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The Hydrolysis of Diethyl Monothioloxalate and Its Reaction with Nucleophiles[1]

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The reactivity of diethyl monothioloxalate (EtSCOCOOEt) toward hydrolysis and attack by other nucleophiles was studied in order to determine whether such compounds might be useful pharmacologically to generate oxalyl thiolesters ($RSCOCOO^-$) intracellularly, and whether they have characteristics suitable to be possible physiologically active metabolites of ascorbic acid. At 25°C and pH 7 to 8, EtSCOCOOEt undergoes base-catalyzed hydrolysis (second-order rate constant, $4.09 \times 10^3\ M^{-1}\ s^{-1}$) to give 70% $EtSCOCOO^-$ + EtOH and 30% $EtOCOCOO^-$ + RSH. In other studies it was found that EtSCOCOOEt reacts very rapidly with thiols, such as cysteine and cysteamine (2-aminoethanethiol), but relatively slowly with amines likely to be encountered physiologically. One can estimate from the data that, if RSCOCOOR compounds are present *in vivo*, the transfer of the alkyl oxalyl group (–COCOOR) to other thiols would occur many thousands of times before hydrolysis to less active derivatives would result. Under physiological conditions the halftime for the transfer to thiols is on the order of milliseconds, whereas for hydrolysis the halftime is minutes. If RSCOCOOR compounds were present physiologically, such characteristics make them very possible metabolic effectors; they could alter the catalytic activities of enzymes by modifying enzymic thiols. Because of this possibility, and because a reasonable mechanism for their formation from ascorbic acid is available, it is suggested that an RSCOCOOR-type compound should be seriously considered as a possible metabolite of this vitamin.

INTRODUCTION

Recent studies in our laboratory (*1–16*) have suggested that oxalyl thiolesters ($RSCOCOO^-$) are probably very important mammalian metabolites. They are formed as the direct product of the suspected physiological reaction catalyzed by L-hydroxy acid oxidase (*1–6*) and they could be formed by further metabolism of the suspected physiological reactions catalyzed by D-amino acid oxidase and D-aspartate oxidase (*1, 7–10*). Initially we summarized (*1, 11, 12*) a considerable body of circumstantial evidence that either the products of these peroxisomal oxidase reactions or their further metabolites may be involved in controlling animal metabolism, and may be acting as part of the intracellular messenger system

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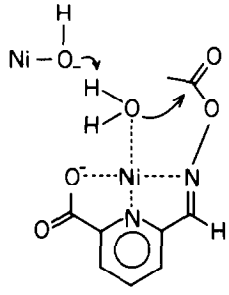
for some hormones, especially insulin. Very recently more direct evidence for these possibilities has been obtained. Thus, oxalyl thiolesters have now been identified as normal constituents of animal tissues (*13*) and they have been shown to alter the catalytic activities of several important animal enzymes (*14–16*) when the oxalyl thiolesters are present at known physiological concentrations.

If oxalyl thiolesters are as important to animal metabolism as it now appears, then consideration should be given both to methods for modifying their levels pharmacologically and to other pathways by which they might be formed *in vivo*. The present research grew out of such considerations. Pharmacological methods for increasing the intracellular concentrations of oxalyl thiolesters may be of particular importance because of our correlations (*1*) indicating that they may be general metabolic inhibitors and that growth factors may lower their concentrations. Thus, methods for increasing their concentrations in cells undergoing uncontrolled growth (as, for example, in cancer) might be therapeutically useful. It is unlikely that the intracellular concentration of oxalyl thiolesters would be increased by treating biological tissues directly with oxalyl thiolesters because the transfer of such charged compounds across the cell membrane would probably not occur. One way to avoid this problem is to use some neutral derivative that can cross cell membranes and be converted into an oxalyl thiolester when it is inside the cell. An ester (RSCOCOOR) of the oxalyl thiolester is a derivative that might serve this purpose. The hydrolytic behavior of such compounds had not previously been characterized so part of the present work was concerned with determining how rapidly they hydrolyze, and whether the initial hydrolytic step proceeds to give the oxalyl thiolester and alcohol or the normal oxygen ester and thiol.

One suspects that, *in vivo*, oxalyl thiolesters would eventually be hydrolyzed to oxalate. It is known that oxalate is derived mainly from two sources in animals (*1, 17, 18*), either from glyoxylate (precursors of which are glycine and closely related metabolites) or from ascorbic acid. Presumably the peroxisomal oxidase reactions contribute to the formation of oxalate from glyoxylate, because the suspected *in vivo* reactants for these enzymic reactions are various nucleophile–glyoxylate adducts (*1–10*). Because oxalyl thiolesters are thus likely intermediates in at least some of the pathways from glyoxylate to oxalate, one wonders whether they might be intermediates in the pathway from ascorbic acid to oxalate as well. The available evidence is certainly consistent with such a possibility. Carbon-isotope tracer studies indicate that the oxalate is derived from carbon atoms 1 (the lactone carbon) and 2 of the ascorbic acid and that glyoxylate is not an intermediate in the reaction. Such a transformation could very well be catalyzed by an oxygenase because the overall reaction has characteristics similar to typical oxygenase reactions. Based on what is known about the mechanisms of other well-studied oxygenases (*19–21*), the most likely mechanism for the direct formation of oxalate (without an oxalyl thiolester as an intermediate) is shown on the left side of Eq. [1], while on the right side the slight alteration in this mechanism that would lead to oxalyl thiolester products is shown. As can be seen, both mechanisms are similar so the formation of oxalyl thiolester products is as likely by such a mechanism as the direct formation of oxalate itself. In fact, the mecha-

proposed for the bimolecular participation of amines in the aminolysis of esters or for the bimolecular participation of hydroxide ion in the alkaline hydrolysis of anilides (*22*).



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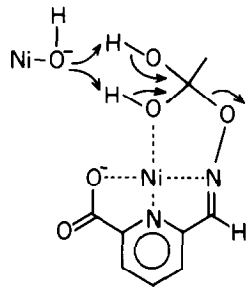

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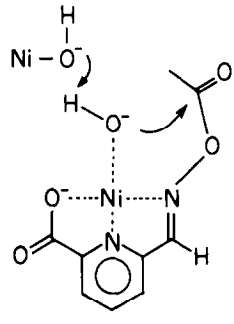

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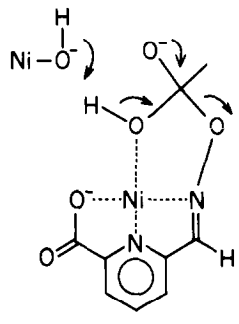

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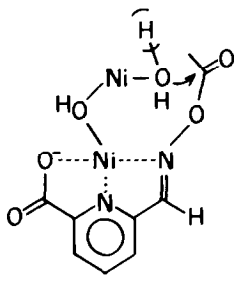

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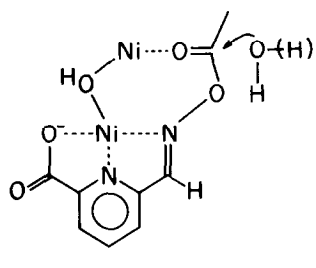

N

In the k_{cat}^{A} and k_{cat}^{B} paths of the Zn(II)-catalyzed hydrolysis of **3**, several lines of evidence have been obtained in support of mechanism **C** (*5*). Analogous mechanisms **M**/**N** are also consistent with the kinetic data obtained for the k_{cat}^{A} and k_{cat}^{B} paths of the Ni(II)-catalyzed hydrolysis of **3**.[3] Although it is not possible to differentiate mechanisms **M**/**N** from **I**–**L** rigorously, **M**/**N** might be preferred by analogy with the Zn(II)-catalyzed reaction. In this regard, it is noteworthy that the mechanisms for the k_{cat}^{W} and k_{cat}^{OH} paths are identical for the catalysis by bivalent metal ions Cu(II) and Ni(II), as discussed above.

distilled and then passed through a Millipore (Milli Q) reverse-osmosis water-purification system. Diethyl monothioloxalate was synthesized from ethyl oxalyl chloride and ethanethiol as follows. To 6.83 g (0.05 mole) of ethyl oxalyl chloride in a round-bottomed flask immersed in an ice bath was added 3.10 g (0.05 mol) ethanethiol in small quantities, following which the solution was refluxed until evolution of HCl ceased. Upon distillation under reduced pressure (62–64°C and 0.25 mm Hg) 4.5 g (55% yield) of the thiolester was obtained. Its characteristics are as follows: uv maximum (ethanol) 269 nm (ε, 3.35 mM^{-1} cm^{-1}); NMR, δ 1.2 and 1.3 (two overlapping triplets, rel. area 6), 2.9 (quartet, rel. area 2), 4.3 (quartet, rel. area 2); analysis, found (calculated in parentheses): C, 44.49 (44.44); H, 6.44 (6.17); S, 19.66 (19.95).

Methods. Routine uv 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 (*5*). 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 EtSCOCOOEt (usually 0.17 to 0.18 mM) were monitored by following the decrease in absorbance at 275 nm. All reactions were performed under pseudo-first-order conditions 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. The reactions were initiated by adding a 100-μl aliquot of an ethanol solution of the thiolester (the thiolester undergoes negligible reaction over a period of several hours in ethanol at 25°C) to a solution of the other components that had been temperature equilibrated at 25°C for at least 10 min in the sample cuvette (total volume, 3 ml) of the spectrophotometer. 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

Hydrolysis of EtSCOCOOEt

When EtSCOCOOEt is placed in aqueous solution at room temperature and pH around neutral, the absorption at 269 nm due to EtSCOCOOEt rapidly decreases and is ultimately replaced by a 260-nm-absorbing species as illustrated in Fig. 1. Since a good isosbestic point is observed, the reaction is thus the simple conversion of EtSCOCOOEt to products with no buildup of intermediates. The 260-nm-absorbing species is undoubtedly $EtSCOCOO^-$ and is stable to hydrolysis at neutral pH (*23*). From pH 7.0 to 8.0 (100 mM sodium phosphate buffers, 25°C) the rate of disappearance of the absorbance due to EtSCOCOOEt is strictly first order in hydroxide ion; the second-order rate constant for the reaction under these conditions is 4.09×10^3 M^{-1} s^{-1} (pK_w taken as 13.97). No effect of buffer concentration on the rate was detected when its concentration was varied from 10 to 100 mM (at pH 7.4).

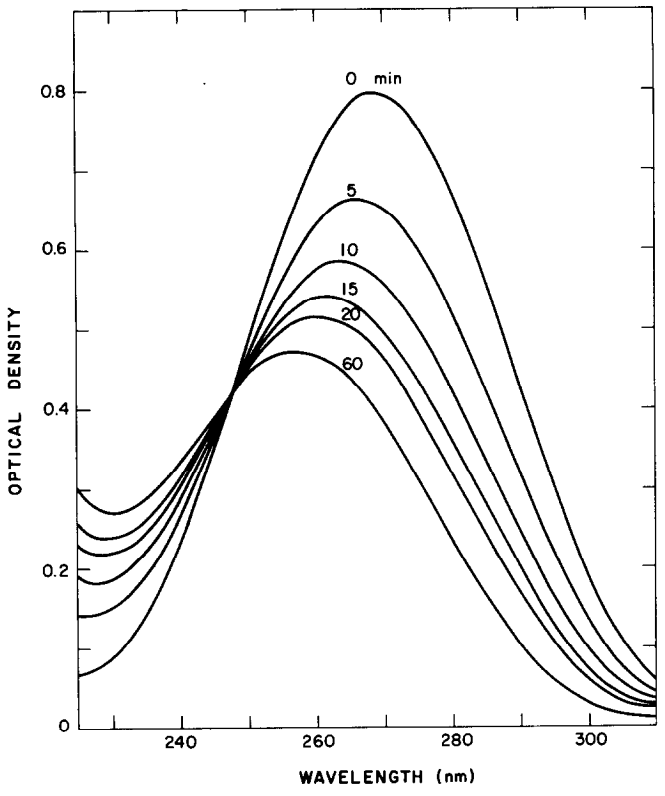

FIG. 1. The uv spectral changes occurring during the hydrolysis of EtSCOCOOEt (0.25 mM) at pH 7.4 (100 mM sodium phosphate buffer) and 25°C. The reaction was initiated by adding the EtSCOCOOEt in 0.10 ml ethanol to 2.9 ml buffer; the initial scan was started at 320 nm within 10 s after initiation and the other scans at the times indicated (scanning rate 200 nm/min). Repeated scans after 60 min indicate no change with time from the 60-min spectrum.

Assuming an extinction coefficient at 260 nm of 2.9 mM^{-1} cm^{-1} for $EtSCOCOO^-$ (*14*), one calculates from a number of experiments performed from pH 7.0 to 8.0 (100 mM sodium phosphate buffer, 25°C) that the yield of thiolester is independent of pH and is 74 ± 2%. Since the yield is less than 100%, some of the hydrolysis must proceed to give $EtOCOCOO^-$ and EtSH. This was confirmed when the solutions following reaction were titrated with Ellman's reagent (*5*). By this method a 31 ± 1% yield of thiol, again independent of pH, was detected. Consequently, in this pH region, the hydrolysis of EtSCOCOOEt occurs as indicated in Eq. [2]. From a combination of the kinetic and product data, one can calculate that k_0 is 2.88 × 10^3 M^{-1} s^{-1} and k_S is 1.21 × 10^3 M^{-1} s^{-1} at 25°C.

$$EtSCOCOO^- + EtOH \underset{70\%}{\overset{k_0[OH^-]}{\longleftarrow}} EtSCOCOOEt \underset{30\%}{\overset{k_S[OH^-]}{\longrightarrow}} EtOCOCOO^- + EtSH \quad [2]$$

Reaction of EtSCOCOOEt with Other Nucleophiles

Given in Table 1 are some kinetic data for the reaction of various nucleophiles with EtSCOCOOEt. Over the range of nucleophile concentrations tested, all of the reactions are second order, first order in EtSCOCOOEt, and first order in the nucleophile. Except for the glycine methyl ester case, the various reactions were studied under pH conditions where the hydroxide ion-catalyzed hydrolysis is negligible so a plot of k_{obs} versus the concentration of the nucleophile is linear and goes through the origin. For the glycine methyl ester reaction, such a plot is also linear but does not go through the origin; the second-order rate constant for this nucleophile was calculated from the slope of such a plot. At pH 7.8, where 25 mM glycine methyl ester causes the rate of disappearance of the absorption due to EtSCOCOOEt to increase by about 38% over that due to hydrolysis alone, glycine itself at a 25 mM concentration causes no detectable change in the rate.

The effects of pH on the various reactions indicate that it is the particular ionized form of the nucleophile illustrated in the table that is the reactant in each case (this was not checked for glycine methyl ester but the form given in the table is almost certainly the reactant in this case). The second-order rate constants, k_N and k_T, are defined by the following: rate = k_{obs}[EtSCOCOOEt] = k_N[N]

TABLE 1

KINETIC DATA FOR THE REACTION OF VARIOUS NUCLEOPHILES WITH EtSCOCOOEt[a]

Nucleophile	pK_a[b]	Range investigated[c]		k_N[d] (M^{-1} s^{-1})	k_T[d,e] at pH 7.4 (M^{-1} s^{-1})
		pH	concn (mM)		
$^+NH_3CH_2CH_2S^-$	8.35[f]	4.0–5.6 (5)	1–3 (5)	1.68×10^4	1690
$^+NH_3CHCH_2S^-$ (COO$^-$)	8.50[g]	4.0–5.6 (5)	1–3 (5)	1.33×10^4	950
NH_2OH	6.04[h]	4.8–5.7 (4)	2–10 (5)	6.64	6.36
$NH_2CH_2COOCH_3$	7.75[i]	7.8	5–25 (5)	0.072	0.022
HOO^-	11.65[i]	6.3–7.2 (5)	1–5 (5)	3.79×10^5	21.3
HO^-	13.97[j]	7.0–8.0 (5)		1.21×10^3[k]	3.3×10^{-4}[l]

[a] Reaction conditions: 25°C; 100 mM sodium phosphate buffer for reactions run from pH 5.7 to 8.0 and 100 mM sodium acetate buffers for reactions run from pH 4.0 to 5.6.

[b] pK_a for the conjugate acid of the nucleophile. Ref. (*23*) contains literature references to these values except for glycine methyl ester which was taken from Ref. (*24*).

[c] The numbers in parentheses refer to the number of different conditions investigated within each range.

[d] Defined in the text.

[e] Calculated from data obtained over the pH range indicated in column 3.

[f] Microscopic ionization constant obtained at 23°C and low (<0.05 M) ionic strength.

[g] Microscopic ionization constant obtained at 25°C and ionic strength of 0.5 to 1.0 M.

[h] Obtained at 25°C and ionic strength of 1.0 M.

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

[j] Value for pK_w at 25°C.

[k] Value is k_S, i.e., the constant for attack at the thiolester carbonyl.

[l] First-order rate constant ($k = k_N[OH^-]$), in s^{-1}, for attack at the thiolester carbonyl.

[EtSCOCOOEt] = k_T[N_T][EtSCOCOOEt], where [N_T] is the total concentration of all ionized and unionized forms of the nucleophile at pH 7.4 and [N] is the concentration of the particular ionized form of the nucleophile that is given in the table; its concentration, at the various pH's where the primary data were obtained, was calculated using the pK_a values given (in the case of cysteine the microscopic pK_a value for ionization at the thiol group is given in the table, but others (*23*) were also used in calculating the concentration of the reacting nucleophile at each pH). The k_N value is a measure of the inherent reactivity of each nucleophile, whereas k_T indicates how reactive the various types of nucleophiles are under conditions similar to those encountered physiologically. It is recognized that several of the pK_a values used for the calculations of k_N were not obtained at the same ionic strength and temperature 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.

A detailed product study of the various reactions of EtSCOCOOEt with nucleophiles was not carried out, but it is expected that all of the reactions involve attack at the thiolester carbonyl group (except for the hydroxide reaction where some of the attack occurs at the other carbonyl as considered previously). The observation that the thiolester uv absorption is lost in each case is consistent with this conclusion. The high reactivity of cysteine and cysteamine (2-aminoethanethiol) relative to other amines indicates that the nucleophile in the former cases is the sulfur rather than the nitrogen. The initial step in such a reaction would not lead to any significant change in uv absorption. However, by analogy to similar reactions (*10, 23*), it is expected that a rapid intramolecular S to N transfer of the acyl group would occur and, thus, that the overall reaction in each of these cases is that shown in Eq. [3].

$$\mathrm{EtSCOCOOEt} + \mathrm{NH_2CHZCH_2SH} \underset{\mathrm{EtSH}}{\overset{\text{slow}}{\rightleftharpoons}} \mathrm{NH_2CHZCH_2SCOCOOEt} \xrightarrow{\text{fast}} \mathrm{HSCH_2CHZNHCOCOOEt} \quad [3]$$

$$\mathrm{Z = H \text{ or } COO^-}$$

If 0.16 mM EtSCOCOOEt is placed in a pH 7.8 solution (100 mM sodium phosphate buffer, 25°C) containing 0.7 to 5.7 mM *N*-acetylcysteamine and the thiolester absorption followed with time, the first-order decay of the absorption gives a k_{obs} of 3.8×10^{-3} s^{-1}. This rate constant was found to be independent of the concentration of *N*-acetylcysteamine as long as it is in excess; in the absence of *N*-acetylcysteamine under similar conditions, the k_{obs} for EtSCOCOOEt hydrolysis is 2.76×10^{-3} s^{-1}. Presumably the reason for the increase in rate in the presence of *N*-acetylcysteamine is because it reacts rapidly with EtSCOCOOEt to give EtSH and $CH_3CONHCH_2CH_2SCOCOOEt$ and that the k_{obs} of 3.8×10^{-3} s^{-1} is for the hydrolysis of this latter compound. Taking into account the known pK_a of 9.38 for *N*-acetylcysteamine (*23*), one can estimate from the kinetic data for other thiols (Table 1) that the halftime for the initial thiolester exchange reaction would be only about 1 s with 1 mM *N*-acetylcysteamine at pH 7.8. The product ratio from the overall hydrolysis reaction was not determined, but it is expected that hydrox-

ide attack at the ester carbonyl of $CH_3CONHCH_2CH_2SCOCOOEt$ would occur at essentially the same rate as observed with EtSCOCOOEt. Consequently, the increase in the rate of hydrolysis seen with $CH_3CONHCH_2CH_2SCOCOOEt$ is probably due to increased attack at the thiolester carbonyl. With the foregoing assumptions, one thus calculates that k_S for $CH_3CONHCH_2CH_2SCOCOOEt$ is 2.7×10^3 M^{-1} s^{-1} at 25°C, in other words, a little more than twice the k_S for EtSCOCOOEt. Presumably the reason for the different reactivities of the two thiolesters is related to the fact that the pK_a for *N*-acetylcysteamine is 9.38 (*23*), whereas for EtSH it is 10.61 (*25*). Hawkins and Tarbell (*26*) and Douglas and Yaggi (*27*) have noted a similar dependence on the pK_a of the leaving group for the attack of hydroxide ion on a series of acetyl thiolesters.

DISCUSSION

It is well documented (*28–33*) that rate constants for hydroxide ion-catalyzed hydrolysis of thiolesters are comparable to, but usually slightly less than, rate constants for the analogous reactions of oxygen esters. Although such results appear to contradict the frequently stated dictum that thiolesters are much more reactive toward nucleophiles than the corresponding oxygen esters, and although, to the present authors' knowledge, a satisfactory explanation for the effect has not been put forward, the fact that thiolesters and oxygen esters do tend to react with hydroxide at comparable rates makes it not too surprising that the reaction of EtSCOCOOEt with hydroxide should be found to give a 70:30 mixture of $EtSCOCOO^-$ and $EtOCOCOO^-$. Such a result implies that the electronic effects of the COSEt and COOEt groups are similar, and that too is precedented by previous observations that MeOCOCOOMe (*31*) and MeSCOCOSMe (*30*) react with hydroxide at comparable rates, the former having a rate constant of 2.95×10^4 M^{-1} s^{-1} at 25°C and the latter a rate constant of 5.4×10^3 M^{-1} s^{-1} at 30°C. The present rate constant of 4.09×10^3 M^{-1} s^{-1} for hydroxide attack on EtSCOCOOEt at 25°C is very similar to these values.

In its reaction with nucleophiles (Table 1), EtSCOCOOEt reacts about two orders of magnitude more rapidly than previously found (*23*) for similar reactions of an oxalyl monothiolester of general structure $RSCOCOO^-$, where RSH is *N*-acetylcysteamine. Since the pK_a of the leaving group EtSH in EtSCOCOOEt reactions is considerably higher (10.61) than that (9.38) of the thiol in the latter case, the effect of esterification of the other carboxyl group on the reactivity of the thiolester is actually even greater; several groups (*27, 34*) have noted that the rate of nucleophilic reactions at thiolester sites is dependent on the pK_a of the leaving group, especially if loss of that group as thiolate is involved in the rate-determining step of the reaction. For the reaction of EtSCOCOOEt with the two thiols listed in Table 1 that would be the case because of their lower pK_a's.

As stated in the introduction, the main goal of this investigation was to characterize the reactivity of a dialkyl monothioloxalate (RSCOCOOR) so that one would have a better understanding of the probable *in vivo* reactions of such compounds if they were used pharmacologically or were normally produced by

biological systems. Relative to this goal, the present study allows several conclusions to be made. First, the observation that EtSCOCOOEt hydrolyzes readily at neutral pH to give mainly $EtSCOCOO^-$ by carbonyl–oxygen cleavage means that viable approach to generating $RSCOCOO^-$ intracellularly would be to treat cells or tissues with a neutral RSCOCOOR; such a compound could pass through the cell membranes but would soon hydrolyze, even nonenzymically, to give the desired $RSCOCOO^-$. The halftime for the hydrolysis of EtSCOCOOEt at pH 7.4 and 25°C is approximately 10 min. At 37°C the reaction would presumably occur at least twice as rapidly, but, in any event, the time scale for the reaction under physiological conditions is minutes rather than seconds or hours. Clearly the rate, as well as the position of cleavage (carbonyl–oxygen or carbonyl–sulfur cleavage), could be altered by a judicious choice of groups to attach to the oxygen or sulfur. The identity of the group attached to sulfur would be largely irrelevant to the ultimate effect of the oxalyl thiolester in the cell because the oxalyl group would be transferred to some physiological thiol within seconds to minutes (*23*).

The main conclusions to be derived from the investigation of EtSCOCOOEt reactivity with other nucleophiles (Table 1) are that thiolesters with this general structure would react with physiological thiols many thousands of times before they are hydrolyzed; that the reactivity of amine nucleophiles, similar to those encountered *in vivo*, is too low for the reaction to have any metabolic significance; and that the reaction with hydrogen peroxide, although relatively fast, would probably also not be important under usual physiological conditions because the concentration of H_2O_2 in cells is normally very low (an exception might be during the oxidative burst, for example, in leukocytes). The easiest way to consider these possibilities is to use the k_T values given in Table 1 in conjunction with probable *in vivo* concentrations of the various nucleophiles. Taking amines first, it can be seen that hydroxylamine is relatively reactive, but, since it is not encountered *in vivo*, such reactivity has no metabolic significance. The reactivity of glycine methyl ester is expected to be similar to that of *N*-terminal residues on proteins and peptides which would probably be present in cells at a concentration of 1 to 5 mM. At that concentration the hydrolysis of EtSCOCOOEt would occur considerably more rapidly than its reaction with such residues so the reaction would not be of importance *in vivo*. The concentration of amino acids in cells would be higher but, as reported here, no reactivity of EtSCOCOOEt with amino acids at 25 mM concentration could be detected. The general conclusion, therefore, is that, if dialkyl monothioloxalates were formed in, or applied to, cells, they would react too slowly with physiological amines for the nonenzymic reaction to have any metabolic significance.

The thiol concentration in cells is probably on the order of 10 mM, and, although not all physiological thiols would react at the same rate as cysteine or cysteamine, their reactivities would be comparable. Consequently, if EtSCOCOOEt were present in cells at pH 7.4 and 25°C, the halftime for its reaction with some thiol would be on the order of 0.05 s. At 37°C with a more physiological thiol (such as glutathione or coenzyme A) as part of the thiolester rather than EtSH, the reaction would probably proceed an order of magnitude more rapidly. Since, therefore, the reaction of RSCOCOOR with thiols under physiological conditions would occur in

the time scale of milliseconds, while the hydrolysis occurs only in minutes, the alkyl oxalyl part (–COCOOR) of such molecules would visit many thousands of thiol molecules before the diester is ultimately hydrolyzed to less reactive products.

A large number of enzymes are known to have reactive thiol groups that when modified cause the catalytic activity of the enzyme to be altered. The present results thus make it probable that if RSCOCOOR compounds were produced *in vivo* they might act as metabolic effectors by modifying enzymic thiols in a mechanism similar to that which has been proposed (*1, 14, 23*) for $RSCOCOO^-$. The main difference in the two types of derivatives is that RSCOCOOR compounds transfer the oxalyl group about two orders of magnitude more rapidly than the negatively charged $RSCOCOO^-$ compounds (*23*). That would be the case both in the initial reaction of any individual thiol molecule and in the further reaction of the initially formed product. An intriguing possibility that presents itself is that perhaps an enzyme modified by RSCOCOOR might hydrolyze off the alcohol part, thus effectively trapping the enzyme in a modified form with the more slowly reacting negatively charged oxalyl function. If this were near a positively charged group on the enzyme, then the modified form would be even more stabilized.

Because of the above considerations, and because there is a reasonable mechanism for the formation of RSCOCOOR derivatives from ascorbic acid (Eq. [1]), it seems imperative to examine whether they might be metabolites of this vitamin. Such a study remains for the future. The specific RSCOCOOR that would be produced from ascorbic acid would have the ester derived from the alcohol group at the 2-position of threonic acid ($R'CH(OH)COO^-$). As such, this negatively charged ester might induce additional specificity for various enzymes.

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