

# Carcinogenicity of *N*-Nitroso-3,4-dichloro- and *N*-Nitroso-3,4-dibromopiperidine in Rats<sup>1</sup>

William Lijinsky and H. Wayne Taylor

Carcinogenesis Program, Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830

## SUMMARY

The carcinogenic potencies of 3,4-dichloro- and 3,4-dibromonitrosopiperidine were compared with that of nitrosopiperidine by feeding to groups of 15 male rats in drinking water. A treatment of 15 weeks with a total of 0.5 mmole of the dichloro compound led to death of all animals before 24 weeks with tumors of the tongue, pharynx, esophagus, nonglandular stomach, nasal turbinates, trachea, bronchi, and bronchioles. Treatment of 27 weeks with the dibromo compound, a total of 1.0 mmole, caused death of all the animals by 41 weeks, with the same types of tumors. One-half of the rats treated with an almost 3-fold higher daily dose of nitrosopiperidine, 3.9 mmoles total, were alive at 40 weeks, and all were not dead until 55 weeks. Most of these animals died with tumors of the tongue, pharynx, esophagus, and nonglandular stomach and with squamous cell tumors and olfactory carcinomas of the nasal cavity, but there were no tumors of the respiratory tree. Substitution of chlorine or bromine in nitrosopiperidine greatly increased the carcinogenicity of the compound.

## INTRODUCTION

As part of our investigation of the effect of substituents in cyclic nitrosamines on their carcinogenic activity we have fed 2 halogenated derivatives of nitrosopiperidine in drinking water to rats, at equimolar doses. The tumor response of the animals is compared with that to similar treatment with nitrosopiperidine.

## MATERIALS AND METHODS

### Chemicals

***N*-Nitroso-1,2,3,6-tetrahydropyridine.** 1,2,3,6-Tetrahydropyridine, 30 g (0.36 mole) (Aldrich Chemical Co., Milwaukee, Wis.), was dissolved in 60 ml (1 mole) of acetic acid diluted with 50 ml water + 100 g ice. To the solution were added 50 g (0.72 mole) sodium nitrite. A yellow oil

started to separate as an upper layer, and after 2 hr standing solid NaOH was added with cooling until the solution was slightly alkaline. The nitrosamine was extracted with 3 × 75 ml of methylene chloride, and the combined extracts were shaken with 20 ml 5 N HCl. After the solution was dried with anhydrous sodium carbonate, the solvent was removed in a stream of nitrogen at room temperature. The yield was 33 g (82% theoretical) of light brown oil, which was pure enough to be used in the next step. The molar absorptivity was 101 at 339 nm in water, and the mass spectrum and NMR<sup>2</sup> spectrum were consistent with the above structure, without significant impurity.



Calculated: C 53.56%, H 7.19%, N 24.98%

Found: C 53.58%, H 7.07%, N 24.95%

**3,4-Dichloronitrosopiperidine.** *N*-Nitroso-1,2,3,6-tetrahydropyridine, 8 g, was dissolved in 30 ml methylene chloride. Chlorine generated by dropping 60 ml 10 N HCl onto 10 g potassium permanganate and washed and dried by passing through water and then sulfuric acid was bubbled into the solution which was cooled in ice water. Heat was generated and the increase in weight due to absorption of chlorine was approximately 10 g. The solution was evaporated in a stream of nitrogen at room temperature to remove the solvent and dissolved chlorine. The residue was a dark brown oil, which partially crystallized in the freezer. Cold methanol (20 ml) was added with stirring, which precipitated a colorless solid. This was filtered off and washed with cold methanol, giving 2.7 g of crystals, m.p. 81–82°. The substance gave only 1 band on thin-layer chromatography (chloroform) on silica gel ( $R_f$  0.92), and the mass spectrum showed a strong molecular ion at  $m/e$  182 and a pattern consistent with the presence of 2 atoms of chlorine in the molecule. The molar absorptivity was 88 at 345 nm in water. The NMR spectrum (CDCl<sub>2</sub>, 100 MHz), while complex, fully supported the assigned structure. In particular an axial chlorine at C-3 was indicated by the following data for the *alpha*-axial protons from 1 of the 2 conformers: 3.76δ, d of d, H-2-*syn-ax*,  $J_{gem} = 14.5$  Hz,  $J_{2ax,3eq} = 3.3$  Hz; 3.29δ, d of d of d, H-6-*syn-ax*,  $J_{gem} = 14$  Hz,  $J_{6ax,5ax} = 10.5$  Hz,  $J_{6ax,5eq} = 4.3$  Hz. (The spectrum will be discussed in

<sup>1</sup> Research supported by the Carcinogenesis Program of the National Cancer Institute and the Energy Research and Development Administration under contract with Union Carbide Corporation.

Received June 5, 1975; accepted July 30, 1975.

<sup>2</sup> The abbreviation used is: NMR, nuclear magnetic resonance.

more detail elsewhere.) Two further crops of crystals were obtained by evaporating the mother liquor and cooling. The total yield was 5 g (40%).



Calculated: C 32.81%, H 4.40%, N 15.31%, Cl 38.74%

Found: C 32.89%, H 4.33%, N 15.27%, Cl 38.69%

Unlike aliphatic nitrosamines containing halogens (e.g., nitroso-2,2'-dichlorodiethylamine), dichloronitrosopiperidine was quite stable in water, and there was no indication of decomposition after 1 week standing in dilute aqueous solution.

**3,4-Dibromonitrosopiperidine.** Nitrosotetrahydropyridine, 6.6 g, was dissolved in 50 ml methylene chloride. Approximately 10 g of bromine were added a little at a time while the solution was stirred in a bath of ice water. The reaction was vigorous and the bromine was initially decolorized. Addition of bromine was stopped when the solution remained permanently brown. The solvent and most of the bromine were removed by evaporation in a stream of nitrogen. A red liquid remained which crystallized in the freezer. Approximately 20 ml of cold methanol were added, and the almost colorless crystals were filtered and washed with cold methanol. The yield was 6.3 g (40%) of colorless solid, m.p. 86–87.5°.



Calculated: C 22.08%, H 2.96%, N 10.30%, Br 58.77%

Found: C 22.06%, H 2.90%, N 10.26%, Br 58.84%

The compound gave a single band on thin-layer chromatography (chloroform,  $R_F$  0.90). The mass spectrum showed a strong molecular ion at  $m/e$  270, with a pattern corresponding to 2 bromine atoms per molecule. The molar absorptivity was 92 at 344 nm in water. The complex NMR spectrum ( $\text{CDCl}_3$ , 100 MHz) was similar to that of the dichloro compound and supported the structural assignment: 3.83 $\delta$ , d of d, H-2-*syn-ax*,  $J_{gem} = 15$  Hz,  $J_{2ax,3eq} = 3$  Hz; 3.22 $\delta$ , d of d of d, H-6-*syn-ax*,  $J_{gem} = 14.5$  Hz,  $J_{6ax,5ax} = 10.5$  Hz,  $J_{6ax,5eq} = 4$  Hz. (The spectrum will be discussed in more detail elsewhere.) Like the dichloro compound dibromonitrosopiperidine was quite stable in dilute aqueous solution.

### Animal Treatments

The 3 nitrosopiperidines were given in drinking water to a group of 15 male Sprague-Dawley rats of the colony of this laboratory housed 3 in a plastic cage in conditions identical with those previously described (2). They were approximately 8 weeks old at the start. Solutions were prepared by dissolving the nitrosamines in 10 ml warm alcohol and diluting to 4 liters with distilled water. The concentrations were dichloronitrosopiperidine, 62 mg/liter, and dibromonitrosopiperidine, 100 mg/liter, both solutions being approximately 0.35 mM. The concentration of nitrosopiperidine was 100 mg/liter (0.88 mM). Each cage of 3 rats was given 60 ml of solution on each of 5 days/week; on the other 2 days tap

water was given. The solutions were administered until animals started to die from the treatment (the toxicity of the halogenated compounds was not known, although it was expected that they would both be more toxic than nitrosopiperidine). At this point treatment stopped and the animals either were kept until they died naturally or were killed when moribund. Dead animals were subjected to a complete necropsy, and organs with tumors or other lesions were fixed for histological examination.

### RESULTS

The 2 halogenated derivatives of nitrosopiperidine caused death of the test animals much earlier than did nitrosopiperidine, although the dosages of the derivatives were smaller. Treatment with dichloronitrosopiperidine was stopped at 15 weeks, after 2 of the animals were dead with tumors. All of the rats in this group were dead at the 24th week of the experiment (Table 1). One animal treated with dibromonitrosopiperidine died at 6 weeks and another died at 12 weeks without tumors. The 3rd animal died at 22 weeks with tumors. The treatment on this group was discontinued after 27 weeks. Fourteen animals in this group were dead at 40 weeks, and the remaining one died at 41 weeks (Table 1). In comparison, more than one-half of the animals given nitrosopiperidine were still alive after 40 weeks of continuous treatment (Table 1). Numbers of tumor-bearing animals in the different groups are shown in Table 1.

Tumors induced by the halogenated derivatives were squamous cell tumors of the esophagus and nonglandular stomach (approximately 90% in both groups) and of the trachea, bronchi, and bronchioles (approximately 33% in both groups). Approximately one-half of these tumors were invasive squamous cell carcinomas, and one-half were benign squamous papillomas. Squamous cell carcinomas of the nasal cavity were present in 2 animals treated with dichloronitrosopiperidine. In the group that received nitrosopiperidine, squamous cell tumors of the tongue, pharynx, and esophagus were present in greater than 90% of the animals, while squamous tumors of the nasal cavity occurred in approximately 36%. Olfactory carcinomas (3), which were not induced by the halogenated derivatives, were present in approximately 60% of the nitrosopiperidine group (Table 2). In addition, a hemangioendothelial sarcoma was present in the liver of one animal in the group that received nitrosopiperidine (Table 2). No tumors of the respiratory or gastrointestinal tracts occurred in 56 nitrite-treated control animals in this laboratory.

### DISCUSSION

Dichloro- and dibromonitrosopiperidines were much more potent carcinogens than nitrosopiperidine in male Sprague-Dawley rats. Both halogenated compounds killed the rats with tumors more quickly than did nitrosopiperidine after a lower dose had been administered. A total dose of 1 mmole of dibromonitrosopiperidine was more effective

Table 1  
Survival times and tumor incidence in male rats receiving 3,4-dichloronitrosopiperidine, 3,4-dibromonitrosopiperidine, and nitrosopiperidine

Compound	Period of treatment (wk)	Total dose (mmoles)	Survivors at wk							Tumor-bearing animals
			0	10	20	30	40	50	60	
3,4-Dichloronitrosopiperidine (62 mg/liter)	15	0.5	15	15	7	0 <sup>a</sup>				15
3,4-Dibromonitrosopiperidine (100 mg/liter)	27	1.0	15	14	13	10	1			13
Nitrosopiperidine (100 mg/liter)	44	3.9	14	14	14	13	8	2	0	14

<sup>a</sup> All died by 24th week.

Table 2  
Numbers and types of tumors induced in male rats by 3,4-dichloronitrosopiperidine, 3,4-dibromonitrosopiperidine, and nitrosopiperidine

Compound	Effective no. of animals	No. of animals with tumors of <sup>a</sup>					Other
		Tongue, pharynx, esophagus, non-glandular stomach	Trachea, bronchi, bronchioles	Nasal cavity			
				Squamous cell	Olfactory carcinoma		
3,4-Dichloronitrosopiperidine	15	13	6	2	0	0	
3,4-Dibromonitrosopiperidine	15	12	5	0	0	0	
Nitrosopiperidine	14	13	0	5	8	1 hemangioendothelial sarcoma of the liver	

<sup>a</sup> Some animals had tumors in more than 1 location.

than 3.9 mmoles of nitrosopiperidine, while 0.5 mmole of dichloronitrosopiperidine was still more effective. On the basis of these results, 3,4-dichloronitrosopiperidine must be considered one of the most potent carcinogens known for rats. One halogenated nitrosamine has been previously tested, 2-chloroethylmethyl nitrosamine, which Druckrey *et al.* (1) found to be less stable, much more toxic, and more carcinogenic than ethylmethyl nitrosamine.

Olfactory carcinomas of the nasal cavity were not induced by either halogenated compound, although this tumor was induced in high incidence by all of the other carcinogenic nitrosopiperidines that we have tested (3, 4).

The 2 halogenated nitrosopiperidines reported here are not notably unstable, and it cannot be supposed that their increased carcinogenic activity is due to their decomposition into some highly reactive species. A more likely explanation relates to our previous studies with cyclic nitrosamines in which methyl substitution in the positions  $\beta$  to the nitroso function greatly increases the carcinogenicity of nitrosomorpholine (5) and dinitrosopiperazine (2). We suggest that halogen substitution in that position has a similar but greater effect than methyl substitution and that the effect is an increase in the activation of hydrogen at the carbon atoms  $\alpha$  to the nitroso function. While we do not understand the nature of this presumably electronic effect, it is consistent

that the less electronegative bromine has a lesser effect than does chlorine in increasing the carcinogenicity of nitrosopiperidine.

## ACKNOWLEDGMENTS

We wish to thank Dr. G. M. Singer for obtaining the NMR spectra and Dr. F. C. Hartman for suggesting that we test halogen-substituted nitrosopiperidines.

## REFERENCES

1. Druckrey, H., Preussmann, R., Ivankovic, S., and Schmähl, D. Organotrope carcinogene Wirkungen bei 65 verschiedenen *N*-Nitroso-Verbindungen an BD-Ratten. *Z. Krebsforsch.*, 69: 103-201, 1967.
2. Lijinsky, W., and Taylor, H. W. Carcinogenicity of Methylated Dinitrosopiperazines in Rats. *Cancer Res.*, 35: 1270-1273, 1975.
3. Lijinsky, W., and Taylor, H. W. Tumorigenesis by Oxygenated Nitrosopiperidines. *J. Natl. Cancer Inst.*, in press.
4. Lijinsky, W., and Taylor, H. W. Carcinogenicity of Methylated Nitrosopiperidines. *Intern. J. Cancer*, in press.
5. Lijinsky, W., and Taylor, H. W. Increased Carcinogenicity of 2,6-Dimethylnitrosomorpholine Compared with Nitrosomorpholine in Rats. *Cancer Res.*, 35: 2123-2125, 1975.