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# BIOCHEMICAL STUDIES OF SIX NITROGEN-CONTAINING HETEROCYCLES IN RAT TISSUES*

KIRK T. KITCHIN,†‡ JANICE L. BROWN† and WILLIAM LIJINSKY§

† Environmental Toxicology Division, Health Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, NC 27711; and § BRI-Basic Research Program, NCI-Frederick Cancer Research Facility, Frederick, MD 21701, U.S.A.

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**Abstract**—Female rats were dosed orally with one-fifth the $LD_{50}$ of either 1-nitrosopiperidine (a carcinogen), cyclohexylamine, piperidine, 4-carboxy-1-nitrosopiperidine, 4-cyclohexyl-1-nitrosopiperidine or 2,6-dimethyl-1-nitrosopiperidine at 21 and 4 hr before they were killed. The five noncarcinogenic compounds had no effects on any experimental variables examined [hepatic DNA damage, ornithine decarboxylase (ODC) activity, serum alanine aminotransferase (SGPT) activity, cytochrome P-450 and glutathione content]. After administration of 40 mg/kg of 1-nitrosopiperidine, marked hepatic DNA damage and a 3- to 7-fold increase in the activity of hepatic ODC were observed. 1-Nitrosopiperidine (120 mg/kg, 3/5 $LD_{50}$) caused DNA damage in rat liver and esophagus but not in leukocytes. This higher dose of 1-nitrosopiperidine also increased hepatic ornithine decarboxylase activity by 9-fold. Thus, this hepatic biochemical assay system correctly identified the one carcinogen and the five noncarcinogens in this series of six nitrogen-containing heterocycles.

Single cell-based systems for predicting carcinogenicity were enthusiastically advanced in the 1970s, but are now recognized to have several problems and limitations [1]. Use of whole animals to determine a compound's potential to cause cancer (or not to cause cancer) offers several advantages over cell-based systems. For example, some pharmacokinetic (absorption, distribution and excretion) and pharmacodynamic (metabolism, endocrine effects and organ–organ interactions) factors are absent from single cell-based systems. In developing carcinogenesis assay systems for risk assessment, it is important to (a) correctly identify noncarcinogens as well as carcinogens [1], and (b) distinguish between carcinogenic initiators and tumor-promotors. Linear models of risk assessment may be appropriate for initiators, while threshold models of risk assessment have been proposed for promoters [2, 3].

The alkaline elution technique to determine DNA damage is useful not only *in vitro* but also *in vivo* as a marker for carcinogenic initiation. In a validation study of ninety-one compounds, this technique correctly identified 92% of the carcinogens tested and 85% of the noncarcinogens [4]. After chemically induced necrosis, non-specific DNA damage may occur by activation of lysosomal endonucleases [4]. Promoters of carcinogenesis have fewer biochemical or morphological markers than does carcinogenic initiation. Most promoters induce ornithine decarboxylase *in vivo* [5–10]. Induction of this marker enzyme has been considered necessary though not sufficient to cause promotion.

Promoters of hepatocarcinogenesis are usually hepatic cytochrome P-450 inducers. Further, depletion of hepatic glutathione in the rat is known to increase susceptibility to covalent binding of reactive intermediates to cellular macromolecules, cellular necrosis [11] and carcinogenesis [12]. Therefore, SGPT‖, hepatic cytochrome P-450 and reduced glutathione levels were also measured in this study.

This biochemical assay system has differentiated between carcinogens and noncarcinogens, primarily of the halogenated hydrocarbon class [13–15]. The overall purpose of this study was to confirm and extend this observation to a second chemical class. Liver and esophagus are target tissues for carcinogenesis by 1-nitrosopiperidine, whereas blood is not [16, 17]. Five noncarcinogenic nitrogen-containing heterocycles [cyclohexylamine, piperidine, 4-carboxylnitrosopiperidine, 4-cyclohexyl-1-nitrosopiperidine and 2,6-dimethyl-1-nitrosopiperidine (Fig. 1)] were selected for study. Their biochemical effects were determined in liver, esophagus and blood and compared to those of 1-nitrosopiperidine.

## METHODS

4-Carboxy-1-nitrosopiperidine, 4-cyclohexyl-1-nitrosopiperidine and 2,6-dimethyl-1-nitrosopiperidine were from the NCI-Frederick Cancer Research Facility. Piperidine (Aldrich Chemical Co., Milwaukee, WI; 98%), 1-nitrosopiperidine (Sigma Chemical Co., St Louis, MO) and cyclohexylamine (Aldrich Chemical Co.; 97%) were diluted with

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‡ Send correspondence to Dr Kitchin (MD-74) at the above address.

‖ Abbreviations: SGPT, serum alanine aminotransferase; ODC, ornithine decarboxylase; and HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate.

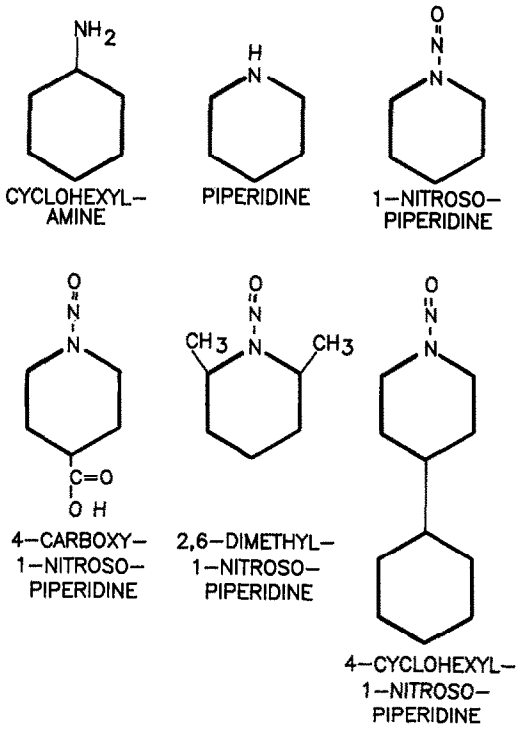


Fig. 1. Structures of the six nitrogen-containing heterocycles.

0.9% saline solution. The solutions containing piperidine and cyclohexylamine were adjusted to pH 7.0 before use.

Ninety-day-old female Sprague–Dawley rats (CD strain) were obtained from Charles River Laboratories (Wilmington, MA) and housed three per cage. Rats were dosed orally with one-fifth the published $LD_{50}$ (NIOSH, Registry of Toxic Effects of Chemical Substances) of these compounds in two doses 21 and 4 hr before they were killed. These times were chosen because changes in ODC activity, glutathione content and DNA damage occur within 4 hr of chemical treatment [4, 6, 18], while changes in hepatic cytochrome P-450 and SGPT activity are observed within 21 hr after chemical exposure [19, 20]. The doses used were 142 mg/kg of cyclohexylamine, 80 mg/kg of piperidine, 56 mg/kg of 4-carboxy-1-nitrosopiperidine, 49 mg/kg of 2,6-dimethyl-1-nitrosopiperidine, 66 mg/kg of 4-cyclohexyl-1-nitrosopiperidine (the doses of the last three compounds are equimolar to 40 mg/kg of 1-nitrosopiperidine) and either 40 or 120 mg/kg of 1-nitrosopiperidine (3/5 $LD_{50}$). Control rats received saline or water alone.

Leukocytes were separated from erythrocytes by layering 4 ml of a 50% solution of rat blood in physiological saline on top of 3 ml of LSM Lymphocyte Separation Medium (Litton Bionetics Inc., Kensington, MD) and centrifuging for 15 min at 500 *g* at room temperature.

To prepare subcellular fractions, liver tissue (1.5 g) was homogenized in 6 ml of ice-cold, pH 7.5, buffer containing NaCl (136 mM), KCl, (5.4 mM), HEPES (20 mM), dithioerythritol (5 mM), EDTA (4 mM) and pyridoxal-5′-phosphate (0.08 mM). Liver samples were homogenized with six strokes of a size C Potter–Elvehjem homogenizer (clearance 0.15 to 0.23 mm) operated at 300 rpm. After a 10-min settling period at 4°, 75 μl of the whole liver homogenate was used for alkaline elution.

Esophagus tissue, prepared with a Potter–Elvehjem homogenizer or by pushing esophagus tissue through a stainless steel screen [21], displayed high degrees of DNA damage induced by these mechanical procedures. Therefore, a new *in situ* method of tissue digestion and lysis (on the filter) was developed to minimize these artifacts. About one-seventh of a rat esophagus was cut into as small pieces as possible with hand scissors and directly placed into 5 ml of physiological buffered saline above the alkaline elution filter holder. Then, two 5-ml solutions of physiological buffered saline were pumped through the filter (flow rate of 0.25 ml/min). To begin tissue digestion and lysis, a 5-ml solution containing 2% sodium dodecyl sulfate (SDS) and 0.5 mg/ml of proteinase K at pH 9.7 was then pumped through the filter (flow rate of 0.1 ml/min). Next, 10 ml of 0.02 M EDTA containing 0.06% Sarkosyl at pH 10 was pumped through the filter (flow rate of 0.125 ml/min). A final 5 ml of 2% SDS containing proteinase K (pH 9.7) was pumped partly through the filter to complete this special procedure for *in situ* esophagus lysis.

An alkaline elution procedure which minimizes protein adsorption to the filter was employed [22]. Three modifications to the Stout and Becker [23] procedure were utilized. Sarkosyl (0.06%) was added to the first EDTA wash. To increase the buffering capacity and better stabilize the pH, 5 mM phosphate buffer ($pK_{a_3} = 12.32$ for phosphoric acid) was added to the pH 12.10 eluting solution. In methyl methanesulfonate treated cells, a 48-hr delay in the SDS-lysis step of alkaline elution increases the sensitivity of detecting DNA damage 10-fold [24]. In our research laboratory, the 48-hr delay step has greatly increased the sensitivity of detecting rat liver DNA damage caused by either 1,2-dibromoethane [14] or dimethylnitrosamine. The increased sensitivity of the DNA damage assay using the 48-hr delay has been interpreted as being due to either changes in DNA unfolding [24] or as the detection of additional DNA alkaline-labile sites and single strand breaks [25]. The more sensitive 48-hr delay in the SDS-lysis step procedure was used for alkaline elution studies. Alkaline elution data are expressed as the fraction of DNA eluted from the filter during the 14-hr period (i.e. 1.00 = 100% DNA eluted).

Assays for ODC activity [26], glutathione content [27, 28], cytochrome P-450 content [29] and SGPT activity [20] were performed by standard methods. For these four assays the high speed supernatant, whole homogenate, microsomes and serum were utilized. The postmitochondrial supernatant (20,000 *g*, 20 min) was spun at 193,000 *g* for 40 min to produce the microsomal fraction and the high speed supernatant. Since the subcellular fractionation method used in this study gives a low yield of microsomal protein per gram liver, the cytochrome P-450 contents per gram liver reported here are lower

than many other published values.

Statistical analysis of the data was done with analysis of variance. Statistically significant differences found by a Tukey's test were then further evaluated with a Student's *t*-test.

## RESULTS

Prior to sacrifice no animals died and no obvious clinical signs of toxicity were visually noted. 1-Nitrosopiperidine at 40 mg/kg caused a 7-fold increase in hepatic ODC activity and substantial DNA damage in liver but not in leukocytes (Table 1). Rat hepatic cytochrome P-450 levels, hepatic glutathione and SGPT values were not altered by 1-nitrosopiperidine. Cyclohexylamine (142 mg/kg) and piperidine (80 mg/kg) did not alter any of the six biochemical parameters (Table 1).

A closer noncarcinogenic analogue of 1-nitrosopiperidine (4-carboxy-1-nitrosopiperidine) was tested subsequently, but did not cause statistically significant effects in any of the seven variables (Table 2). A higher dose of 1-nitrosopiperidine (120 mg/kg) was concurrently used in an attempt to demonstrate DNA damage in leukocytes as well as liver. In rats given the carcinogen 1-nitrosopiperidine, DNA was damaged significantly in liver (240% increase in the fraction of DNA eluted from the filter) and esophagus (58% increase) but not in leukocytes (Table 2). Hepatic ODC activity was increased 9-fold.

Two additional noncarcinogenic [17] analogues of 1-nitrosopiperidine (the 4-cyclohexyl- and 2,6-dimethyl-derivatives) were given to rats. Neither compound caused any alterations in the liver or esophagus (Table 3). In contrast, at equimolar doses the carcinogen 1-nitrosopiperidine caused hepatic DNA damage and increased hepatic ODC activity.

## DISCUSSION

At doses of one-fifth the $LD_{50}$, none of the five noncarcinogens showed any biochemical effects whatsoever. Administration of 1-nitrosopiperidine caused high, intermediate and no detectable DNA damage in liver, esophagus and leukocytes (which are frequently proposed as a biological monitor of chemical exposure) respectively. Compared to liver, only limited DNA damage occurred in esophagus (a tissue in which tumors arise) at a dose of 120 mg/kg. The limited amount of DNA damage observed in esophagus may be due to a lesser blood flow or metabolic activation than is present in liver. In rat and human hepatocytes, 1-nitrosopiperidine (1.0 to 3.2 mM) caused dose-related responses in both DNA damage (as measured by alkaline elution) and unscheduled DNA synthesis [30]. 1-Nitrosopiperidine also caused hepatic ODC induction and thus is putatively classified by this biochemical assay system as both an initiator and a promoter. 1-Nitrosopiperidine is an initiator and at minimum a complete carcinogen *in vivo* [16]. None of the six compounds examined here has been studied extensively for tumor promoting activity *in vivo*. At the doses used in this study hepatic cytotoxicity, as monitored by SGPT activities, was not observed.

No published reports of alkaline elution data from

Table 1. Effects of three nitrogen-containing heterocycles on six biochemical variables

| Treatment | Blood alkaline elution (fraction of DNA eluted) | Liver alkaline elution (fraction of DNA eluted) | Liver ornithine decarboxylase (nmol $CO_2$/g liver/hr) | Liver glutathione (μmol/g) | Liver cytochrome P-450 (nmol/g) | Serum alanine aminotransferase (I.U./l.) |
|---|---|---|---|---|---|---|
| Saline | 0.070 ± 0.021 (8) | 0.100 ± 0.010 (8) | 1.44 ± 0.32 (8) | 3.94 ± 0.39 (8) | 3.52 ± 0.36 (8) | 11.1 ± 0.8 (8) |
| Cyclohexylamine (142 mg/kg) | 0.105 ± 0.055 (8) | 0.110 ± 0.008 (8) | 1.59 ± 0.21 (8) | 3.77 ± 0.27 (8) | 4.11 ± 0.32 (8) | 12.2 ± 1.4 (8) |
| Piperidine (80 mg/kg) | 0.049 ± 0.011 (7) | 0.101 ± 0.009 (7) | 3.13 ± 0.82 (7) | 3.34 ± 0.33 (7) | 4.13 ± 0.33 (7) | 12.3 ± 2.1 (6) |
| 1-Nitrosopiperidine (40 mg/kg) | 0.079 ± 0.021 (8) | 0.320 ± 0.015* (8) | 10.4 ± 1.56* (8) | 3.42 ± 0.29 (8) | 2.46 ± 0.26 (8) | 10.9 ± 1.1 (8) |

Adult female rats were given orally the indicated doses of the three compounds 21 and 4 hr before being killed. Values are the means ± SE for the number of rats indicated in parentheses.

* $P < 0.001$.

Table 2. Effects of 1-nitrosopiperidine and 4-carboxy-1-nitrosopiperidine on seven biochemical variables

| Treatment | Esophagus alkaline elution (fraction of DNA eluted) | Blood alkaline elution (fraction of DNA eluted) | Liver alkaline elution (fraction of DNA eluted) | Liver ornithine decarboxylase (nmol $CO_2$/g liver/hr) | Liver glutathione ($\mu$mol/g) | Liver cytochrome P-450 (nmol/g) | Serum alanine aminotransferase (I.U./l.) |
|---|---|---|---|---|---|---|---|
| Saline | 0.191 ± 0.017 (17) | 0.067 ± 0.021 (10) | 0.235 ± 0.04 (6) | 1.50 ± 0.74 (7) | 4.02 ± 0.60 (7) | 3.19 ± 0.30 (5) | 15.8 ± 1.7 (7) |
| 4-Carboxy-1-nitrosopiperidine (56 mg/kg) | 0.144 ± 0.015 (12) | 0.040 ± 0.013 (12) | 0.211 ± 0.017 (8) | 1.77 ± 0.50 (8) | 3.98 ± 0.45 (8) | 2.36 ± 0.16 (6) | 16.3 ± 1.5 (8) |
| 1-Nitrosopiperidine (120 mg/kg) | 0.302 ± 0.029* (19) | 0.117 ± 0.038 (11) | 0.798 ± 0.038* (8) | 15.1 ± 2.27* (8) | 3.97 ± 0.46 (8) | 2.01 ± 0.38† (6) | 13.9 ± 1.7 (8) |

Adult female rats were given orally the indicated doses of 1-nitrosopiperidine or 4-carboxy-1-nitrosopiperidine, 21 and 4 hours before being killed. Values are the means ± SE for the number of rats indicated in parentheses.
*† Significantly different vs saline: * $P < 0.01$, and † $P < 0.05$.

Table 3. Effects of three 1-nitrosopiperidines on six biochemical variables

| Treatment | Esophagus alkaline elution (fraction of DNA eluted) | Liver alkaline elution (fraction of DNA eluted) | Liver ornithine decarboxylase (nmol $CO_2$/g liver/hr) | Liver glutathione ($\mu$mol/g) | Liver cytochrome P-450 (nmol/g) | Serum alanine aminotransferase (I.U./l.) |
|---|---|---|---|---|---|---|
| Water | 0.159 ± 0.026 (10) | 0.111 ± 0.017 (9) | 0.91 ± 0.33 (9) | 4.68 ± 0.31 (9) | 2.38 ± 0.23 (9) | 13.6 ± 0.5 (9) |
| 1-Nitrosopiperidine (40 mg/kg) | 0.258 ± 0.042 (8) | 0.242 ± 0.031* (6) | 3.22 ± 0.40* (5) | 4.38 ± 0.20 (6) | 1.64 ± 0.19 (6) | 17.6 ± 2.4 (6) |
| 2,6-Dimethyl-1-nitrosopiperidine (49 mg/kg) | 0.194 ± 0.075 (4) | 0.114 ± 0.012 (9) | 1.13 ± 0.44 (9) | 4.50 ± 0.25 (9) | 2.88 ± 0.21 (9) | 17.9 ± 1.6 (9) |
| 4-Cyclohexyl-1-nitrosopiperidine (66 mg/kg) | 0.193 ± 0.088 (5) | 0.117 ± 0.011 (5) | 0.65 ± 0.21 (5) | 4.55 ± 0.45 (5) | 2.27 ± 0.23 (5) | 16.5 ± 1.7 (5) |

Adult female rats were given orally equimolar doses of the nitrogen-containing heterocycles 21 and 4 hr before being killed. The vehicle was water. Values are means ± SE for the number of rats indicated in parentheses.
* $P < 0.01$.

esophagus were found (e.g. Ref. 31). Conventional published methods for determination of DNA damage by alkaline elution could not be used for esophagus tissue. In this study we report an *in situ* digestion and lysis method which prepares rat esophagus tissue for subsequent alkaline elution procedures. This new experimental procedure may be useful in other tissues where conventional tissue preparation methods for alkaline elution do not work adequately.

Substituents in 1-nitrosopiperidine affect both the carcinogenic potency and the target organs in rats. At the 4-position, methyl substitution does not alter potency, but 4-chloro-nitrosopiperidine is a considerably more potent carcinogen than 1-nitrosopiperidine and the target organ remains the esophagus. On the other hand, 4-hydroxy-1-nitrosopiperidine and nitroso-4-piperidine induce liver tumors as well as tumors of the esophagus, but their carcinogenic potency is similar to that of 1-nitrosopiperidine [17]. 4-Phenyl-1-nitrosopiperidine and 4-*t*-butyl-1-nitrosopiperidine are less potent than 1-nitrosopiperidine, but the phenyl derivative induced mainly liver tumors. 4-Cyclohexyl-1-nitrosopiperidine and 4-carboxy-1-nitrosopiperidine (nitrosoisonipecotic acid) were not carcinogenic. 2-Methyl-1-nitrosopiperidine was a less potent carcinogen than 1-nitrosopiperidine and 2,6-dimethyl-1-nitrosopiperidine was not carcinogenic, presumably because of steric hindrance caused by the methyl groups [17]. Explanation of the different effects of substituents at the 4-position on the carcinogenicity of 1-nitrosopiperidine is lacking.

While 1-nitrosopiperidine was mutagenic in the Ames Salmonella assay, 4-cyclohexyl-1-nitrosopiperidine, 2,6-dimethyl-1-nitrosopiperidine and 4-carboxy-1-nitrosopiperidine (nitrosoisonipecotic acid) were not significantly mutagenic in the Ames assay. Experimentally, 4-carboxy-1-nitrosopiperidine does not cause DNA damage in rat liver, esophagus or blood, ODC induction in rat liver (this study), or tumors in rats [17]. 2,6-Dimethyl-1-nitrosopiperidine does not cause DNA damage in rat liver or esophagus, ODC induction in rat liver (this study) or tumors in rats [17].

Among the six nitrogen-containing heterocycles studied, this biochemical assay system identified the one carcinogenic molecule and the five non-carcinogens as well. Piperidine [4], cyclohexylamine [32], 4-carboxy-1-nitrosopiperidine [17], 4-cyclohexyl-1-nitrosopiperidine [17] and 2,6-dimethyl-1-nitrosopiperidine [17] are not carcinogenic *in vivo* nor were they positive in this assay system. This report confirms and extends prior similar experimental results with halogenated aliphatic compounds [14, 15], halogenated aromatic compounds [13, 33] and aromatic promoters of hepatocarcinogenesis [9].

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