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MUTAGENICITY OF THIONITRITES IN THE AMES TEST

THE BIOLOGICAL ACTIVITY OF THIYL FREE RADICALS

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Abstract—Thiols, such as glutathione and cysteine, are mutagenic in the Ames test, using *Salmonella typhimurium* strain TA 100 and rat kidney S-9 preparation [Glatt *et al. Science* **220**, (1983)]. Formation of thiyl free radicals, RS$^{\cdot}$, has been implicated in this effect. We have prepared thionitrite (nitrosylmercaptan) derivatives of glutathione and other thiols. These unstable derivatives decompose by homolysis, yielding RS$^{\cdot}$ radicals. Glutathione thionitrite is mutagenic to strains TA 100 and TA 102, in the absence of activation by mammalian S-9 preparations. We suggest that this mutagenicity is evidence for the role of thiyl free radicals as biological reactive intermediates. Since alkyl nitrites readily convert thiols to thionitrites, our findings have implications for the toxicology of nitrosating drugs, such as amyl nitrite.

Glutathione (GSH) and other non-protein sulfhydryls play an important role in cellular defence against oxidative damage [1]. The GSH/glutathione peroxidase system reduces H_2O_2 and organic hydroperoxides to alcohols [2]. Sulfhydryls are excellent nucleophiles, and trap reactive intermediates such as epoxides and quinones to form covalent adducts [3]. In some cases, such as the benzidine/peroxidase system, GSH acts as both a reducing agent and a nucleophile [4, 5]. *N*-Acetylcysteine [6] and GSH [7] inhibit chemical mutagenesis induced by agents such as 4-nitroquinoline-*N*-oxide and benzidine in Ames test strains of *Salmonella typhimurium*. Administration of the cysteine precursor L-2-oxothiazolidine-4-carboxylate protects against the toxic effects of acetaminophen and, possibly, other agents [8].

In view of the interest in using sulfhydryls as antidotes to carcinogens and toxicants, careful attention must be given to the report of Glatt *et al.* [9] that GSH and cysteine are mutagenic in the Ames test. This mutagenicity was dependent upon activation by S-9 preparations, and it could not be prevented by the addition of catalase or superoxide dismutase [9]. Thus, production of active oxygen species via the autoxidation of the sulfhydryl is not responsible for the mutagenic effect, and the authors suggested that "Thiol free radicals are good candidates for the active species . . ." [9]. Cysteine activation was observed with either rat kidney or liver S-9, whereas GSH was activated by kidney S-9 only [9]. The subcellular localization of the activating factors in rat kidney also differed: cysteine was activated by cytosol, and less effectively by microsomal preparation, whereas GSH was activated only by microsomal preparation [9]. The enzymatic basis of these differences is not understood. In a recent publication from the same laboratory [10], cysteine and penicillamine (β,β-dimethylcysteine) were shown to be mutagenic in *S. typhimurium* strains TA 100 and TA 92. The L-enantiomers of these compounds were considerably more mutagenic than the D-enantiomers. L-Penicillamine showed marked mutagenicity even in the absence of S-9 fraction [10].

Several groups of researchers have applied the technique of electron spin resonance (ESR) spin-trapping to the characterization of thiyl free radicals. The thiyl radical spin adduct of DMPO (5,5-dimethyl-1-Δ-pyrroline-*N*-oxide) was detected in aerobic solutions of cysteine [11] and attributed to metal-catalyzed autoxidation; the same spin adduct is produced via horseradish peroxidase-catalyzed oxidation of cysteine [12]. Oxidation of GSH by horseradish peroxidase occurs by a one-electron pathway, and the GS$^{\cdot}$ radical can be trapped with DMPO [13]. In contrast, GSH oxidation by the enzyme glutathione peroxidase, which reduces H_2O_2 and organic hydroperoxides, does not produce detectable thiyl radical intermediates [13].

Thiyl radicals are generated during the horseradish peroxidase-catalyzed oxidation of *p*-phenetidine in the presence of GSH or cysteine [14], presumably via the one-electron oxidation of the thiol by the *p*-phenetidine radical. The subsequent fates of the GS$^{\cdot}$ radical are not fully understood, but probably include dimerization to give oxidized glutathione (GSSG) and reaction with O_2 to give oxygenated products [15].

The reaction of sulfhydryls with alkyl nitrites produces thionitrites (nitrosylmercaptans):

$$\text{R—SH} + \text{R}'\text{—ONO} \rightarrow \text{R—S—N=O} + \text{R}'\text{—OH}$$

The decomposition of thionitrites occurs spontaneously at room temperature, and a radical mechanism has long been suspected:

$$\text{R—S—N=O} \rightarrow \text{RS}^{\cdot} + \text{NO} \rightarrow \tfrac{1}{2}\ \text{R—SS—R} + \text{NO}$$

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This mechanism was verified by an ESR spin-trapping investigation [15]. Thus, the nitrosation of thiols to produce thionitrites constitutes a simple system for the generation of thiyl free radicals.

The formation of thionitrites in biological systems has been proposed as a mechanism for the activation of guanylate cyclase by glyceryl trinitrate or $NaNO_2$ [16]. Törnqvist *et al.* [17] have suggested that nitrosation of cysteine may be responsible for the mutagenicity of methyl nitrite. We hypothesized that alkyl thionitrites should be direct-acting (S-9 independent) mutagens, since these compounds produce thiyl radicals via a spontaneous chemical decomposition at room temperature. Experiments using *S. typhimurium* strains TA 100 and TA 102 have confirmed this hypothesis, and are reported here.

METHODS

$NADP^+$ and GSH were purchased from Boehringer Mannheim Canada, Dorval, P.Q. *N*-Acetylcysteine was purchased from the Sigma Chemical Co., St. Louis, MO, U.S.A. Triphenylmethyl mercaptan, *tert*-butyl nitrite, 2-aminofluorene (S-9 dependent control) and 2-nitrofluorene (S-9 independent control) were purchased from the Aldrich Chemical Co., Milwaukee, WI, U.S.A. *N*-Acetylcysteine, triphenylmethyl mercaptan, 2-mercaptoethanol and *tert*-butyl nitrite were prepared in dimethyl sulfoxide (DMSO), and GSH was prepared in H_2O. Solutions of thiols and nitrites were prepared fresh for each experiment.

S. typhimurium strains TA 98, TA 100, and TA 102 were gifts from Dr. B. Ames, University of California at Berkeley, and were maintained as frozen permanents at −80°. An innoculum from a freshly thawed frozen permanent (4 μl/ml of nutrient broth) was used for each experiment. These overnight cultures were grown in Oxoid nutrient broth No. 2, at 37°, with continuous shaking, for about 12 hr, until absorbance at 650 nm reached 1.0; the cultures were then kept on ice until used. One extra hour of growth was required for strain TA 102.

The thionitrite derivatives were prepared immediately before the test. Triphenylmethyl thionitrite was synthesized using benzene as solvent for the reaction of triphenylmethyl mercaptan with excess *tert*-butyl nitrite [18]. The stable green cystalline product was isolated by removal of solvent under vacuum and prepared as a DMSO solution. The nitrosations of 2-mercaptoethanol and *N*-acetylcysteine were carried out in DMSO. For each reaction, *tert*-butyl nitrite solution was added dropwise to a solution of the thiol. The progress of the nitrosation reactions was monitored by optical spectroscopy, since these unstable thionitrites cannot be isolated in pure form. The red color of the product ($\lambda_{max} \simeq 550$ nm) increased linearly with concentration of *tert*-butyl nitrite added to the thiol solution, and reached a plateau at approximately 1:1 molar ratio. The molar absorptivities of the thionitrites were estimated from the absorbances of the 1:1 mixtures used in the Ames test, assuming 100% yield: $\varepsilon_{552} = 20\ M^{-1}\ cm^{-1}$ (2-mercaptoethanol thionitrite) and $\varepsilon_{550} = 21\ M^{-1}\ cm^{-1}$ (*N*-acetylcysteine thionitrite). These values are consistent with literature values for other alkyl thionitrites [19]. For these compounds, the red color of the product reached maximum intensity within 5 min, and remained stable for greater than 30 min. The solutions were used for the Ames test within 10 min.

GSH is only slightly soluble in DMSO; therefore, a solution of *tert*-butyl nitrite in DMSO was added dropwise to a solution of GSH in water. The reaction proceeded more slowly than in the previous case, and maximum color was observed after approximately 30 min. A 2:1 molar ratio of nitrite : GSH gave maximum yield. The molar absorptivity was estimated as $\varepsilon_{548} = 15\ M^{-1}\ cm^{-1}$; this probably represents about 75% yield only, by comparison with the values quoted above. Therefore, the concentrations quoted in the results may be slightly overestimated, for this compound. The 2:1 preparation was used in the Ames test.

Serial dilutions of the thionitrite and thiol solutions were prepared in DMSO, just prior to use in the Ames test. GSH was diluted in water. Incubations contained 0.5 ml phosphate buffer, pH 7.4, 0.1 ml bacterial culture, and 0.1 ml or less of the mutagen solution. The mixture was incubated at 37° for 30 min before addition of top agar and plating.

Aroclor-induced rat hepatic S-9 was purchased from Intermedico, Willowdale, Ontario, and used at a protein concentration of 1 mg/plate. The method for the determination of toxicity was as described [20]. Plates were incubated for 2 days (TA 98, TA 100) or 3 days (TA 102) at 37°, and counted with a 3M electronic colony counter. Data points represent the mean ± S.D. of at least three plates and are not corrected for background. The dose axis is logarithmic.

RESULTS

Alkyl thionitrites serve as a convenient source of thiyl radicals in solution [15]. Most thionitrites cannot be prepared in pure form, due to their instability; the only known exceptions are triphenylmethyl thionitrite and *N*-acetyl-penicillamine thionitrite [21], in which the α-carbon is fully substituted. We wished to study the biological properties of thionitrites as a test of the postulated mutagenicity of thiyl free radicals [9]. Thionitrite derivatives of *N*-acetylcysteine, 2-mercaptoethanol, and GSH were prepared by treatment of the thiol with *tert*-butyl nitrite at room temperature, as recommended by Doyle *et al.* [18], and used in the Ames test without isolation from the reaction mixture. Spectroscopic measurements showed that the nitrosation reactions proceeded nearly to completion, under the conditions used (see Methods); however, a residue of unreacted nitrite and thiol probably remained. Thus, in each experiment, we compared the thionitrite preparation with the parent thiol and *tert*-butyl nitrite.

Triphenylmethyl thionitrite was isolated as a solid and dissolved in DMSO for testing. At a dose of 0.5 μmole/plate, no mutagenic effect was observed. Above this dose, the yield of revertants decreased and toxicity could be observed as a thinning of the background lawn of his⁻ colonies. Beyond 2 μmoles/plate the compound was not soluble under the test

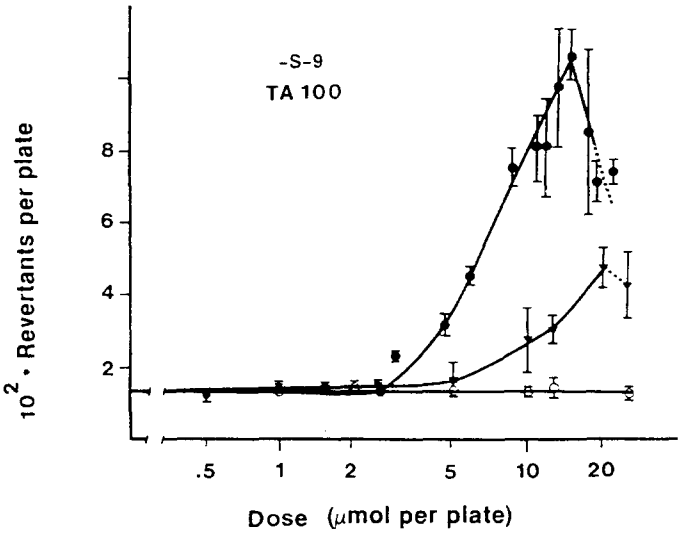

Fig. 1. Mutagenicity of glutathione thionitrite-TA 100. The Ames test procedures were as described in the text. Data are shown for glutathione (○), *tert*-butyl nitrate (▼), and glutathione thionitrite (●). Data are pooled from two separate experiments.

conditions. Results with *N*-acetylcysteine thionitrite were also inconclusive, since toxicity was evident at doses of 5 μmoles/plate and above.

GSH thionitrite had more favorable solubility and toxicity properties. GSH was not mutagenic in strain TA 100 in the absence of S-9 activation (Fig. 1), consistent with previous reports [9]. (The mutagenicity of GSH was first observed using activation by rat kidney S-9 [9].) *tert*-Butyl nitrite was mutagenic at doses of 10 μmoles and above, yielding a little more than a doubling of the spontaneous reversion frequency (Fig. 1). This effect has been reported previously, using strain TA 1535, a precursor strain of TA 100 [22]. Toxicity was observed at 25 μmoles (slight) and 50 μmoles (obvious).

The substantial mutagenicity of GSH thionitrite is apparent in Fig. 1. The rise in the yield of revertant colonies was observed in the range of 2–5 μmoles, and reached a maximum of about 1000 revertants at 15 μmoles; above this dose, the yield declined and toxicity became apparent. The possible residue of unreacted *tert*-butyl nitrite in the preparation of GSH thionitrite cannot account for the observed mutagenicity, which was greater both in maximum number of revertants and yield per μmole of compound.

The effect of addition of hepatic S-9 is shown in

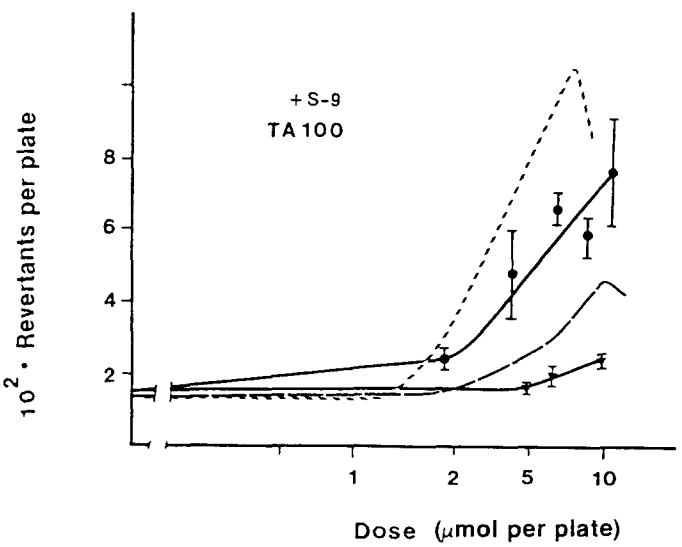

Fig. 2. Effect of S-9 fraction on thionitrite mutagenicity-TA 100. Conditions were as in Fig. 1, except for the addition of hepatic S-9 preparation. Data shown are for glutathione thionitrite (●) and *tert*-butyl nitrite (▼). Also shown for comparison are the curves obtained in the absence of S-9 (Fig. 1): short dashes: glutathione thionitrite; long dashes: *tert*-butyl nitrite. Glutathione was non-mutagenic in the presence or absence of hepatic S-9 (data not shown).

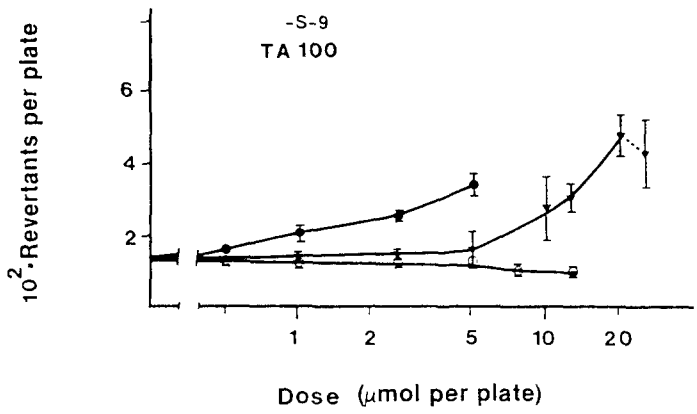

Fig. 3. Mutagenicity of 2-mercaptoethanol thionitrite-TA 100. Data shown are for 2-mercaptoethanol (○), *tert*-butyl nitrite (▼—same data as in Fig. 1), and 2-mercaptoethanol thionitrite (●). No S-9 preparation was used. Data are pooled from two separate experiments.

Fig. 2; the results from Fig. 1 are shown as dashed lines. The mutagenicities of both *tert*-butyl nitrite and GSH thionitrite are somewhat diminished by the addition of the mammalian activating system.

The results of studies with 2-mercaptoethanol are shown in Fig. 3. 2-Mercaptoethanol was non-mutagenic; indeed, the thiol caused a reproducible dose-dependent decrease in the yield of spontaneous revertants, as has been noted by Zeiger and Pagano [23]. The thionitrite derivative of 2-mercaptoethanol gave a maximum yield of 332 ± 31 revertants at 5 μmoles/plate, more than twice the spontaneous background of 135 ± 16. Thus, 2-mercaptoethanol thionitrite can be considered mutagenic by the criterion of doubling the spontaneous reversion rate. 2-Mercaptoethanol does not cause bacterial toxicity at doses of 25 μmoles/plate (our data) or even 71.5 μmoles/plate [23]. In contrast, 2-mercaptoethanol thionitrite was toxic at doses of 7.5 μmoles (partial kill) and 12.5 μmoles (complete kill), as judged by microscopic examination of the background lawn.

Tester strain TA 102 was developed by Ames and colleagues in 1982 [24]. This strain contains only AT base pairs in the mutated triplet codon, and is sensitive to mutagenesis by X-rays, H_2O_2, mitomycin C, and other oxidative chemicals [24]. We found that this tester strain grows more slowly than TA 100,

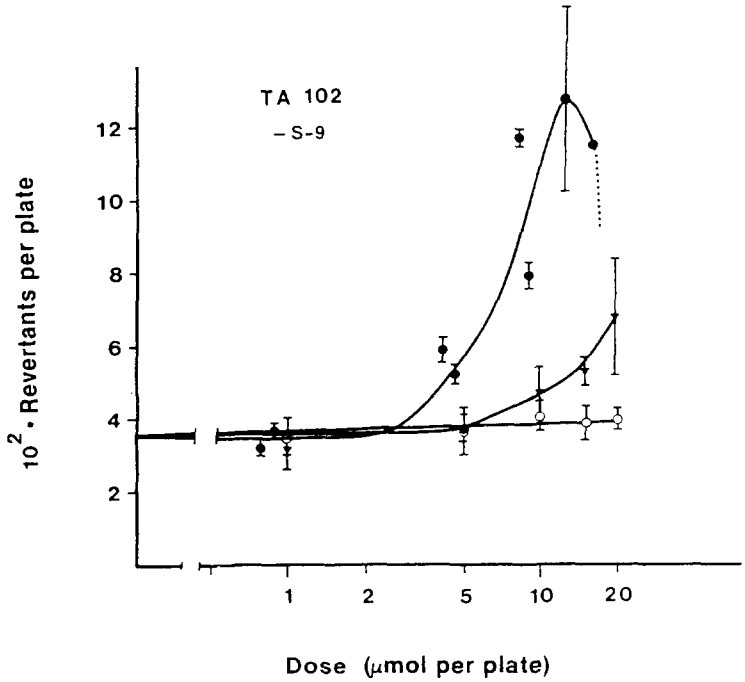

Fig. 4. Mutagenicity of glutathione thionitrite-TA 102. Data shown are for glutathione (○), *tert*-butyl nitrite (▼), and glutathione thionitrite (●). No S-9 preparation was used. Data are pooled from two separate experiments.

and therefore incubated the plates for 3 rather than 2 days. The spontaneous mutation rate under these conditions was 358 ± 40, within the reported range of 300 ± 60 [25, *]. We studied the response of this strain to GSH thionitrite (Fig. 4). GSH produced an insignificant rise in reversion rate to about 400. *tert*-Butyl nitrite induced about the same number of revertants in TA 102 as in TA 100, although this increase is measured against a higher spontaneous rate in TA 102. GSH thionitrite mutagenicity rose to 1284 revertants at 12.5 μmoles, and then decreased due to toxicity.

Studies with strain TA 98 were negative.

DISCUSSION

The formation of thiyl radicals has been proposed as the mechanism of the observed mutagenesis of GSH [9]. We have found that GSH thionitrite, a precursor of GS· radical, is a direct-acting mutagen of comparable potency to GSH activated by rat kidney S-9 [9]. Homolysis of alkyl thionitrites, yielding RS· and NO, is the only significant reaction of this class of compounds. At the elevated temperatures used in the Ames test, this homolytic decomposition should be rapid. Thus, our experiments are probably as direct an examination of the activity of thiyl radicals as can be arranged, and support the view that such radicals may be biological reactive intermediates. Törnqvist *et al.* [17] studied the mutagenicity of methyl nitrite, and suggested that nitrosation of thiols may play a role. Our results are consistent with this suggestion.

Glatt *et al.* [9, 10] have reported the mutagenicity of thiol compounds activated by rat liver S-9, rat kidney S-9, and in the absence of activating systems. Generally, kidney S-9 is the best activating system; both microsomal and cytosolic fractions have some activating capacity [9]. Our results do not necessarily imply that thiyl radical formation is the only mechanism for production of thiol-derived mutagens. However, since thiyl radicals can be generated by many spontaneous and enzymatic processes, they may play a central role in thiol bioactivation.

Ignarro and colleagues [16, 26] have suggested that thionitrites may be involved in the relaxation of smooth muscle by amyl nitrite and glyceryl trinitrate, via an effect on guanylate cyclase. In view of the widespread abuse of amyl nitrite as a stimulant [27], further study of the biological consequences of the nitrosation of thiols seems warranted.

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* B. N. Ames, unpublished circular, 10 August, 1983.

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