

## Research Section

### CARCINOGENESIS IN RATS BY CYCLIC *N*-NITROSAMINES CONTAINING SULPHUR\*

**Abstract**—The effects of chronic exposure to three sulphur-containing heterocyclic *N*-nitrosamines were determined after repeated oral administration to female Fischer 344 rats. Nitrosothiazolidine did not significantly affect the survival of the rats or the incidence of tumours at a total dose of 3.5 mmol. Nitrosodithiazine, an analogue of nitrosothiazolidine which contains an extra sulphur atom inserted between the carbons of its CH<sub>2</sub>—CH<sub>2</sub> moiety, produced only three tumours (two of the nasal mucosa) in a group of 20 rats at a total dose of 1.75 mmol/rat. Nitrosothialdine, the all-*cis* 2,4,6-trimethyl analogue of nitrosodithiazine, was a potent carcinogen that significantly shortened the lifespan and produced oesophageal tumours in 70% of treated rats as well as numerous tumours of the tongue and liver; this outcome was unexpected because  $\alpha$ -methyl substitution in other heterocyclic nitrosamines usually reduces or eliminates tumorigenicity. The results extend the data base on the carcinogenic activity of molecules containing both divalent sulphur and the nitrosamino function. The lack of significant carcinogenicity of nitrosothiazolidine in this study suggests that its presence in the human food supply presents a relatively minor risk.

#### INTRODUCTION

There are numerous potential sources of exposure to sulphur-containing *N*-nitroso compounds. *N*-Nitrosothiazolidine (Fig. 1) and its derivatives may be ingested as contaminants of cured meats and fish (Massey *et al.* 1985; Pensabene & Fiddler, 1983; Sen *et al.* 1986). *N*-Nitrosothiazolidine-4-carboxylic acid and its 2-methyl derivatives have commonly been detected in human urine (Ohshima *et al.* 1984; Tsuda *et al.* 1986). Other sulphur-containing *N*-nitroso compounds can be formed from pesticides, drugs, and foodstuff constituents.

The possible cancer risk associated with exposure to sulphur-containing *N*-nitroso compounds is difficult to estimate using currently available knowledge. Of several hundred such compounds reported in the literature, only one having the structure of a nitrosamine appears to have been tested for carcinogenicity: *N*-nitrosothiomorpholine (Fig. 1) differed in target organ from its oxygen analogue (Garcia *et al.* 1970). Furthermore *N*-nitrosomorpholine was mutagenic whereas *N*-nitrosothiomorpholine was not (Andrews & Lijinsky, 1984). This isolated result suggests that the presence of divalent sulphur in the heterocyclic ring may alter a nitrosamine's biological properties, and that results for a larger number of

examples will be required before meaningful risk assessments or structure-activity correlations can be made for such compounds. This paper extends knowledge in this area by reporting the results of

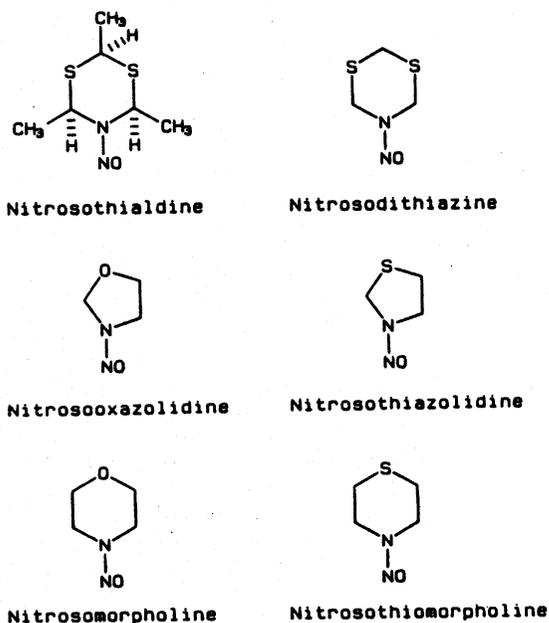


Fig. 1. Structures of the cyclic, sulphur-containing *N*-nitrosamines tested, and those of related compounds.

\*This paper is dedicated to the memory of Leon Golberg, friend and wise teacher in toxicology, whose rare wit as Editor of this Journal was so refreshing.

long-term oral administration of three sulphur-containing heterocyclic nitrosamines. One of these, nitrosothiazolidine, is a contaminant of the human food supply.

#### MATERIALS AND METHODS

**Chemicals\*.** *N*-Nitrosothiazolidine was prepared as previously described (Miller *et al.* 1985), as was *N*-nitrosothialdine (Hansen *et al.* 1981). The UV spectrum of the latter compound in ethyl acetate had  $\lambda_{\max}(\epsilon)$  386 nm (61).

*N*-Nitrosodithiazine was prepared from 1,3,5-dithiazine (formothialdine) synthesized by the method of Levi (1929) from formaldehyde and ammonium sulphide in aqueous medium. Formothialdine was a waxy solid; mass spectrometric data were as follows: (electron impact using a Finnigan 330 mass spectrometer fitted with a Finnigan 6000 MS data system):  $m/z$  (% relative intensity) 123 ( $M^+ + 2, 5$ ), 122 ( $M^+ + 1, 3$ ), 121 ( $M^+, 56$ ), 76 (23), 75 (100). A two-phase system consisting of 40 g (0.33 mol) 1,3,5-dithiazine in 200 ml dichloromethane and 42 g (0.61 mol) sodium nitrite dissolved in 400 ml water was cooled to 0°C. A solution of 36 ml (0.6 mol) glacial acetic acid in 50 ml water was added dropwise to the cold mixture. When the addition was complete, the ice-bath was removed and the reaction mixture was stirred at room temperature overnight. The organic layer was separated and the aqueous portion was extracted with dichloromethane. The combined organic extracts were washed with 10% sodium hydroxide, filtered through a pad of anhydrous magnesium sulphate, and evaporated *in vacuo*. The heterogeneous residue was extracted with ethyl acetate; the organic layer was filtered and evaporated on a rotary evaporator to give 24 g of a yellow oil. Thin-layer chromatography on silica gel indicated that at least six different compounds were present in the mixture. The nitrosodithiazine was separated and purified by column chromatography on silica gel. Elution with ether gave 5.45 g (11%) of crystalline product, with the following characteristics: MP, 99–101°C; UV (ethyl acetate)  $\lambda_{\max}(\epsilon)$ , 380 nm (69); <sup>1</sup>H-nuclear magnetic resonance (CDCl<sub>3</sub> containing 0.5% tetramethylsilane using a Nicolet NT-300 spectrometer),  $\delta$  4.16 (s, 2H), 4.86 (s, 2H), 5.59 (s, 2H); mass spectrometry,  $m/z$  (% relative intensity) 152 ( $M^+ + 2, 1$ ), 150 ( $M^+, 8$ ), 122 (2), 120 (25), 46 (15), 45 (21), 42 (100), 30 (20). Found: C 24.22, H 4.10, N 18.46, S 42.39%; C<sub>3</sub>H<sub>6</sub>N<sub>2</sub>OS<sub>2</sub> requires C 24.00, H 4.00, N 18.67, S 42.67%.

**Animals and treatment.** Each compound was administered to a group of 20 female F344 rats of the colony of the Frederick Cancer Research Facility, bred and maintained in cages of four within a barrier. The rats were fed sterilized Purina Autoclavable Rodent Laboratory Chow (mash) (Ralston Purina Co., Inc., St Louis, MO) *ad lib.*, and were 7 wk old at the beginning of treatment. The rats treated with *N*-nitrosothialdine and *N*-nitrosodithiazine also had access to acidified (with hydrochloric acid to pH 2.5)

tap-water *ad lib.* The animals treated with *N*-nitrosothiazolidine were given 80 ml of an aqueous solution containing 41 mg *N*-nitrosothiazolidine/litre for each cage on 5 days of each week; on the remaining 2 days they were given tap-water *ad lib.* Treatment lasted 104 wk.

Treatment with *N*-nitrosothialdine and *N*-nitrosodithiazine consisted of twice weekly administration by gavage of 0.2 ml of solutions of the compounds in ethyl acetate:corn oil (1:2, v/v). Rats were treated with *N*-nitrosothialdine (17.5 mg/ml) for 43 wk, with *N*-nitrosodithiazine (14 mg/ml) for 47 wk, or with vehicle only (controls) for 57 wk. No additional group of untreated controls was included, since contemporary groups of untreated F344 rats are continuously maintained, and typical results have been reported elsewhere (Lijinsky *et al.* 1981).

After treatment the rats were allowed to die naturally, except for a few that were killed when moribund. Three controls survived until wk 130 and were then killed. Complete autopsies were carried out on each rat; all lesions and major organs and tissues were removed, fixed in formalin, embedded in paraffin, processed and stained with haematoxylin and eosin for histological examination. Statistical analysis of the resulting data on tumour incidence was performed using Fisher's exact test (one-tailed).

#### RESULTS

The survival times and incidence of tumours in the groups of treated and control rats are given in Table 1. The two cyclic *N*-nitrosamines containing two sulphur atoms in the ring had very different effects. *N*-Nitrosothialdine (Fig. 1) was clearly carcinogenic, since it considerably reduced the lifespan of the animals treated with it and gave rise to tumours that were not usually seen in control F344 rats. Most of the rats treated with *N*-nitrosothialdine had such neoplasms, the most common being in the oesophagus ( $P < 0.01$ ) and tongue ( $P = 0.02$ ); a third of the rats treated with this nitrosamine also had liver neoplasms.

In contrast, *N*-nitrosodithiazine (Fig. 1), the analogue of *N*-nitrosothialdine but lacking the three methyl substituents, seemed devoid of carcinogenic activity, even though the total dose was larger (because equimolar doses were given for a longer time) than that of the carcinogen, *N*-nitrosothialdine. *N*-Nitrosodithiazine did not reduce the lifespan of the treated rats, nor did it induce tumours of types not normally seen in control rats; the two tumours of the nasal mucosa do not constitute a statistically significant result ( $P = 0.24$ ), although they might suggest a possible weak carcinogenic effect.

Administration of *N*-nitrosothiazolidine in drinking-water for 104 wk led to no reduction in lifespan compared with that of control rats, and no tumours were seen that could be related to the treatment; the incidence of one hepatocellular adenoma in the liver of one of the treated rats was not statistically significant ( $P = 0.50$ ). For comparison, the results of analogous treatment with nitrosothiazolidine's oxygen analogue, *N*-nitrosooxazolidine (Fig. 1), are also shown in Table 1, although they have been presented previously (Lijinsky & Reuber, 1982). The

\*References to brand names or firms does not constitute endorsement by the US Department of Agriculture over others of a similar nature not mentioned.

Table 1. Survival and tumour incidence in female F344 rats given sulphur-containing *N*-nitrosamines

Compound (method of administration)	Total dose (mmol/rat)	Incidence of neoplasms															
		Number of survivors at wk:															
		Median wk of death															
		Liver															
Hepatocellular:																	
Cholangioma																	
Haemangiosarcoma																	
Oesophagus																	
Squamous cell tumours																	
Total Carcinoma																	
Tongue																	
Squamous cell tumours																	
Total Carcinoma																	
Nasal Carcinoma																	
<i>N</i> -Nitrosothiazolidine (in drinking-water)	3.5	20	20	20	19	16	4*	110	0	1	0	0	0	0	0	0	0
<i>N</i> -Nitrosooxazolidine† (in drinking-water)	1.7	20	20	20	5	0		53	19	0	0	15	0	0	0	0	0
<i>N</i> -Nitrosothialdine (by oil gavage)	1.6	20	20	20	13	3	0	70	0	4	3	0	0	14	6	7	1
<i>N</i> -Nitrosodithiazine (by oil gavage)	1.75	20	20	20	20	18	10	98	1	0	0	0	0	0	0	0	2
Control (by oil gavage)	—	20	20	20	20	18	16	108	0	0	0	0	0	0	0	1	1

\*All surviving rats were killed at wk 110-122.

†Re-evaluation of slides from the experiment described by Lijinsky & Reuber (*Carcinogenesis* 1982, 3, 911).

conditions used for testing the three sulphur-containing nitrosamines listed in Table 1 were chosen so as to be comparable with those of the *N*-nitrosooxazolidine study, in that the four compounds were administered at weekly doses that were equimolar.

## DISCUSSION

All three sulphur-containing compounds tested showed marked differences in carcinogenicity from their oxygen-containing or unsubstituted analogues.

Methylation at the  $\alpha$ -carbon atoms of cyclic nitrosamines such as *N*-nitrosopiperidine and *N*-nitrosopyrrolidine usually reduces or eliminates carcinogenic activity (Lijinsky, 1984), but with *N*-nitrosodithiazine and *N*-nitrosothialdine, just the opposite was observed. *N*-Nitrosodithiazine did not significantly increase tumour incidence, whereas its triply methylated analogue, nitrosothialdine, was a potent carcinogen and affected a variety of organs.

The results for the latter two compounds also contrast in part with expectations based on data for their acyclic counterparts. *N*-Nitrosodithiazine and *N*-nitrosothialdine can be regarded as sulphur-containing derivatives of *N*-nitrosodimethylamine and *N*-nitrosodiethylamine, respectively, which have been  $\alpha$ -oxidized at both sides of the nitrosamino function. However, the biological effects of nitrosodithiazine bear no resemblance to those of nitrosodimethylamine, a very potent carcinogen. It is possible that the relative absence of activity of *N*-nitrosodithiazine results from the fact that the dealkylation step, which normally activates nitrosamines to their ultimately carcinogenic form, can lead to an alkylthiomethylating, rather than a methylating, agent; alkylation of DNA by such a species, e.g. at the 0-6-position of a guanine residue, would yield an altered base whose 0-6 alkyl substituent is in the formaldehyde oxidation state, and thus is susceptible to hydrolytic or other spontaneous repair. If this rationale is correct, *N*-nitrosodioxazine should be similarly weakly or noncarcinogenic. Unfortunately, despite repeated attempts to do so, neither this compound nor the oxygen analogue of *N*-nitrosothialdine could be prepared. The only product isolated in the attempted *N*-nitrosodioxazine synthesis, which involved the reaction of formaldehyde and ammonia under different reaction conditions, was 1,3,5-trinitrosohexahydro[*s*]triazine. It is well known that formaldehyde-ammonia condensation produces hexamethylenetetramine through the initial intermediate, 1,3,5-hexahydrotriazine (Nielsen *et al.* 1979; Richmond *et al.* 1948); thus isolation of oxygen-containing condensation products was not possible.

*N*-Nitrosothialdine did not follow the pattern suggested by *N*-nitrosodithiazine, in that its carcinogenic activity was similar to that of its acyclic analogue (*N*-nitrosodiethylamine) in the sites and types of neoplasms induced (liver and oesophagus), although in terms of reduced survival of the treated rats it was less potent than *N*-nitrosodiethylamine (Lijinsky *et al.* 1981). *N*-Nitrosothialdine has not been detected in foods, but thialdine has been identified as a component of beef flavour volatiles (Brinkman *et al.* 1972; Wilson *et al.* 1973). Nitrosation in the vapour

phase by nitrogen oxides could favour the formation of this nitrosamine, and might occur under certain cooking conditions.

In the case of *N*-nitrosothiazolidine, the oxygen analogue has been prepared and tested. The contrast here was also striking. *N*-Nitrosooxazolidine (Fig. 1) was a very potent carcinogen: it induced liver neoplasms in all of the rats treated with it for 50 wk (Lijinsky & Reuber, 1982) and all of the treated rats died by wk 67.

The difference between *N*-nitrosooxazolidine and *N*-nitrosothiazolidine in carcinogenic effect contrasts sharply with the difference between *N*-nitrosomorpholine and its sulphur-containing analogue, *N*-nitrosothiomorpholine. Both of these latter compounds are carcinogens of similar potency in rats when administered in drinking-water (Garcia *et al.* 1970; Garcia & Lijinsky, 1972), although they induce different types of neoplasm. *N*-Nitrosomorpholine induced a high incidence of liver tumours—hepatocellular carcinomas and, especially at higher doses, haemangiosarcomas (Lijinsky & Reuber, 1982) and many tumours of the oesophagus. On the other hand, *N*-nitrosothiomorpholine at two different doses induced a high incidence of neoplasms of the oesophagus, but no liver neoplasms were observed (Garcia *et al.* 1970). These differences suggest that the presence of sulphur in the ring greatly affects activation to a liver carcinogen, but does not affect activation to an oesophageal carcinogen in the same way. Since *N*-nitrosooxazolidine had a similar potency to *N*-nitrosomorpholine in inducing neoplasms in the rat liver, but did not induce any tumours in the oesophagus at the same concentration, it is likely that the activation of both of these oxygen-containing cyclic nitrosamines may be similar in the liver but different in the oesophagus. On the other hand, it seems that whatever mechanisms are responsible for the carcinogenic effects of nitrosooxazolidine or nitrosothiomorpholine do not apply to the noncarcinogenic nitrosothiazolidine. At present it is not possible to specify the mechanism by which these cyclic nitrosamines induce tumours. The currently accepted mechanisms of genotoxicity are difficult to reconcile with the relative carcinogenic potencies of these compounds, since the oxygen-containing cyclic nitrosamines are strongly mutagenic to bacteria with hepatic microsomal activation, whereas both nitrosothiomorpholine and nitrosothiazolidine are inactive (Andrew & Lijinsky, 1984; Miller *et al.* 1985), as also are the two other sulphur-containing cyclic nitrosamines included in this study (*N*-nitrosothialdine and *N*-nitrosodithiazine) when tested under the same conditions.

We conclude that the presence of divalent sulphur can compound the difficulty of predicting the carcinogenicity of *N*-nitrosamines from data for analogous compounds such as the oxygen-containing derivatives. Differences might result in part from the sulphur atom's relative susceptibility to attack by oxidizing agents and electrophiles, with the resulting chemical changes having potentially activating or deactivating effects. The unexpectedly greater carcinogenicity of *N*-nitrosothialdine over its unmethylated analogue may result from the unusual steric consequences of methyl substitution, including marked

deviations from planarity of the  $>N-N=O$  system (Hansen *et al.* 1981). Biological activity may also be related to specialized effects, such as the ability of a compound having the oxidation state of nitroso-dithiazine to decompose by a variety of conceivable pathways to formaldehyde, a carcinogen for the nasal mucosa in F344 rats (Starr & Gibson, 1985); it may be important that nasal tumours were observed in two of the animals given *N*-nitrosodithiazine in our experiments. Clearly, more information will be required before the effects of sulphur substitution on nitrosamine carcinogenesis can be rationalized, a task of importance if reliable structure-activity correlations and risk assessments for untested compounds are to be made.

However, our data do permit some preliminary conclusions regarding the possible significance for public health of one sulphur-containing nitrosamine to which people are exposed. The absence of treatment-related neoplasms in rats given *N*-nitrosothiazolidine, which has been found at low concentrations in cured meats (Pensabene & Fiddler, 1983; Sen *et al.* 1986), suggests that its presence in the diet gives rise to little cancer risk compared with the other potentially carcinogenic nitrosamines that are present. Further testing of nitrosothiazolidine at higher doses is desirable since there is a possibility that a carcinogenic effect might be observed under different conditions, for example in different animal species.

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