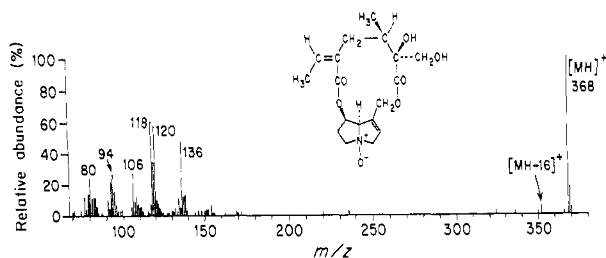

Figure 1. FAB mass spectrum of retrorsine *N*-oxide (**5**).

the characteristic ion series, m/z 136, 120, 119, 93, and 80, seen in the EI mass spectra of all macrocyclic diesters of retronecine (Crout, 1969). Similar lower ion series are also observed in the EI mass spectra of nonmacrocyclic tertiary amine (Pedersen and Larsen, 1970), *N*-oxides (Culvenor et al., 1975; Abdullaev et al., 1974), and pyrrole (Mattocks, 1969) derivatives of retronecine esters. By analogy to the structures proposed for these ions, it is rationalized that the prominent ion series in the FAB spectra is also derived from the pyrrolizidine ring system.

In the EI mass spectra of retronecine *N*-oxide derivatives, a dual fragmentation pathway is observed that first involves loss of the *N*-oxide oxygen to give the tertiary base (M – 16) and the pyrrolic dehydro alkaloid (M–17, M–18), which act as precursors to the lower ion series (Culvenor et al., 1975; Abdullaev et al., 1974). These pathways may be operating under FAB conditions as well. In addition to the $(M + H - 16)^+$ ion noted above, a small ion at $(M + H - 18)^+$ with 2–3% relative abundance is also observed in the FAB mass spectra.

Presently macrocyclic diester pyrrolizidine alkaloid *N*-oxides are often routinely reduced in the isolation process to their tertiary bases (Phillipson and Handa, 1978; Huizing and Malingré, 1979). This is done at least in part to facilitate the analysis or structural elucidation of the parent *N*-oxide. Using FAB mass spectrometry, one can directly analyze the parent *N*-oxide.

Registry No. 1, 35337-98-5; 2, 38710-25-7; 3, 13268-67-2; 4, 38710-26-8; 5, 15503-86-3.

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Nitrite Inhibition of Acyl Transfer by Coenzyme A via the Formation of an *S*-Nitrosothiol Derivative

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At 25 °C and at pH 6 and below, coenzyme A reacts with nitrite in aqueous media to form an *S*-nitrosothiol (thionitrite) derivative. Spectroscopic observation shows that in a pH 2 buffer the reaction essentially reaches completion after 0.5 h. Raising the pH to 6 decreases, but does not completely suppress, both the rate and the extent of reaction. The derivative formed is stable with respect to hydrolysis over a wide range of pH (1–13). In the presence of phosphotransacetylase, this *S*-nitrosothiol derivative, in contrast to coenzyme A itself, is incapable of accepting activated acetyl groups. These results suggest a possible coenzyme A related mechanism that may contribute, in part, to the inhibition of the outgrowth of bacteria such as *Clostridium botulimum* in processed food products.

The mechanism by which nitrite inhibits bacterial growth is still unknown. Recent work with aerobic bacteria has shown that oxidative phosphorylation may be inhibited by the presence of nitrite (Rowe et al., 1979; Yarbrough et al., 1980). On the other hand, nitrite was found to inhibit the phosphorylation reactions associated with the phosphoroclastic system in the extracts of *Clostridium sporogens* (Woods et al., 1981). A recent electron spin resonance study with *Clostridium botulinum* (Reddy et al., 1983) suggests that the interaction between nitrite and proteins containing iron–sulfur clusters may be the source of the effects on the energy metabolism processes mentioned above. After treatment with nitrite, bacterial cultures of *Clostridium perfringens* were found to exhibit substantially decreased activity of glycolytic enzymes, e.g., glyceraldehyde-3-phosphate dehydrogenase and aldolase (O'Leary and Solberg, 1976). However, whether nitrite interacts directly or indirectly with these enzymes remains unresolved.

Under appropriate conditions, nitrite reacts with simple thiol compounds to produce *S*-nitrosothiol (thionitrite) derivatives (Byler et al., 1983; Kresze and Uhlich, 1959; Oae et al., 1978). Accompanying the nitrite-induced decrease of glycolytic enzymatic activities, there is also a significant decrease of free –SH concentration in soluble

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cellular constituents in bacterial cultures (O'Leary and Solberg, 1976). Although Buchanan and Solberg (1972) suggested that nitrite might inhibit the growth of *Staphylococcus aureus* by interacting with CoA, no one has ever systematically investigated the direct interaction of nitrite with this key metabolite. In the present study, we demonstrate that CoA indeed reacts with nitrite to form a *S*-nitrosothiol derivative and that this derivative (CoASNO), unlike the parent CoA, cannot function as an acyl group transfer reagent. Possible implications of this finding with respect to the bacterial-inhibition properties of nitrite are discussed.

EXPERIMENTAL SECTION

Spectroscopic Measurements. The formation of CoASNO from CoA and nitrite in buffers with different pH values was followed spectroscopically at the characteristic wavelengths of thionitrite compounds (Byler et al., 1983; Kresze and Uhlich, 1959; Oae et al., 1978). The extent of reaction was determined from the absorption of the product, which has calculated molar extinction coefficients of 888 and 16.9 M^{-1} cm^{-1} at 332 and 547.5 nm, respectively. Because thionitrites are sensitive to UV irradiation (Byler et al., 1983), all the experiments were run under minimal incandescent light at 25 °C. All spectrometric measurements were performed with a Beckman DU-7 spectrometer.

Thin-Layer Chromatography (TLC). Separation of CoA- and glutathione (GSH)-related compounds were achieved by modifying a procedure (Shimizu, 1970) that was originally developed for paper chromatographic techniques. Solutions of the compounds were applied to cellulose-coated thin-layer glass plates (20 × 20 cm, 0.25 mm) and developed with ethanolic ammonium acetate [ethanol–0.5 M ammonium acetate (pH 4.0, 4:3 v/v] at 25 °C. CoA and its related compounds were visualized with UV light. GSH and its derivatives were located with ninhydrin spray.

Acyl Group Transfer Reaction. The extent of acyl transfer from acetyl phosphate to CoA catalyzed by phosphotransacetylase was determined from the amount of unreacted acetyl phosphate by the procedure of Abiko (1970). In this method, the unreacted acetyl phosphate was used to convert hydroxylamine to hydroxamate, which in the presence of $FeCl_3$ produces red complexes that may be quantitated by absorbance measurements at 510 nm. Calculated amounts of CoA or CoASNO were added to 0.8 mL of 25 mM Tris buffer, pH 8.0, containing 6 μmol of acetyl phosphate and 5 units of phosphotransacetylase. After 5 min at room temperature (25 °C), 0.2 mL of 0.25 M arsenate was added and incubated for exactly 15 min. After incubation with arsenate, 2.0 mL of freshly made hydroxylamine–acetate buffer [prepared by mixing 28% hydroxylamine hydrochloride, 14% NaOH, and 0.1 M sodium acetate, pH 5.4 (1:1:2 v/v)] was added and allowed to stand for 10 min. Finally, 1 mL each of 4 M HCl, 12% trichloroacetic acid, and 5% $FeCl_3$ in 0.1 M HCl was added in this sequence for color development.

Permeability of Vesicle Membranes to Potassium Salts. Soybean phospholipid (asolectin) vesicles were prepared by sonication at 4 °C in 0.8 M sucrose containing 10 mM potassium 2-(*N*-morpholino)ethanesulfonate (MES) at pH 6.0, according to an established procedure (Tu et al., 1982). A 0.5-mL aliquot of vesicle suspension (80 mg/mL) was then quickly added to 2.0 mL of each of the three potassium salt solutions, which also contained 10 mM MES at the same pH. The swelling of the vesicles was followed by the absorbance change at 450 nm. The rate of swelling of the vesicles, as determined by absorbance changes at 450 nm, was used to gauge the extent to which salts enter the interior aqueous media at 25 °C.

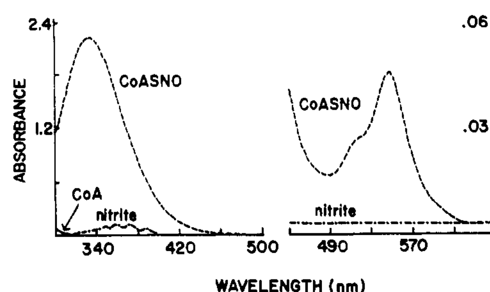

Figure 1. Absorption spectrum of CoASNO from 300 to 650 nm (4× ordinate scale expansion from 450 to 650 nm). 7.5 μmol of CoA was mixed with 9.0 μmol of $NaNO_2$ in 3.0 mL of pH 2.0 buffer (50 mM KCl–HCl) at room temperature in the dark. The spectrum of the solution was continuously monitored until no further change was noted. The spectrum of the solution at equilibrium is shown by (---) (CoASNo). The spectra of unreacted nitrite and CoA at the above concentration are shown by (–·–) and (—), respectively. CoA has negligible absorption above 320 nm. The molar extinction coefficients (ε) of CoASNO at 332, 512, and 547.5 nm were estimated as 888, 10.4, and 16.9, respectively.

Reagents. All the chemicals used in the present study were of highest purity commercially available.

RESULTS

Spectroscopic Evidence for *S*-Nitrosothiol Formation. Although, in general, nitrite may react with hydroxyl, amino (primary and secondary), and sulfhydryl groups (Kresze and Uhlich, 1959; Turney and Wright, 1959), the formation of the *S*-nitrosothiol is the most rapid and has the largest equilibrium constant (Aldred et al., 1982; Byler et al., 1983; Challis, 1973). In the present study, the addition of a slight excess of nitrite to CoA (molar ratio, 1.2:1) solution at pH 2.0 resulted in the formation of a pale orange-red compound. The reaction proceeded to completion within 0.5 h as is evident from thin-layer chromatography described below under Separation of the *S*-Nitrosothiol Derivative of Coenzyme A. The absorption spectrum of this compound (Figure 1) is characteristic of an *S*-nitrosothiol derivative (CoASNO). While organonitrites, *N*-nitrosamines, and thionitrites all absorb strongly near 330 nm, only the –SNO chromophore also exhibits bands in the visible region (ca. 510 and 545 nm) (Kresze and Uhlich, 1959). Because $A_{332}/A_{547.5}$ (=49) for CoASNO (Figure 1) very nearly equals $\epsilon_{336}/\epsilon_{543}$ (=51) for pure *S*-nitrosocysteine (Byler et al., 1983), we have strong evidence that only the sulfhydryl groups of CoA have reacted with nitrite under the present conditions. The formation of appreciable amounts of other organonitrites would substantially increase this ratio. In addition, the absorption spectrum of CoASNO remains essentially unchanged in the pH range of 1.5–13 (data not shown). Stability with respect to hydrolysis is another characteristic of thionitrites (Kresze and Uhlich, 1959) that contrasts markedly to the behavior of analogous alkyl nitrite (RONO) species.

Stoichiometry of the Reaction. Although the spectroscopic data suggest that only the –SH group reacts with nitrite in the present experiment, determination of the reaction stoichiometry is required to strengthen such a claim. By keeping the total molarity of the reactants constant, but varying the mole fraction of CoA and nitrite, we obtained maximum absorbance changes with a molar ratio of CoA to nitrite as 1.0. As shown in Figure 2, the absorbance of both 332 and 547.5 nm reach maxima at this ratio. Since (1) the nitrosation as determined at 332 nm and the formation of thionitrite as measured at 547.5 nm

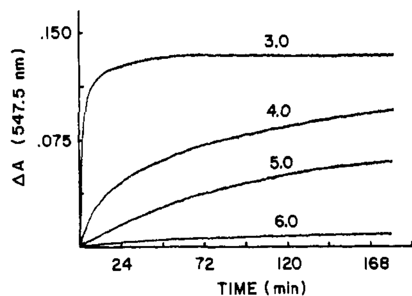

Figure 2. pH dependence of the rate and extent of CoASNO formation. 25 μmol of CoA was mixed with 30 μmol of $NaNO_2$ in 3 mL each of four different pH media. The formation of CoASNO was followed by the absorbance increase at 547.5 nm. Since the spectroscopic properties of CoASNO are independent of pH (see the text), the extent of conversion of CoA to CoASNO at equilibrium (data not shown except for pH 3.0) in buffers of pH 3.0, 4.0, and 5.0 (50 mM phthalate) and 6.0 (50 mM phosphate) was estimated as 95, 85, 59, and 11%, respectively. The net absorbance increase (547.5 nm) at equilibrium of the reaction mixture at pH 3.0, 4.0, 5.0, and 6.0, was 0.1342, 0.1192, 0.0830, 0.0157, respectively.

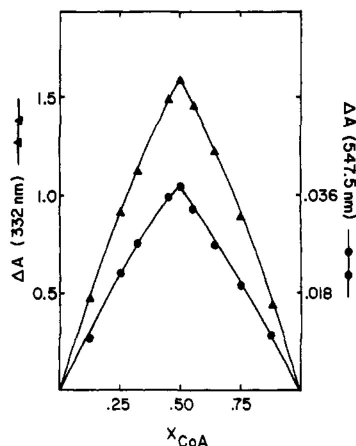

Figure 3. Stoichiometry of the reaction. The reaction between CoA and nitrite was allowed to reach an equilibrium state in a pH 3.0 buffer (50 mM phthalate) at 25 °C. In the experiments, the initial total concentration of CoA and nitrite was kept as 3.8 mM but with a different molar ratio of these two reactants. The observed net absorbance increases at 332 nm (nitrosation) and 547.5 nm (thionitrosation) were plotted against the initial mole fraction of CoA. The mole fraction X_{CoA} is defined as [CoA]/([CoA] + [nit]) in which the concentration terms represent the initial values; [nit] is the total initial stoichiometric concentration of nitrite species (HNO_2 + NO_2^-).

have the same stoichiometry and (2) and ratio of absorbance increase at these two wavelengths remains nearly constant ($A_{332}/A_{547.5} = 49$), we conclude that only CoASNO was formed under the present conditions.

pH Dependence of the Reaction. Recent work on a related model system shows that the reaction between cysteine and nitrite is strongly pH dependent (Byler et al., 1983). This result suggests that the primary species that reacts with the –SH group is probably nitrous acid. A related study (Kresze and Winkler, 1963) at much lower pH (near 0) implies that under such acidic conditions $H_2NO_2^+$ may also play a role. In the present study, we also observed qualitatively that both the reaction rate and the extent of reaction decreases as the pH of the medium increases (Figure 3). Despite the low equilibrium concentration of undissociated HONO ($pK_a = 3.5$) at pH 6.0, and reaction still proceeds to an observable extent at this pH. We assume that the reaction obeys the equation

$$CoA + H^+ + NO_2^- \rightleftharpoons CoASNO + H_2O \quad (1)$$

Table I. Reaction between CoA and GSNO

solution	observed, relative R_f[b]
CoA	1.0
GSH	0.93
CoASNO	0.77
GSNO	0.59
CoASSCoA	0.83
GSSG	0.54
CoASSG	0.66
CoA + GSNO[a]	1.0, 0.93, 0.77, and 0.59
CoASNO + GSH[a]	1.0, 0.93, 0.77, and 0.59

[a] The thionitrite derivatives were synthesized as described in Figure 1 except that a slight excess of GSH or CoA was used. The pH of the reaction solution was then adjusted to 6.5 before the addition of an equal amount of free CoA or GSH. After 20 min at room temperature, the mixture was applied to the same thin-layer plate and developed. [b] The observed R_f (0.85) value of CoA was defined to be 1.0.

This assumption is based on a previous investigation on the formation of the thionitrite derivative of cysteine (Byler et al., 1983). Using the procedure developed in that investigation, the apparent equilibrium constant based on molar concentrations at the equilibrium state may be represented by

$$K_c = [CoASNO]/([H^+][nit][CoA]) \quad (2)$$

where [nit] is the total stoichiometric concentration of unreacted nitrite species (HNO_2 + NO_2^-) at equilibrium. Thus, the data shown in Figure 3 provide an estimated apparent equilibrium constant of $(1.8 \pm 0.5) \times 10^7$ in this pH range.

Separation of the *S*-Nitrosothiol Derivative of Coenzyme A. The separation of CoA and its related analogues by paper chromatography has been reported (Shimizu, 1970). In the present study, we used cellulose-coated glass plates to separate unreacted CoA from CoASNO as described under Experimental Section. Reaction mixtures (pH 2.0) prepared as in Figure 1 were withdrawn and applied to the plate at different time intervals after initial mixing. As the reaction proceeds, a new spot with a lower migration rate (R_f relative to CoA = 0.77) was formed. After 0.5 h only the new spot was observed on the plate.

Acyl Transfer Reaction. The ability of CoA to accept and then subsequently to donate acyl groups plays a role of paramount importance in many biological processes. It is of interest to examine the effect of S-nitrosation on this key, metabolic activity of CoA. In the procedure of Abiko (1970) for the measurement of acyl group transfer to CoA, excess acetyl phosphate is allowed to react with hydroxylamine to form hydroxamate, which forms stable complexes with ferric ion. Thus, a more complete acyl transfer reaction will yield less ferric complex. The decrease of the absorbance at 516 nm is proportional to the amount of CoA added to the reaction mixture. Typically, the presence of 0.28, 0.55, and 1.10 μmol of CoA in the solution containing 6 μmol of acetyl phosphate decreased the absorbance from 0.7090 to 0.6776, 0.6397, and 0.5540, respectively. On the other hand, the addition of 0.55 and 1.10 μmol of CoASNO synthesized under the conditions described in Figure 1 only slightly changed the absorbance to 0.7060 and 0.7035, respectively. Thus, these results demonstrate that CoASNO is essentially inactive in accepting acyl groups from acetyl phosphate.

Reaction of CoA with Other *S*-Nitrosothiol Compounds. Under appropriate conditions, *S*-nitrosothiol compounds may transnitrosate (via transfer of the –NO group) other chemicals having the –SH moiety (Oae et al.,

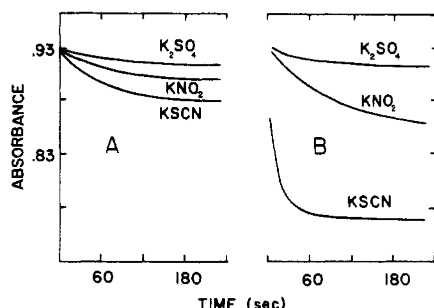

Figure 4. Swelling of asolectin vesicles induced by solute movement. (A) The trace represents the swelling of the vesicles after being added to 100 mM K_2SO_4, 150 mM KNO_2, or 150 mM KSCN. (b). The experimental conditions wre identical with that of (A) except that the vesicles were allowed to incubate that valinomycin (5 μg/mg of lipid) before addition to the salt solutions. Similar swelling patterns were also observed at pH 5.0 and 7.0.

1978). In particular, as shown in Table I, the addition of S-nitrosoglutathione (GSNO) to a solution of CoA results in the transfer of the -NO group. The intensities of the UV absorption of the CoA and CoASNO spots detected by TLC upon mixing CoA and GSNO or CoASNO and GSH were approximately the same. Thus, the extent of transnitrosation for these two systems is qualitatively the same. Although, in some situations, S-nitrosothiols may interact with one another to form disulfide derivatives (Oae et al., 1978), under the present experimental conditions, we did not observe the formation of any disulfides; as indicated in Table I, TLC would identify such species by their distinctive R_f values.

Membrane Permeability of Nitrite. For the reactions studied to be effective in bacterial inhibition, it is essential that nitrite in some form be able to penetrate biomembranes. The relative permeability of charged species toward membranes may be conveniently determined by the rate of swelling of vesicles as measured by the change in light scattering of the suspension (Mitchell and Moyle, 1969). Using this technique, we have measured the swelling of soybean phospholipid (asolectin) vesicles induced by the entry of three different potassium salts: KSCN, KNO_3, and K_2SO_4 (Figure 4A). When valinomycin, a K^+ -specific ionophore, was included, the observed relative rate of swelling remains unchanged (Figure 4B). The results show that nitrite ion has a somewhat lower permeability than that of thiocyanate ion but substantially higher value than that of sulfate ion.

DISCUSSION

CoA, like other -SH-containing compounds within a cell, can react with nitrite either directly or indirectly via reaction with such nitrosating agents as other thionitrite compounds, to form the relatively stable CoASNO. In vivo, the direct reaction between whatever nitrite permeates the membrane and -SH groups may be limited but certainly not completely precluded by the nearly neutral pH of bacterial cytoplasm. In nitrite-processed meats, however, where the pH characteristically ranges from about 5.5 to 6.0 (Hamm and Hofmann, 1966), the direct reaction between nitrite and extracellular but permeable -SH-containing compounds should occur. The indirect reaction, on the other hand, is not pH dependent and can occur even under alkaline conditions if some preformed thionitrite or other neutral nitrosating agent is present. In either case, once formed the S-nitroso derivative of coenzyme A, in contrast to the parent compound, is incapable of accepting acyl groups. In view of the importance of CoA-mediated acyl transfer reactions in biological processes, observed inhibition of bacterial growth by nitrite may be attributable, in part, to the formation of the thionitrite derivative of CoA.

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Registry No. CoA, 85-61-0; nitrite, 14797-65-0; phosphotransacetylase, 9029-91-8.

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