Lung Tumors in Rats Treated with *N*-Nitrosoheptamethyleneimine and *N*-Nitrosooctamethyleneimine* (33694)

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(Introduced by Philippe Shubik)

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The conversion of an *N*-nitrosamine to an alkylating agent which alkylates nucleic acid *in vivo* was first demonstrated by Magee and Farber (1). It has been further demonstrated that neoplasia occurs in organs in which such a reaction takes place (1) and studies of some cyclic *N*-nitrosamines carried on in this laboratory confirm these observations (2). A direct correlation between nucleic acid alkylation and neoplasia, however, has never been established. As part of these investigations, the tumorigenicity of a series of cyclic nitrosamines was examined. It seemed probable that, as with the open chain nitrosamines (3), increasing molecular weight would correlate with decreasing tumorigenic potency, and the program was designed to explore this possibility.

Nitrosoazetidine (4), nitrosopyrrolidine (3), nitrosopiperidine (3) and, more recently, nitrosohexamethyleneimine (5) have all been tested and the next higher homologs, nitrosoheptamethyleneimine and nitrosooctamethyleneimine, have been synthesized and tested by administration to rats in solution in drinking water. Both compounds gave rise to a high incidence of carcinoma of the lung at the two dose levels used, as well as to carcinomas and papillomas of the esophagus. Surprisingly, and in contrast with nitrosohexamethyleneimine, neither of the two higher homologs gave rise to any tumors of the liver.

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Materials and Methods. *N*-Nitrosoheptamethyleneimine was prepared from 60 g of heptamethyleneimine (Aldrich Chemical Co., Milwaukee), which was mixed with 70 ml of water and 30 ml of concentrated HCl and cooled in ice. Approximately 20 ml of ether were added and 35 g of sodium nitrite were added in small portions with stirring. After standing overnight, slowly reaching room temperature, and addition of more ether, the brown upper layer was separated, washed with water, dried over anhydrous potassium carbonate and the ether was removed by warming in a stream of nitrogen. Crude yield: 56 g. The yellow oil was distilled *in vacuo* and the bulk of the material, distilling at 141–142° at 15 mm, was collected. Yield: 49 g. It solidified in the cold room to a pale yellow solid, mp 20–22°. The structure of the compound was confirmed by mass spectrometry and nuclear magnetic resonance spectrometry (NMR); gas chromatography on SE-30 silicone revealed only a single peak.

N-Nitrosooctamethyleneimine was prepared by adding 55 g of octamethyleneimine (Aldrich Chemical Co., Milwaukee) to 40 ml of concentrated HCl in 100 ml of water. The solution was cooled in ice and 40 g of sodium nitrite was added slowly with stirring. After standing overnight the brown oil was separated off and the aqueous layer was extracted with ether. The ether extract was combined with the brown oil, washed with water, dried over anhydrous potassium carbonate and the ether was evaporated, leaving 55 g of crude nitrosamine. This was distilled *in vacuo* and the bulk of the material, distilling at 130–132° (12 mm), was collected as a pale yellow oil, which solidified on standing to a yellow solid, mp 56–57°. Yield: 42 g. The chemical structure was confirmed by mass spectrometry and NMR; a single peak

TABLE I. Survival of Treated Rats.

Compound and sex	Concentration (mg/liter)	No. of animals surviving at week								
		0	10	20	30	40	50	60	70	80
Nitrosoheptamethyleneimine										
♂	200	9	8	6	1	0				
♀	200	11	10	2	0					
♂	50	10	10	10	0					
♀	50	10	10	8	0					
Nitrosooctamethyleneimine										
♂	200	10	10	10	10	3	0			
♀	200	10	10	10	10	5	1	0		
♂	50	10	10	10	10	9	4	3	2	0
♀	50	10	10	10	9	4	0			

was detected in a gas chromatogram of the compound on SE-30 silicone.

The acute toxicity of each nitrosamine was determined by *per os* administration of olive oil solutions by gavage into groups of 4 male rats (doses ranged from 100 to 1600 mg/kg), and observing the number of deaths within 48 hr. Occasionally an animal died with convulsions, as has been observed with other cyclic nitrosamines (2).

Two concentrations, 50 mg/liter and 200 mg/liter, of either of the nitrosamines were given to groups of 10 male and 10 female MRC rats, bred randomly in this laboratory. The nitrosamines were weighed, dissolved in a few milliliters of ethanol and poured into the appropriate volume of water; this ensured complete solution of the compounds. Each cage of 5 animals was given 100 ml of solution each night, 5 nights a week, contained in a brown bottle (this minimized photodecomposition of nitrosamine). During the day tap water was substituted for the solution.

Feeding of the nitrosamine solutions continued until one or more animals in a group became ill or died, whereupon treatment ceased. Complete autopsy was performed on all animals at death (some were killed when moribund) and tissues were fixed for histological examination.

Results. In the acute toxicity tests with both nitrosamines, the animals that died showed severe centrilobular necrosis in the liver, with much congestion, and a greater or lesser degree of congestion in the lungs. The LD_{50} as determined by the method of Weil (6) was 283 mg/kg for nitrosoheptamethyleneimine and 566 mg/kg for nitrosooctamethyleneimine.

The survival rates of the treated animals are given in Table I. Only one animal treated with nitrosoheptamethyleneimine survived beyond 30 weeks after treatment began. Survival was much better in the animals treated with nitrosooctamethyleneimine, although few survived week 50 of the experiment.

The results of the long term feeding tests of both nitrosamines are given in Table II. The tumors commonly observed were papillomas and squamous carcinomas of the esophagus, and papillomas of the associated tongue and soft palate, with a few tracheal tumors. The other tumor commonly observed was squamous carcinoma of the lung. In many instances the tumors seem to originate from large cystic lesions which resemble epidermoid cysts (Figs. 1 and 2). The possibility that tumors developed from bronchiectasis was explored and in at least one case there was evidence of such an origin (Fig. 3). Tumors were located mainly in the middle or external areas of the lungs and were characterized either by nests of squamous cells infiltrating the parenchyma from the wall of the cysts, or as solid nodules (Fig. 4). Foci of squamous metaplasia were quite common, many of which were well differentiated, showing alveolar extension.

The first esophageal tumor was a papilloma

TABLE II. Tumors in Rats Treated with Nitrosoheptamethyleneimine and Nitrosooctamethyleneimine.

Compound	Concentration (mg/iter)	Duration (weeks)	Initial no. of animals and sex	No. of tumor-bearing animals	No. of animals with tumors of				
					Lung	Esophagus Pap	Esophagus Ca	Tongue and soft palate	Other
Nitrosoheptamethyleneimine	200	16	9 ♂	5	4	5	0	0	0
			11 ♀	10	7	6	1	0	2 pap trachea
	50	26	10 ♂	7	4	4	1	4	1 pap trachea
			10 ♀	7	4	4	1	3	3 pap trachea
Nitrosooctamethlyeneimine	200	34	10 ♂	5	0	4	1	3	1 ca nose
			10 ♀	9	5	8	0	2	1 ca trachea
	50	36	10 ♂	9	3	6	1	1	0
			10 ♀	7	3	6	1	3	0

seen in a female that died in the seventh week of treatment with 200 mg/liter of nitrosoheptamethyleneimine; there was extensive squamous metaplasia of the bronchial epithelium in the lungs of this animal. The first lung tumor (a squamous carcinoma) was observed in a female of the same group that died 16 weeks after treatment began. In the group treated with the lower dose of nitrosoheptamethyleneimine the first lung tumor and the first esophageal tumor were both observed in week 22 of the experiment.

In the group treated with nitrosooctamethyleneimine at 200 mg/liter no lung tumors were observed in the males, although they were found in half of the females. The first esophageal tumor was seen in a male that died at 34 weeks and the first lung tumor at 37 weeks. The corresponding findings in the group fed the lower dose were the first lung tumor (in a female) at 42 weeks and the first esophageal tumor (in a male) at 33 weeks.

Apart from a single squamous carcinoma in the nose of a male fed nitrosooctamethyleneimine, no other tumors were observed. Although the livers of the animals that died early were sometimes abnormal and congested, in no case was a liver tumor seen. Many animals, treated with either nitrosamine, bore more than one type of tumor, but in some animals either lung carcinomas or esophageal tumors occurred alone.

Discussion. Nitrosoheptamethyleneimine is a potent lung carcinogen in MRC rats, and also produces a high incidence of esophageal tumors, but no liver tumors. The cyclic nitrosamine with only one carbon atom less, nitrosohexamethyleneimine, also gives rise to many esophageal tumors, but is a potent liver carcinogen and has no tumorigenic effect on the lung (5). Nitrosooctamethyleneimine produces results very similar to those of nitrosoheptamethyleneimine, but appears to be somewhat less potent than the latter. Whereas the cyclic compound nitrosohexamethyleneimine is very similar to its aliphatic analog nitrosodi-*n*-propylamine (3) in the tumor pattern that it elicits, nitrosooctamethyleneimine differs sharply from its open chain analog nitrosodi-*n*-butylamine (which gives rise to tumors of liver, esophagus, and urinary bladder).

It is possible, but unlikely, that the animals treated with nitrosohepta- and nitrosooctamethyleneimine died of other causes before liver tumors had time to develop. Some of the animals survived to 50 weeks or more without detectable lesions in the liver. That single high doses of the two nitrosamines cause rapid death of rats with extensive liver necrosis, and yet chronic treatment with the same two compounds fails to produce liver tumors or other liver lesions might indicate that there is no connection between the acute toxic effect of the nitrosamines and their tumorigenic action.

Tumors of the esophagus are commonly induced by a great variety of nitrosamines,

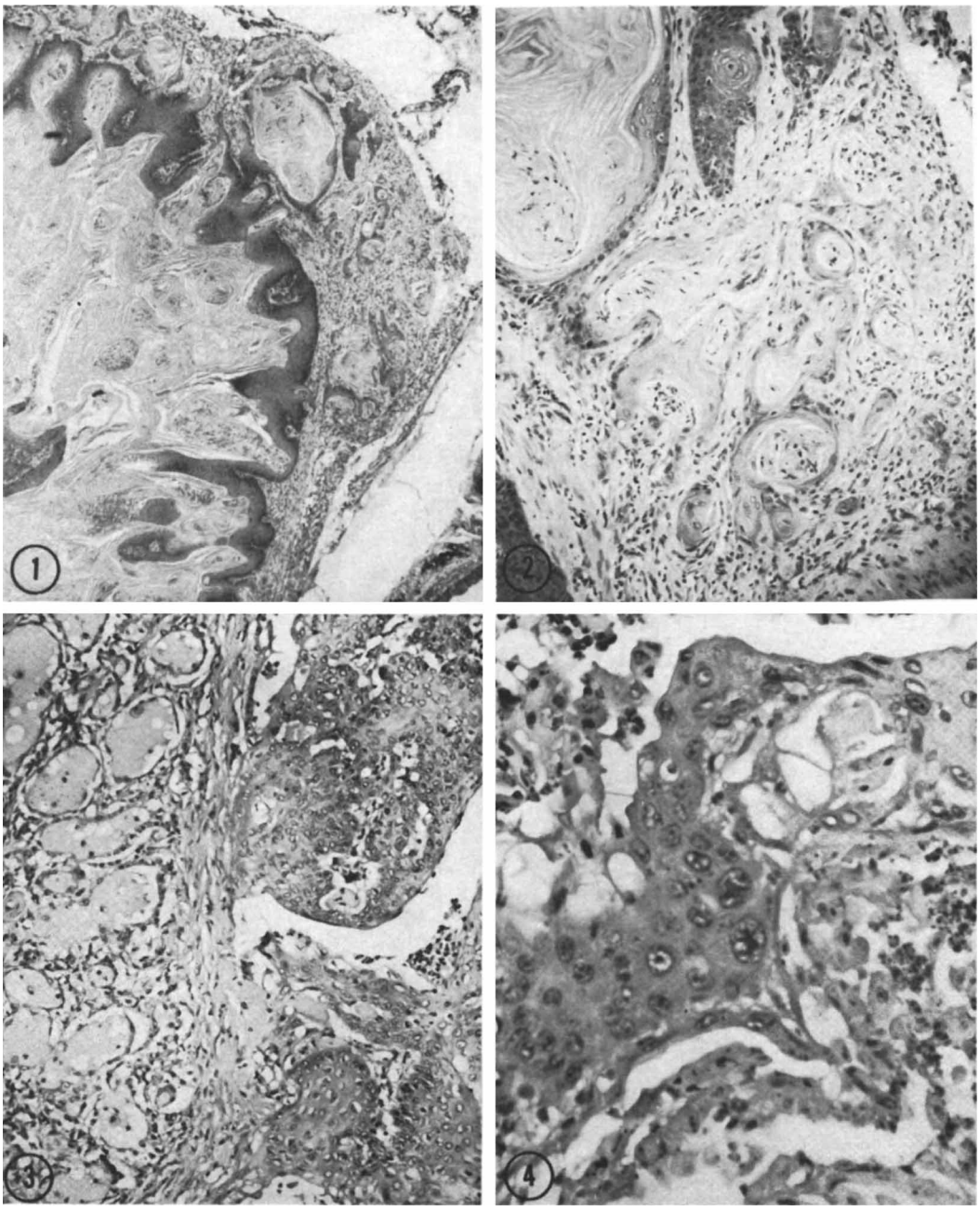

FIG. 1. Epidermoid cyst-like structure from which squamous carcinoma extends into adjacent tissues; ×35.

FIG. 2. Islands of squamous carcinoma invading connective tissue, (detail of 1); ×120.

FIG. 3. Squamous carcinoma attached to bronchial wall; ×130.

FIG. 4. Nuclear pleomorphism in squamous carcinoma cells; ×300.

including aliphatic, aromatic (nitrosomethylaniline) and cyclic (nitrosopiperidine, nitrosohexamethyleneimine, -heptamethyleneimine, and -octamethyleneimine) nitrosamines. On the other hand, while nitrosopiperidine and nitrosohexamethyleneimine give rise to liver tumors and no lung tumors, the two higher homologs, nitrosohepta- and nitrosooctamethyleneimine, give rise to lung tumors but not to liver tumors. The tumorigenic effect of these compounds is not explained by the enzymatic conversion of these chemically very similar compounds to an alkylating agent, which then alkylates DNA in the organ in which the alkylating intermediate is produced. The relation between alternative biochemical interactions and carcinogenesis is now being investigated.

Summary. Two new cyclic nitrosamines, nitrosoheptamethyleneimine and nitrosooctamethyleneimine, have been prepared and tested biologically by feeding to rats in drinking water, at two concentrations, 50 and 200 mg/liter. Both nitrosamines produced a high incidence of squamous carcinomas in the lung and of squamous tumors in the esophagus. The lower dose of the compounds did not seem less effective than the 200 mg/liter concentration. Nitrosooctamethyleneimine appeared to be a less potent tumorigen than nitrosoheptamethyleneimine, the latter giving rise to lung tumors after less than 16 weeks treatment, and to esophageal tumors after 7 weeks. Although large single doses of both nitrosamines caused rapid death of the animals with severe necrosis of the liver, there were no tumors or other lesions of the liver in animals treated chronically.

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