be the case were our findings (*1–6*) that glyoxylate thiohemiacetals, formed nonenzymically from thiols and glyoxylate, are excellent substrates for mammalian L-hydroxy

[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 Service.

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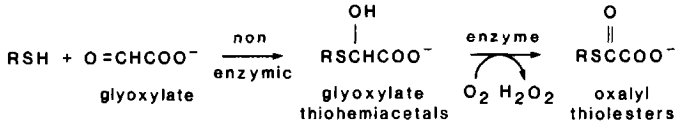
acid oxidase, that oxalyl thiolesters are the products of such reactions (Eq. [1]),



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and that the reactions show characteristics indicating that they are probably occurring *in vivo*. Although currently the reaction of Eq. [1] is the best-defined route to oxalyl thiolesters, it is likely that they are formed by other pathways as well. For example, oxalyl thiolesters could also arise from further metabolism of the thiazoline-2-carboxylate products of the suspected physiological reactions catalyzed by D-amino acid oxidase and D-aspartate oxidase (*6–10*). A considerable body of evidence (*6, 11, 12*) suggests that the products (or their further metabolites) of these various peroxisomal oxidase reactions are involved in controlling animal metabolism, and possibly may be functioning as part of the intracellular messenger system for some hormones, especially insulin.

Regardless of the mechanism for their formation, it has now been determined by direct measurement (*13*) that oxalyl thiolesters are present in animal cells. Furthermore, their probable involvement in controlling animal metabolism has been confirmed by our very recent findings (*14–16*) that the catalytic activities of several metabolically important enzymes are affected by such compounds when present at known physiological concentrations. Given the probable metabolic importance of the oxalyl thiolesters, it seemed imperative to characterize their nonenzymic reactivity, especially with nucleophiles, so that one could gain a better understanding of how they might be affecting the activities of various enzymes. Such a nonenzymic study is reported here.

There is an extensive literature on the reactions of nucleophiles with thiolesters (*17–30*) but virtually none of these studies has been concerned with determining the reactivities of oxalyl derivatives. The single exception is that the base-catalyzed hydrolysis of $EtSCOCOO^-$ has been investigated (*31*). It will be seen that some of the characteristics of oxalyl thiolester reactions are similar to those of other derivatives, but that the oxalyl compounds appear to be especially reactive toward thiols. For this reason most of the current investigation is concerned with characterizing the reaction with thiols.

EXPERIMENTAL PROCEDURES

Materials. Unless otherwise noted, commercially available materials, reagent grade or better, were used as received. All water used in this work was glass distilled and then passed through a Millipore (Milli Q) reverse osmosis water purification system. DL-6-Mercaptooctanoic acid and 8-mercaptooctanoic acid were prepared by E. J. Brush following the procedures outlined by White (*32*). Dihydrolipoic acid and pantetheine were obtained by reduction of their oxidized forms (*4*). *N*-Oxalylcysteamine was synthesized as previously described (*10*).

S-Oxalyl-*p*-thiocresol (TL-S-Ox)[3] was prepared by the general method of Stolle (*33*) with some modifications. To a stirred ice-cold solution of 7 g (0.06 mol) oxalyl chloride in 100 ml anhydrous ether was slowly added over 2 h 4.7 g (0.04 mol) *p*-thiocresol dissolved in 100 ml anhydrous ether. After warming to room temperature and refluxing for 1 h, the solvent was evaporated under reduced pressure. To the residue were added seven 0.3-ml aliquots of water, each followed by vigorous shaking and venting of the liberated HCl. Following evaporation under reduced pressure with gentle heating, a yellow solid formed. After 25 ml of petroleum ether was added, the solid was filtered from the solution, dried under reduced pressure, and sublimed. At 0.05 mm Hg, unreacted *p*-thiocresol was collected at 40°C and the light yellow needles of TL-S-Ox (acid form) at 85°C. The material melted sharply at 101°C (reported melting point: 100°C, Ref. (*33*)) and had less than 2% free thiol as determined by titration. In aqueous solution at pH 7.5, TL-S-Ox absorbs at 227 and 264 nm with extinction coefficients of 13 and 2.5 mM^{-1} cm^{-1}, respectively.

N-Acetyl-*S*-oxalylcysteamine (NAC-S-Ox) was prepared by oxalyl thiolester exchange in a procedure related to that used by Quayle (*34, 35*) to prepare oxalyl coenzyme A. Following the addition of 0.3 g (1.7 mmol) TL-S-Ox to 0.4 g (3.4 mmol) *N*-acetylcysteamine dissolved in 5 ml dichloromethane, the mixture was stirred vigorously for 5 h (subsequent work indicates that ½ h is sufficient) and then the solvent was evaporated under reduced pressure. If an oil resulted, 5 ml of dichloromethane was added and the evaporation repeated. Eventually a white solid was obtained which in the present work was purified by repeated leaching (seven or more times or until no free thiol was detected by titration) with 10-ml quantities of dichloromethane. In later work it has been found that a better way to obtain the crystalline product is to store the dichloromethane solution at −20°C overnight. The compound purified by either procedure melts at 164°C with decomposition (reported melting point (*36*): 159–160°C with decomposition). At neutral pH, NAC-S-Ox has an absorption maximum at 260 nm with an extinction coefficient of 2.9 mM^{-1} cm^{-1} (*14*).

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 previously described (*4*). All 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 reactions of NAC-S-Ox were monitored by following the decrease in the 260-nm absorbance while those of TL-S-Ox were monitored by following the increase in absorbance at 265 nm due to the ionized *p*-thiocresol product (extinction coefficient at 263 nm is 18 mM^{-1} cm^{-1}) that is produced at the pH values employed. EDTA (1 to 3 mM) was added to each reaction solution to minimize thiol autoxidation. However, since the pH of thiol reaction solutions was always less than 7.5, the thiol concentrations were low (1 to 6.5 mM), and the reactions are so fast (they

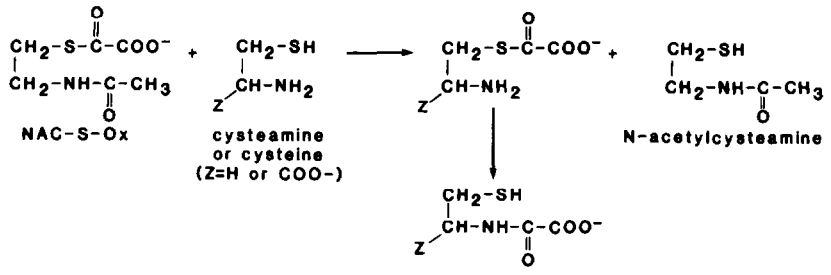
[3] Abbreviations used: NAC-S-Ox, *N*-acetyl-*S*-oxalylcysteamine; TL-S-Ox, *S*-oxalyl-*p*-thiocresol; ED, ethylenediamine.

are over in a few minutes under the conditions employed), thiol autoxidation was not a serious problem. All reactions were run under pseudo-first-order conditions and good first-order kinetics were observed for at least three half-times. The observed first-order rate constants (k_{obs}) were calculated by standard methods, although in some cases a small correction (less than 5%) was made to the optical density due to a linear drift in the baseline at infinite time. All stock solutions and buffers were kept in a water bath maintained at 25.0°C, and then further equilibrated at the same temperature for at least 10 min in the sample cuvette (total volume, 3 ml) of the spectrophotometer prior to initiating the reaction by adding a small aliquot of a solution containing the thiolester. Unless otherwise noted, each reported rate constant is the average of those obtained in at least three identical runs with the reproducibility always being better than ±5%.

RESULTS

Preliminary observations. At 25°C in buffer solutions of pH 6 to 8, NAC-S-Ox is stable for hours to days; no decrease in the 260-nm absorption is detectable over a period of several hours. At higher pH values, however, the absorbance decreases with time due to base-catalyzed hydrolysis.

A preliminary survey of the possible reactivity of various nucleophiles with NAC-S-Ox was carried out by monitoring its 260-nm absorption in the presence of the nucleophile under the following conditions: 25°C, 50 mM sodium phosphate buffer, pH 7.2, containing 3 mM EDTA and sufficient NaCl to give an ionic strength of 0.5 M. Not unexpectedly, hydroxylamine at 50 to 100 mM concentrations causes a decrease with time in the 260-nm absorption under such conditions. Somewhat surprisingly, however, it was found that, whereas 200 mM concentrations of L-alanine caused no detectable decrease in the 260-nm absorption, the presence of L-cysteine or cysteamine (2-aminoethanethiol) at 1 to 3 mM concentrations led to a very rapid decrease in the absorption. Since the amino group of alanine should have approximately the same reactivity as that of cysteine or cysteamine, such results imply that it is the thiol group of the latter compounds that is reacting with NAC-S-Ox. A simple oxalyl transfer to the thiol group of cysteine or cysteamine would not lead to any significant change in the 260-nm absorption. However, one can calculate from other data (*10*) that, for *S*-oxalylcysteamine, the intramolecular transfer of the oxalyl group from sulfur to nitrogen (and thus loss of the 260-nm absorption) would occur with a half-time of approximately 0.2 s under such conditions. A similar transfer involving the cysteine derivative would be expected to occur at about the same rate. Consequently, the reactions that are presumably occurring in the reaction of NAC-S-Ox with cysteine or cysteamine are those summarized in Eq. [2]. Consistent with this formulation is the observation that *S*-methylcysteine shows no detectable reactivity toward NAC-S-Ox under similar conditions. Furthermore, when equimolar quantities of NAC-S-Ox and cysteamine are allowed to react and the products analyzed by thin-layer chromatography (using Fisher silica gel G plates, a developing solvent of acetic acid, concentrated HCl (20 : 1), and visualization with



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iodine/azide spray), the only compounds detected are *N*-oxalylcysteamine (R_f = 0.61) and *N*-acetylcysteamine (R_f = 0.50). After such a reaction no *S*-oxalyl derivative is present as evidenced by the lack of any spots being seen when the plates are sprayed with hydroxylamine reagent (2 M, pH 7.0) followed by gentle warming and spraying with ferric ion (5% $FeCl_3$ in 0.1 N HCl/ethanol). Unreacted *S*-oxalyl compounds show a purple spot when thus treated.

Kinetic data for the reaction of NAC-S-Ox with nucleophiles. Summarized in Table 1 are some quantitative data for the reaction of various nucleophiles with NAC-S-Ox at two different ionic strengths. The ionic strength effects are small in most cases; the largest effect is for the hydroxylamine reaction which probably involves a zwitterionic intermediate. Most of the constants given in the table are second-order rate constants. The constant k_T is defined by the following: rate = k_{obs}[NAC-S-Ox] = k_T[N_T][NAC-S-Ox], where [N_T] is the total concentration of all forms (ionized and unionized) of the nucleophile at the particular pH given. The values of k_T are useful as a measure of the relative reactivities of the various nucleophiles at a pH close to the physiological pH. The constant k_N is defined by rate = k_{obs}[NAC-S-Ox] = k_N[N][NAC-S-Ox], where [N] is the concentration of the actual presumed nucleophile, calculated from known pK_a values.

From data obtained at several different nucleophile concentrations, the reactions involving cysteamine, cysteine, homocysteine, imidazole, hydrogen peroxide, and hydroxide were explicitly shown to be second-order reactions, first order in NAC-S-Ox, and first order in the nucleophile. By analogy, one suspects that the cysteine ethyl ester and penicillamine reactions are also second order, but that was not directly shown since data with these compounds were collected at only one concentration. In the case of hydroxylamine, the reaction is second order at low concentrations of hydroxylamine but changes to third order overall (second order in the hydroxylamine concentration) at higher concentrations. Both the second- and third-order constants, obtained at pH 7.2 and ionic strength 0.5 M, are given in Table 1. With glycine, the reaction is so slow that data were obtained only when the concentration was 1 M. Although a second-order rate constant is reported in the table, no conclusions concerning the order of this reaction can be made until it is studied further. In other experiments not given in the table, it was shown that, at pH 7.2 and ionic strength 0.5 M, the k_T values (assuming the reactions are second order, which they may not be) for alanine, alanine methyl ester, and *S*-methylcysteine are all less than 10^{-3} M^{-1} s^{-1}.

The hydroxylamine reaction proceeds at approximately the same rate from pH

TABLE 1

KINETIC DATA FOR THE REACTION OF VARIOUS NUCLEOPHILES WITH NAC-S-Ox

Reactant (in parentheses is the pK_a of the conjugate acid of the presumed reacting species[a])	Second-order rate constants (M^{-1} s^{-1}) at 25°C			
	Ionic strength = 1.0 M[b]		Ionic strength = 0.5 M[c]	
	k_T[d] (at pH 7.15)	k_N[d] (independent of pH)	k_T[d] (at pH 7.20)	k_N[d] (independent of pH)
Cysteamine (8.35[e])	10.2	170	14.7	220
Cysteine (8.50[e])	10.9	260	16.0	340
Cysteine Et ester (7.45[e])	9.9[f]	76	12.3	94
Homocysteine (9.14[e])	4.1	380	4.6	410
Penicillamine	0.094		0.082	
Hydroxylamine (6.04[g])	0.165	0.178	0.059	0.063
			2.6[h]	3.0[h]
Imidazole (7.10[g])	4.4×10^{-4}[i]	0.00083		
Glycine (9.60[j])	4.1×10^{-5}[k]	0.018		
H_2O_2 (11.65[l])	0.097	2800		
Hydroxide (13.97[m])	5.9×10^{-7}[n]	4.2	4.1×10^{-7}[n]	2.6

[a] Although the listed pK_a values were not all measured at the temperature and ionic strength of the kinetic data, these values were the ones used to calculate the k_N values given in the table.

[b] Reaction solutions contained 10 mM potassium phosphate buffer, 1 mM EDTA, and sufficient KCl to maintain the ionic strength at 1.0 M.

[c] Reaction solutions contained 50 mM sodium phosphate buffer, 3 mM EDTA, and sufficient NaCl to give an ionic strength of 0.5 M.

[d] Defined in the text.

[e] See Table 2 for the definition of these pK_a constants and the conditions under which they were obtained.

[f] Calculated from data obtained at pH 7.37.

[g] Obtained at 25°C and ionic strength 1.0 M (*41*).

[h] Third-order rate constant in M^{-2} s^{-1}.

[i] Ionic strength 1.4 ± 0.2 M.

[j] Obtained at 25°C and low (<0.05 M) ionic strength (*37*).

[k] Second-order rate constant calculated from data obtained at pH 7.35. The order of the reaction was not determined; it was just assumed to be first order in glycine to calculate this constant.

[l] Obtained at 25°C and low (<0.05 M) ionic strength (*42*).

[m] Value for pK_w at 25°C (*43*).

[n] First-order rate constant ($k = k_N[OH^-]$) in s^{-1}, calculated from data obtained at pHs 11.8 to 12.2.

6.5 to 7.3, so presumably both the second- and third-order reactions involve the neutral form of the hydroxylamine. In calculating the k_N values given in Table 1 for the nitrogen nucleophiles, it was assumed that only the neutral amine forms of imidazole and glycine react.

When the rates of the reactions of NAC-S-Ox with cysteine, cysteamine, and hydrogen peroxide were investigated at different pH values (6.5 to 7.5), it was observed in each case that the rate increases with pH, as expected if the actual nucleophile is the thiolate or hydroperoxide ion. Indeed it was found in each of

these cases that a constant k_N was obtained when it was calculated using kinetic data obtained at various pH values and the appropriate pK_a values (see Tables 1 and 2). For the thiolamine reactants, the thiol and ammonium groups have similar pK_a values so that the nucleophile could potentially be either $^+NH_3RS^-$ or NH_2RS^-. It was found that constant k_N values are obtained when it was assumed that $^+NH_3RS^-$ is the reactive species but not when NH_2RS^- is assumed to be the reacting nucleophile (all the kinetic data were collected between pH 6.5 and 7.5). The values of k_N given in Table 1 are thus those obtained assuming that $^+NH_3RS^-$ is the only reacting species when the pH is around 7. In these calculations the microscopic pK_a values given in Table 2 were employed. It is recognized that several of the pK_a values used for the calculations of k_N were not obtained at the same temperature and ionic strength as the kinetic data, so in those cases the reported k_N values should be viewed with that reservation in mind. However, they are still useful as an approximate indication of the reactivity of each nucleophile.

The reaction of ethylenediamine with NAC-S-Ox at pH 7.15 does not follow second- or third-order kinetics or a combination of the two. When the total concentration ([N_T]) of ethylenediamine is varied from 0.1 to 0.4 M, a plot of log k_{obs} vs log[N_T] indicates an approximate order of 1.65. A possible explanation for the results with this compound is that the ethylenediamine forms a complex with NAC-S-Ox and that this complex reacts with another molecule of the ethylenediamine (ED) to lead to products:

$$\text{NAC-S-Ox} + \text{ED} \underset{}{\overset{K}{\rightleftharpoons}} \text{NAC-S-Ox.ED} \xrightarrow{k[\text{ED}]} \text{products.} \qquad [3]$$

It can easily be shown that a plot of $[N_T]/k_{obs}$ vs $1/[N_T]$ should be linear if the reaction occurs as in Eq. [3] and such a plot is indeed linear. Values for K and

TABLE 2

MICROSCOPIC IONIZATION CONSTANTS FOR THIOLAMINES[a]

Compound	pK_a^+	p$K_a^{\pm\prime}$	pK_a^0	p$K_a^{\pm}$	Reference
Cysteamine[b]	8.34			10.75	*37, 38*
Cysteine[c]	8.50	8.85	10.00	10.35	*39*
Cysteine ethyl ester[b]	7.45	6.77	9.09	8.41	*38*
Homocysteine[d]	9.14	9.21	10.52	10.59	*40*
Glutathione[e]	8.93	9.13	9.08	9.28	*44*

[a] The various acid ionization constants are for the following equilibria:

$$\begin{array}{ccc} {}^+NH_3RSH & \overset{K_a^+}{\rightleftharpoons} & {}^+NH_3RS^- \\ K_a^{+\prime} \Big\downarrow\uparrow H^+ & & K_a^{\pm} \Big\downarrow\uparrow H^+ \\ NH_2RSH & \underset{H^+}{\overset{K_a^0}{\rightleftharpoons}} & NH_2RS^-. \end{array}$$

[b] Constants obtained at 23°C and low (<0.05 M) ionic strength.
[c] Constants obtained at 25°C and ionic strength of 0.5 to 1.0 M.
[d] Constants obtained at 25°C and low (<0.05 M) ionic strength.
[e] Constants obtained at 25°C and ionic strength 0.2 to 0.55 M.

k can be obtained from this plot and were found to be 2.8 M^{-1} and 0.018 M^{-1} s^{-1}, respectively, at 25°C, pH 7.15, and ionic strength 1.15 ± 0.1 M. As the pH is increased from 6.5 to 7.5, the rate of the reaction increases, but insufficient data were collected to be able to distinguish which of the three possible ionized and unionized forms of ethylenediamine is involved in each step of the reaction in Eq. [3].

Kinetic data for the reaction of TL-S-Ox with nucleophiles. Because the reaction of NAC-S-Ox with simple thiols (thiols that do not contain a β- or γ-amino group to which the oxalyl group can transfer) could not be followed spectrally, some kinetic data (summarized in Table 3) were collected using TL-S-Ox as the reacting thiolester. With this reactant, rate data for the reaction of simple thiols could be readily measured because of the strong absorbance of the thiocresolate ion product. However, easily analyzable data could not be obtained using the aminothiols as reactants because the rates of the inter- and intramolecular oxalyl transfers now were comparable, and thus the spectral changes with time were complex.

Several of the reactions of TL-S-Ox with thiols were shown to be second order,

TABLE 3

KINETIC DATA FOR THE REACTION OF NUCLEOPHILES WITH TL-S-OX

Nucleophile	pK_a	k_T[a] at pH 6.96[b] (M^{-1} s^{-1})	k_N[a] × 10^{-3} (M^{-1} s^{-1})
Glutathione	8.93[c]	59[d]	5.6
Pantetheine	9.38[e]	18.3[d]	4.8
N-Acetylcysteamine	9.38[f]	14.0	3.7
8-Mercaptooctanoic acid	10.53[g]	1.2	4.5
6-Mercaptooctanoic acid	10.86[h]	0.13	1.0
Dihydrolipoic acid	10.7[i]	1.5	8.2
Hydroxylamine	6.04[j]	2.1[d]	0.0023

[a] Defined in the text.

[b] Reaction conditions: 25°C, 10 mM potassium phosphate buffer containing 1 mM EDTA, ionic strength 1.0 M (KCl).

[c] See Table 2 for the definition of this pK_a constant and the conditions under which it was obtained.

[d] Calculated by extrapolation from data obtained at pHs 5.75 to 6.55.

[e] Estimated to be the same as for *N*-acetylcysteamine at 25°C and ionic strength 1.0 M.

[f] At 25°C and ionic strength 1.0 M (*45*).

[g] Estimated to be the same as for 1-propanethiol at 25°C and ionic strength 1.0 M (*26*).

[h] Estimated to be the same as for 2-propanethiol at 25°C and low (<0.05 M) ionic strength (*46*).

[i] From reference *47*.

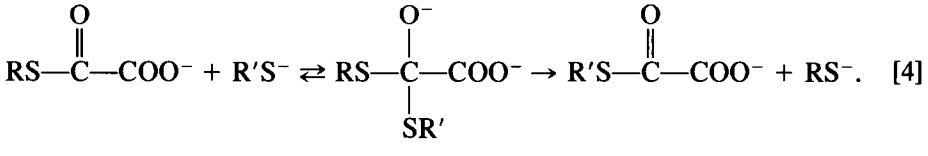
[j] At 25°C and ionic strength 1.0 M (*41*).

and the effect of pH on the glutathione reaction indicated that the thiolate ion is the reacting nucleophile with TL-S-Ox as it is with NAC-S-Ox. Consequently, the primary data for the TL-S-Ox reactions were treated in the same way as was previously done for the NAC-S-Ox data, with k_T and k_N having the same meaning as before. The reaction with hydroxylamine was complicated by some further reaction of the initially formed thiocresolate with hydroxylamine in the system. However, at low hydroxylamine concentrations (less than 6 mM) the second reaction is slow enough that the rate constant for its initial reaction with TL-S-Ox could be estimated. At the low concentrations used, the reaction is strictly second order overall (first order in hydroxylamine); no evidence for a third-order component was obtained.

DISCUSSION

Perhaps the most important result of the current investigation is the general observation that oxalyl thiolesters react so rapidly with thiols. No quantitative data concerning such reactivity had been obtained previously, although Quayle (*34, 35*), in his study of bacterial oxalate metabolism (in which *S*-oxalylcoenzyme A is an intermediate), had noted that the transfer of the oxalyl group from one thiol to another occurs readily. To arrive at an estimate of how rapidly the oxalyl transfer to thiols occurs relative to other acyl group transfers, it is necessary first to consider some of the expected details of the reaction.

From earlier work, especially by Bruice, Jencks, and Hupe and their co-workers (*17–24, 26–30*), one suspects that the oxalyl transfer will proceed with the formation of a tetrahedral intermediate as illustrated in Eq. [4].



Depending on the acidities of RSH and R′SH, either the first or second step of this reaction can be rate determining. If RSH is more acidic (has a lower pK_a) than R′SH, then the first step will be rate determining and the second step fast, whereas if RSH has a higher pK_a than R′SH, then the first step will be a rapid equilibrium and the second step rate determining. For the reactions of TL-S-Ox studied here, the first situation applies since the estimated pK_a for *p*-thiocresol is 6.62 (the value of 6.82 at low ionic strength (*48*) adjusted downward by 0.20 unit (*49*) for a change of ionic strength to 1.0 M) and those of the reacting thiols (Table 3) are higher. On the other hand, for the reported reactions of NAC-S-Ox, the second step will be mainly rate determining because the pK_a (9.38) of *N*-acetylcysteamine is slightly higher than those of the reactants (Table 2). However, at least in the case of homocysteine (microscopic pK_a = 9.14), the rates of both steps will be comparable.

The previous investigation that is closest to the present one involving TL-S-Ox

as the reactant is a study by Hupe and Jencks (*26*), in which they reported rate constants for the reaction of the thiol anion of mercaptoethanol with several acetyl thiolesters having thiol leaving groups with pK_a values of 2.75 to 7.91. Although the authors did not specifically measure the reactivity of *S*-acetyl-*p*-thiocresol, one can estimate from their data that the second-order rate constant (equivalent to k_N) for the reaction of the thiol anion of mercaptoethanol ($pK_a = 9.61$ (*49*)) with *S*-acetyl-*p*-thiocresol at 25°C and ionic strength 1.0 M would be approximately 49 M^{-1} s^{-1}. Since the k_N values for the reaction of TL-S-Ox with thiols of similar acidity are approximately 4 to 5 $\times$ 10^3 M^{-1} s^{-1} (Table 3) under the same conditions, this indicates that the *S*-oxalyl derivative reacts with thiols about two orders of magnitude more rapidly than the *S*-acetyl compound when the rate-determining step is the first one of Eq. [4]. The rate of oxalyl transfer can also be related to that of formyl transfer; it is known that the latter occurs approximately 10^3 times faster from formyl thiolesters than analogous acetyl transfers (*28*). Thus, the oxalyl transfer is only about a factor of 10 slower than the formyl transfer.

One peculiarity of the data in Table 3 is that k_N does not seem to vary as the pK_a of the attacking thiol changes from 8.93 to 10.53 for the series of primary thiols (the first four listed in Table 3). This is in contrast to the results obtained by Hupe and Jencks (*26*) for the reaction of a series of thiols with *S*-acetyl-*p*-nitrothiophenol where a Bronsted β of 0.27 was observed. The reason for the lack of dependence on pK_a is not known but it may just be due to experimental variation over the limited range of pK_a's investigated, or because the pK_a values of some of the reactants have been incorrectly estimated for the particular conditions of the kinetic experiments.

A systematic investigation of the reactivity of an *S*-acetyl alkyl thiolester with alkyl thiols, analogous to the present study involving NAC-S-Ox, has not been reported. Probably the main reason for this is that such reactions are usually difficult to follow spectrally, because they involve very little, if any, change in absorbance as the acyl transfer proceeds. In the experiments reported here, this problem was circumvented by using β- or γ-aminothiols as reactants, in which case the thiolester formed on oxalyl transfer rearranges immediately to the nonabsorbing *N*-oxalyl derivative (Eq. [2]). Although this method limits the range of thiols that can be investigated, it does allow one to obtain some valuable information concerning the reactivity of thiols and thiolesters that are similar to those likely to be encountered physiologically. NAC-S-Ox should be an excellent model for the reactivity of *S*-oxalylcoenzyme A and a reasonable one for *S*-oxalylglutathione, i.e., the *S*-oxalyl derivatives that will be present to the greatest extent *in vivo*. Furthermore, not only are some of the aminothiol reactants present physiologically, but also their thiol pK_a values are similar to those of a number of other biological thiols. Thus, the aminothiol reactivity should be representative of the reactivities of such other thiols.

It is noted from the results given in Tables 1 and 2 that k_N varies with the pK_a of the attacking thiol nucleophile. When the data are plotted according to the Bronsted equation, a β of 0.39 (correlation coefficient 0.96) is obtained from the 0.5 M ionic strength data, and a β of 0.42 (correlation coefficient 0.98) is obtained from the data collected at an ionic strength of 1.0 M. Hupe and Jencks (*26*) report

that, for reactions of thiols with *S*-acetyl-*p*-nitrothiophenol, β is 1.0 when the second step of Eq. [4] is rate determining, whereas it is 0.27 when the first step is rate determining. Probably the reason for the 0.4 value observed here is that the pK_a values of the attacking thiol nucleophiles, although all less than that for *N*-acetylcysteamine, are close enough to it that the rates of the two steps are comparable, and thus a value between the two extremes is obtained. The range in the pK_a values of the attacking thiol nucleophiles is probably too small, and the inaccuracies in the experimental data too great, to detect the curvature in the Bronsted plot expected for such a situation.

Although no systematic study of thiol reactivity toward *S*-acetyl alkyl thiolesters has appeared, Stokes and Stumpf (*25*) reported that the reaction of *S*-acetylcoenzyme A with dithiothreitol proceeds with a second-order rate constant of 0.205 M^{-1} s^{-1} at pH 8.5 and 30°C. Given that the initial pK_a for dithiothreitol is 9.2 (*50*), one thus calculates that k_N for the reaction equals 1.23 M^{-1} s^{-1}. This reaction should be a good model for the *S*-acetyl analog of the homocysteine reaction with NAC-S-Ox, because the pK_a's of dithiothreitol and homocysteine are similar, and coenzyme A thiolesters will have reactivities virtually identical to those of *N*-acetylcysteamine. Consequently, taking into account the different temperatures of the reactions, one concludes that, when the two steps of Eq. [4] are proceeding at comparable rates, the oxalyl transfer to thiols occurs about 500 times more rapidly than the corresponding acetyl transfer.

The question arises whether the high reactivity of the aminothiols with NAC-S-Ox might be the result of a complex being formed by the reactants, due to the formation of a salt bond between the negatively charged oxalyl group and the positive ammonium part of the aminothiol. Such a possibility seems remote for several reasons, the most important one being that oxalyl transfers from TL-S-Ox also proceed much faster than acetyl transfers (complex formation of TL-S-Ox with its reactants is not expected). Furthermore, if complex formation were important to the reactivity of the aminothiols with NAC-S-Ox, then a greater effect of ionic strength than that observed (Table 1) would be expected. Consequently, although the reactivity of NAC-S-Ox with thiols not containing amino groups has not been measured, one is forced to the conclusion that NAC-S-Ox, and thus by extrapolation virtually any other physiological oxalyl thiolester, has high inherent reactivity toward thiols in general.

Pohl *et al.* (*28*) have noted that there is a relation between the equilibrium constant for nucleophile addition to carbonyl compounds and the rate of the corresponding acyl transfer reaction from one thiol to another. Such a relation is not too surprising, especially if the reaction proceeds with the second step of Eq. [4] being rate determining or partially rate determining, because a carbonyl addition (the first step of Eq. [4]) is involved in the overall transfer reaction. A carbonyl addition reaction that would be a suitable model for the oxalyl vs acetyl transfer comparison is the addition of glutathione to glyoxylate vs its addition to acetaldehyde. The formation constant (*4*) for the addition of glutathione to the free aldehyde form of glyoxylate is 114 mM^{-1}, while that for its addition to the free aldehyde form of acetaldehyde is 0.27 mM^{-1}. The difference in these equilibrium constants of over 400-fold thus parallels very closely the calculated difference in

the rates of oxalyl vs acetyl transfer. Consequently, the high rate of the oxalyl transfer reaction, although unexpected, is really not so surprising when viewed in this manner.

A detailed discussion of the limited amount of data reported here on the reactivity of NAC-S-Ox with nucleophiles other than thiols is outside the scope of this article. However, it is clear from a comparison of the present results with those that have been obtained using other thiolesters (*17, 24*) that the unusually high reactivity of NAC-S-Ox with thiols is not mimicked in its reactivity with other nucleophiles. In fact, if anything, NAC-S-Ox appears to react more slowly than expected with oxygen and nitrogen nucleophiles. In any event, for present purposes the most important information to be derived from these reactivity studies is what they indicate concerning the probable nonenzymic reactivity of the oxalyl thiolesters *in vivo*.

Because the reactions of NAC-S-Ox with imidazole, and with non-thiol-containing amino acids and their derivatives, are so slow under typical physiological conditions, it can be concluded that nonenzymic reactions involving such nucleophiles and oxalyl thiolesters will not be metabolically significant. The same probably also holds for nonenzymic reactions of oxalyl thiolesters with oxygen nucleophiles under usual *in vivo* conditions. As is noted from the results in Table 1, the reaction with H_2O_2 proceeds at a reasonable rate, but, because the concentration of H_2O_2 in most cells is so low, it would not usually occur to any significant extent. A possible exception may occur during the oxidative burst, for example, in leukocytes, when the levels of H_2O_2 rise considerably.

In contrast to the situation with oxygen and nitrogen nucleophiles, the nonenzymic reactivity of oxalyl thiolesters with thiols is so high that such reactions occur at a rapid rate under physiological conditions and thus may have considerable metabolic significance. As an illustration of how fast these reactions are proceeding, the results found in this research indicate that, with a typical biological thiol (for example, glutathione or coenzyme A) at a concentration of 1 mM and low (micromolar) concentrations of an oxalyl thiolester, the half-time for the oxalyl transfer to the thiol is only about 1 min at pH 7.2 and 25°C. At the physiological temperature of 37°C, the reaction would be expected to proceed at least twice as fast. The net result, therefore, is that any oxalyl thiolester produced *in vivo* will very rapidly equilibrate with every available thiol, i.e., all thiols that are not prevented from reacting by some constraint, steric or otherwise. Furthermore, the fact that oxalyl thiolesters do not react rapidly with physiological amine and oxygen nucleophiles means that the oxalyl group is free to zip around among the thiols without being drained off prematurely to an unreactive derivative. The oxalyl group of the oxalyl thiolester will, however, eventually encounter a thermodynamic sink when it reacts with an aminothiol, in which case it will be rapidly rearranged to the unreactive (nonenzymically) *N*-oxalyl derivative (Eq. [2]). Evidence that this reaction does indeed occur *in vivo* was recently obtained when *N*-oxalylcysteine was identified as a constituent of kidney homogenates (*13*).

It seems probable that the nonenzymic reaction of oxalyl thiolesters with aminothiols (especially with cysteine but possibly with others as well) may be part of the normal mechanism for controlling the levels of oxalyl thiolesters *in vivo*.

However, the reaction may also be of significance in metabolic states where higher than normal levels of various aminothiols are either produced or used in therapy. For example, it seems possible that some of the metabolic effects associated with the genetic diseases homocystinuria or cystinosis (*51*) may be due to draining off excessive amounts of the oxalyl thiolesters by the higher than normal levels of homocysteine or cysteine in such individuals. Likewise, some of either the beneficial or the side effects of D-penicillamine use as an anti-inflammatory agent (*52*) could be due to a similar reaction. Being a tertiary thiol, penicillamine reacts considerably more slowly with oxalyl thiolesters than primary thiols (Table 1), but, considering that relatively high doses of D-penicillamine are given in therapy, the nonenzymic reaction with *in vivo* oxalyl thiolesters may be significant.

It was originally hypothesized (*1*) that oxalyl thiolesters might affect the catalytic activities of enzymes by covalently modifying some nucleophilic residue on the enzyme surface. However, at that time one could not come to any conclusions concerning what residues might be modified because the relative reactivities of oxygen, nitrogen, and sulfur nucleophiles toward the oxalyl thiolesters were not known. The results of the current research strongly imply that modification of oxygen and nitrogen (including imidazole) sites on enzymes by direct reaction with oxalyl thiolesters is very unlikely unless the reactions are enzyme catalyzed. On the other hand, the current results indicate that modification of enzymic thiol residues should occur readily even by a non-enzyme-catalyzed process. Since the catalytic activities of many enzymes that contain reactive thiol residues are altered when their thiols are modified, the present results have thus focused attention on the possibility that oxalyl thiolesters may function as metabolic effectors by covalently modifying enzymic thiols. Recent work in this laboratory (*14–16*) has demonstrated that oxalyl thiolesters do indeed affect the catalytic activities of enzymes and, at least in one case (*14, 15*), appear to accomplish this by the above mechanism.

ACKNOWLEDGMENTS

The authors thank H. Afeefy for obtaining one of the reported rate constants and W. P. Jencks for some useful critical comments.

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