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Comparison of the Acute Toxicity of N-nitrosocimetidine with Three Structurally Related Carcinogens in the Rat*

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ABSTRACT

The acute toxicity of N-nitrosocimetidine, the nitrosated derivative of the histamine H_2-receptor blocking agent cimetidine, was compared with the toxicities of three structurally related nitroso compounds known to be potent carcinogens, namely N-methyl-N'-nitro-N-nitrosoguanidine, N-methyl-N-nitrosourea, and N-methyl-N-nitrosourethane, and also with the parent drug cimetidine. The acute toxicity of each compound was investigated in 6-week-old female Fischer-344 rats by estimating the median lethal doses via three different routes of administration, and by assessing the sequence of histopathological alterations induced. According to median lethalities, all three known carcinogens were substantially more toxic than nitrosocimetidine whether administered by the intravenous, intraperitoneal, or oral routes. The widest margin of difference was represented by orally administered N-methyl-N-nitrosourea, the median lethal dose being 59 times greater than oral N-nitrosocimetidine. By this method, the acute toxicities of N-nitrosocimetidine and the parent drug cimetidine were virtually identical for each of the three routes of administration. Sequential histological assessment indicated that the three known carcinogens induced specific pathological alterations mainly in organs which were also known to be targets for their carcinogenic activity. In contrast, no tissue lesions of a specific nature were associated with N-nitrosocimetidine or cimetidine in this study. The comparable results with N-nitrosocimetidine and the parent drug provide biological support for previously obtained biochemical data which suggested that N-nitrosocimetidine is rapidly denitrosated to cimetidine in the rat.

INTRODUCTION

Cimetidine (Tagamet), a histamine H2-receptor blocking agent, is widely prescribed for inhibiting acid secretion in patients with gastric or duodenal ulcers (5). However, several clinical reports of an anecdotal association between human gastric carcinoma and cimetidine treatment have led to some concern that this drug might be involved in the causation of cancer (8, 13, 29, 37). Cimetidine is nitrosated *in vitro* in the acid pH range (3) and generates a mutagen when mixed with nitrite in isolated human gastric juice (6). The possibility therefore exists that nitrosation of cimetidine could occur in man *in vivo* with the formation of a potentially carcinogenic end-product.

The chemical structure of nitrosated cimetidine, N-nitrosocimetidine, is identical in part with that of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), and analogous to N-methyl-N-nitrosourea (MNU) and N-methyl-N-nitrosourethane (MNUt). All three latter compounds are known to be strong carcinogens which decompose *in vitro* and *in vivo* to yield the same methylating intermediate. Nitrosocimetidine has been tested and proved positive in a number of short-term assays which demonstrate genotoxicity, and in this respect it parallels the activities of the three structurally related carcinogens. Thus, nitrosocimetidine is capable of inducing mutation in the Ames *Salmonella* assay (15, 28), sister chromatid exchange and chromosome aberrations in the chinese hamster ovary cell culture system (2, 15), mutation and transformation of BHK cells (4), DNA strand breakage in cultured mouse epithelial cells (33), alkylation of DNA both *in vitro* and *in vivo* (10, 18, 20), and DNA-repair replication in human lymphocytes (14).

These results, indicative of DNA-reactivity, appear to be a valid basis for concern regarding the

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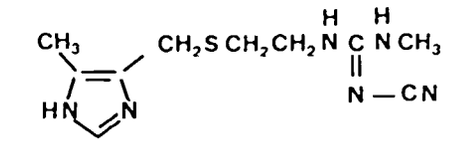
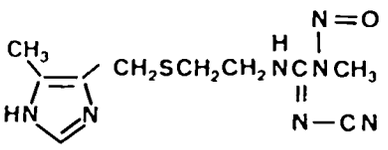
acute and chronic toxicities of this potentially endogenous nitroso compound in human beings. On the other hand, bioassay has suggested that nitrosocimetidine is noncarcinogenic for rats (12, 23, 25). The discrepancy between the positive *in vitro* tests and the negative long-term effects of nitrosocimetidine *in vivo* might be explained by a recent study which indicates that this compound is rapidly converted back to cimetidine in the presence of rat blood (18). If this denitrosation process occurs in the live animal it could be anticipated that the acute toxicity of nitrosocimetidine would parallel that of cimetidine and not the severity of the three carcinogenic agents. Consequently we have assessed the median lethality and acute histopathological response of nitrosocimetidine by various routes of administration, comparing the effects directly with those of MNNG, MNU, MNUt, and cimetidine. In addition, the significance of varying the route of administration in terms of the distribution of early pathological lesions induced by each of the three carcinogens has not been investigated previously. A subsidiary goal of this study therefore was to determine if a correlation existed between the organ sensitivity to acute injury and the site-specificity for the known carcinogenic actions of MNNG, MNU, and MNUt.



Cimetidine



Nitrosocimetidine

FIG. 1.—Chemical structure of nitrosocimetidine and cimetidine.

MATERIALS AND METHODS

Animals. Female Fischer-344 rats were obtained from Charles River, Kingston, N.Y., at 5 weeks of age, and were maintained on a basal diet of Wayne Lab Blox (Allied Mills, Inc., Chicago, Ill.) and tap water for 1 week. Rats receiving the test chemicals by the oral route, but not the other animals, were fasted for 16 hours prior to treatment.

Chemicals. Cimetidine was generously supplied by Smith, Kline, and French Laboratories, Philadelphia, Pa. MNNG was purchased from Sigma Chemical Co., Saint Louis, Mo. MNU was synthesized as previously described (17) and purified following the procedure of Swann and Magee (36). The nitrite salt of nitrosocimetidine (Fig. 1) was generated by nitrosating cimetidine (1, 20). All of these compounds prepared in the laboratory demonstrated melting points identical to those stated in the literature (1, 3, 36), and absorption maxima in the 400-nm range characteristic of the NNO chromophore (3, 26). Solutions of nitrosocimetidine, MNNG, and MNU were prepared from weighed quantities of dry crystals. MNUt was synthesized by the nitrosation of ethyl methylcarbamate (Eastman Organic Chemicals, Rochester, N.Y.) following the procedure outlined by Lijinsky and Taylor (24). The concentration of MNUt in the resulting oil was estimated from volumetrically prepared aqueous solutions utilizing the extinction coefficient noted by Mirvish (26). As described below, stock solutions of the nitroso compounds and of cimetidine were prepared immediately before intraperitoneal and intravenous dosing by first dissolving the agents in dimethyl sulfoxide (DMSO). The compounds were instantly soluble and stable in this solvent. DMSO solutions were diluted with physiological saline for injection. In the light of our results it is important to emphasize that, in the absence of thiol compounds, nitrosocimetidine is stable in aqueous solution; the intact compound has a half-life of greater than 100 hours at room temperature (20). The final percentages of DMSO in the various solutions were dependent on the solubility of the chemicals, as ascertained in preliminary experiments. For oral administration, chemicals were ground to a fine powder in a glass homogenizer, suspended in sesame oil, and given by gastric intubation.

Determination of Median Lethality Values. The median lethal dose for each compound was calculated by the Reed-Muench method (16) with estimation of the 95% confidence limits according to the method of Pizzi (16). This measure of acute toxicity was determined for a single administration of each chemical by three different routes, intragastric, intraperitoneal, and intravenous. Five rats were assigned to each dose-level and these are listed according to route of administration in Table I. The proportion of DMSO used for injection of each test chemical was as follows; MNU, 50% for intravenous administration and 40% for intraperitoneal; MNUt, 10% and 20% respectively; MNNG, 60% for both routes of administration; nitrosocimetidine, 20% for both routes; cimetidine, 50% for both routes. Intra-

TABLE I.—Dose levels of chemicals used for median lethality assay in female rats.[a]

Chemicals	Route of administration		
	Intravenous	Intraperitoneal	Peroral
MNU	50.0 to 282.8 (6 dose levels, $F = \sqrt{2}$)	35.4 to 111.4 (5 dose levels, $F = \sqrt{2}$)	100.0 to 400.0 (5 dose levels, $F = \sqrt{2}$)
MNUt	6.3 to 25.0 (5 dose levels, $F = \sqrt{2}$)	12.5 to 100.0 (7 dose levels, $F = \sqrt{2}$)	100.00 to 400.0 (5 dose levels, $F = \sqrt{2}$)
MNNG	56.6 to 320.0 (6 dose levels, $F = 1.4$)	50.0 to 200.0 (5 dose levels, $F = \sqrt{2}$)	141.4 to 800.0 (6 dose levels, $F = \sqrt{2}$)
Nitrosocimetidine	140.0 to 384.0 (4 dose levels, $F = 1.4$)	280.0 to 768.0 (4 dose levels, $F = 1.4$)	4,000.0 to 11,314.0 (4 dose levels, $F = \sqrt{2}$)
Cimetidine	100.0 to 400.0 (5 dose levels, $F = \sqrt{2}$)	200.0 to 800.0 (5 dose levels, $F = \sqrt{2}$)	2,828.0 to 11,314.0 (5 dose levels, $F = \sqrt{2}$)

[a] Dose levels in mg/kg of body weight; F represents the coefficient used for the calculation of each dose level.

venous injection was performed via the tail vein under ether anesthesia. The animals were weighed at 0, 1, 2, 3, 7, 10, and 14 days after treatment and all survivors were sacrificed at the 14th day.

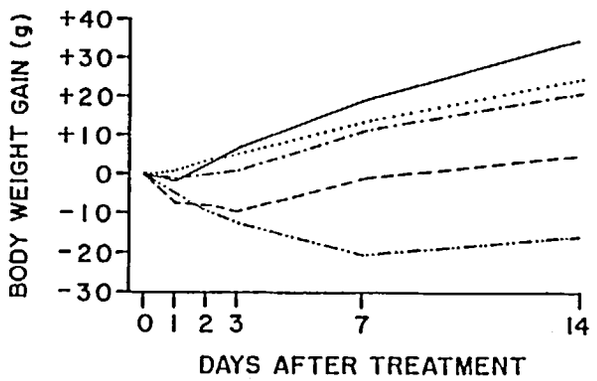
Sequential Histopathology Study. In order to determine the distribution of acute tissue injury along a sequential course, additional groups of 21 rats were given a single dose-level of nitrosocimetidine, MNNG, MNU, or MNUt, administered either intragastrically by stomach tube, or intravenously. For intravenous injection, the dose given for nitrosocimetidine, MNNG, and MNU was 0.65 mmole/kg body weight, but because of severe toxicity, this was reduced to 0.1 mmole/kg for MNUt. The proportion of DMSO used for injection of the various compounds by this route was 10% for nitrosocimetidine and MNU, 2% for MNUt, and 50% for MNNG and the vehicle control. By oral administration, the dose was 1.0 mmole/kg body weight for all compounds.

TABLE II.—Median lethal values of N-nitrosocimetidine, cimetidine, and 3 carcinogenic N-nitroso compounds in female rats.[a]

Chemicals	Route of administration		
	Intravenous	Intraperitoneal	Peroral
NC	243.4 (196.4–301.7)	486.8 (392.8–603.3)	9,513.7[b]
CIM	248.4 (199.8–308.7)	377.5 (206.6–690.3)	9,641.3[b]
MNNG	140.5 (113.0–174.7)	113.9 (86.4–150.1)	377.5 (288.3–494.4)
MNU	74.9 (57.2–98.1)	84.1 (69.2–102.2)	161.0 (129.5–200.2)
MNUt	20.1 (16.2–25.0)	27.9 (21.1–37.0)	237.8 (195.7–289.0)

[a] Dose levels in mg/kg of body weight; values in parentheses are upper and lower 95% confidence limits.
[b] 95% confidence limits could not be established.
NC: nitrosocimetidine; CIM: cimetidine.

Regardless of the route of administration, the volume actually given to each rat was 0.5 ml for 100 g body weight. Three animals from each group were sacrificed at each of the following time intervals after



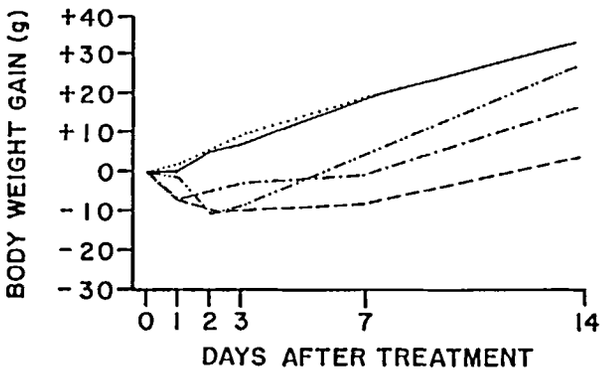


FIG. 2.—Average body weight gain of each group given an intragastric administration of nitrosocimetidine, MNU, MNNG, MNUt, or sesame oil. Nitrosocimetidine, ——; MNU, – – –; MNNG, –·–·–; MNUt, –··–··–; sesame oil, ·····.

FIG. 3.—Average body weight gain of each group given an intravenous administration of nitrosocimetidine, MNU, MNNG, MNUt, or DMSO. Nitrosocimetidine, ——; MNU, – – –; MNNG, –·–·–; MNUt, –··–··–; DMSO, ·····.

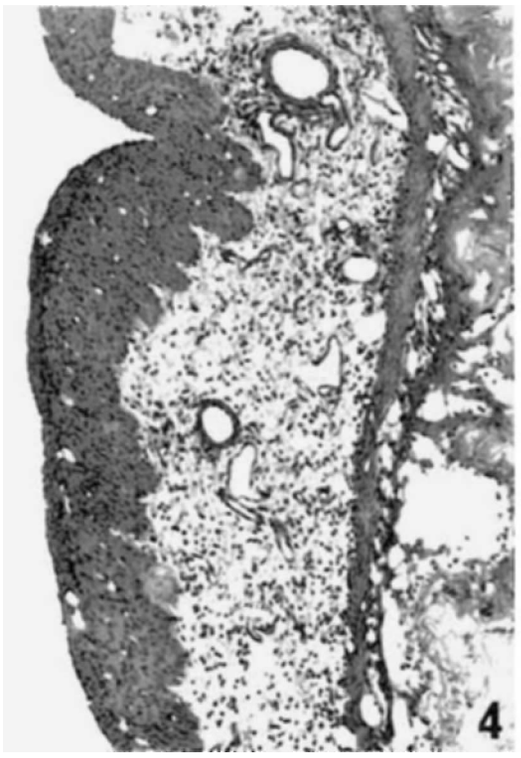


FIG. 4.—Loss of epithelial cells and submucosal edema in the forestomach 3 days after oral MNNG. Hematoxylin and eosin. ×120.

FIG. 5.—Ulceration of the lining of the glandular stomach 2 days after oral MNNG. H&E. ×60.

treatment; 3 hr, 6 hr, 1, 2, 3, 7, and 14 days. Weights were recorded at 0, 1, 2, 3, 7, and 14 days.

For preservation of the tissues, each animal was perfused via the heart under ether anesthesia, first with heparinized saline, and then with 10% neutral buffered formalin solution using a standard tissue perfusion apparatus. Major organs were taken for histological evaluation using hematoxylin and eosin stained sections. The organs of animals dying or sacrificed (without perfusion) in the median lethal assays were also prepared for histology to add to the data generated in the sequential histopathological study.

RESULTS

Body Weight Gain. The average body weight gain over the 14-day period of each of the treatment groups in the sequential histopathological study is shown in Figs. 2 and 3. The body weights of rats treated with nitrosocimetidine by both intragastric and intravenous routes increased at rates which matched or exceeded those of the vehicle-treated controls. In contrast, body weights of rats receiving MNU and MNUt by either route were diminished for up to 1 week recovering to levels substantially lower than those of nitrosocimetidine and control groups. Intravenously administered MNNG also affected body weight but very little by the intragastric route. The most profound effect was caused by intragastric MNUt.

Median Lethality. The median lethal values and 95% confidence limits of the 5 chemicals tested by 3 different routes of administration are listed in Table II. The actual values for nitrosocimetidine and cimetidine were equivalent at each of the 3 routes. By the intravenous and intraperitoneal routes, the only animals killed by these 2 compounds were those dying within several minutes of treatment. After intragastric administration, the highest dose of nitrosocimetidine, 11,314 mg/kg, killed 3 of 5, while 8,000 mg/kg killed 2 of 5 animals, all within 2 days. A dose of 5,657 mg/kg or less was not lethal. This pattern of deaths was almost identical for cimetidine. By contrast the median lethal values for MNNG, MNU, and MNUt were respectively, 25, 59, and 40 times lower than nitrosocimetidine by the intragastric route, 4, 6, and 17.5 times lower by the intraperitoneal route and 2, 3, and 12 times lower by the intravenous route. Unlike the cimetidine derivative, the deaths caused by systemic injection of the three carcinogens were not instantaneous but toxicity related.

Sequential Histopathology Study. The only tissue alterations found during the period of obser-

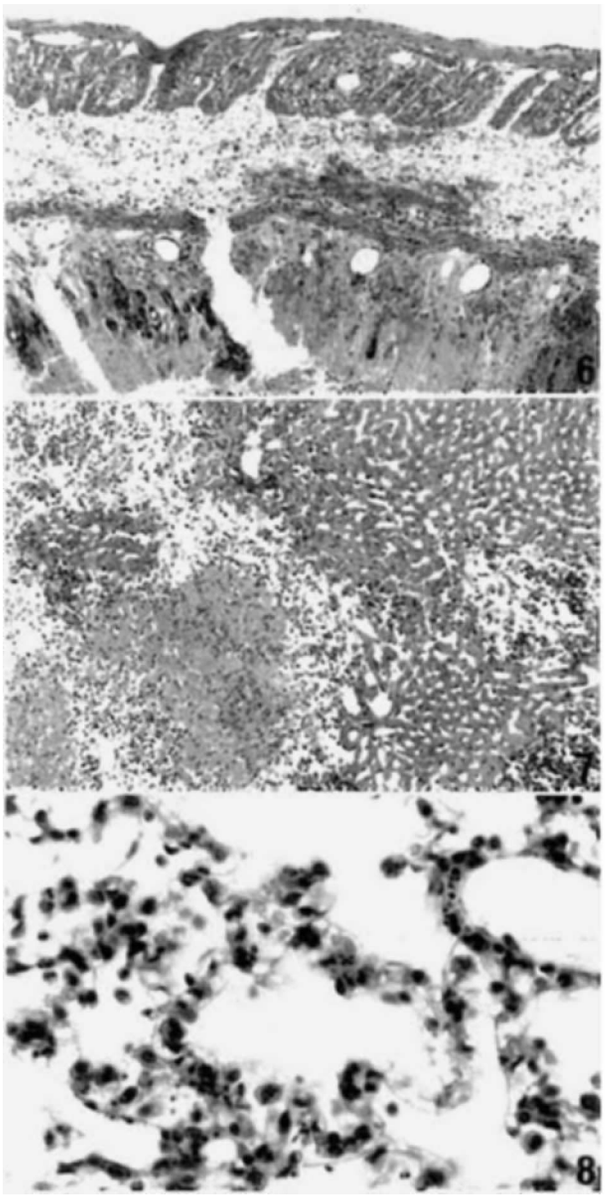


FIG. 6.—Epithelial necrosis and submucosal edema and hemorrhage in the glandular stomach 2 days after oral MNNG. H&E. ×100.

FIG. 7.—Liver necrosis at 3 days after oral MNNG. H&E. ×100.

FIG. 8.—Scattered epithelial necrosis in alveoli of the lung 6 hours after intravenous MNNG. H&E. ×460.

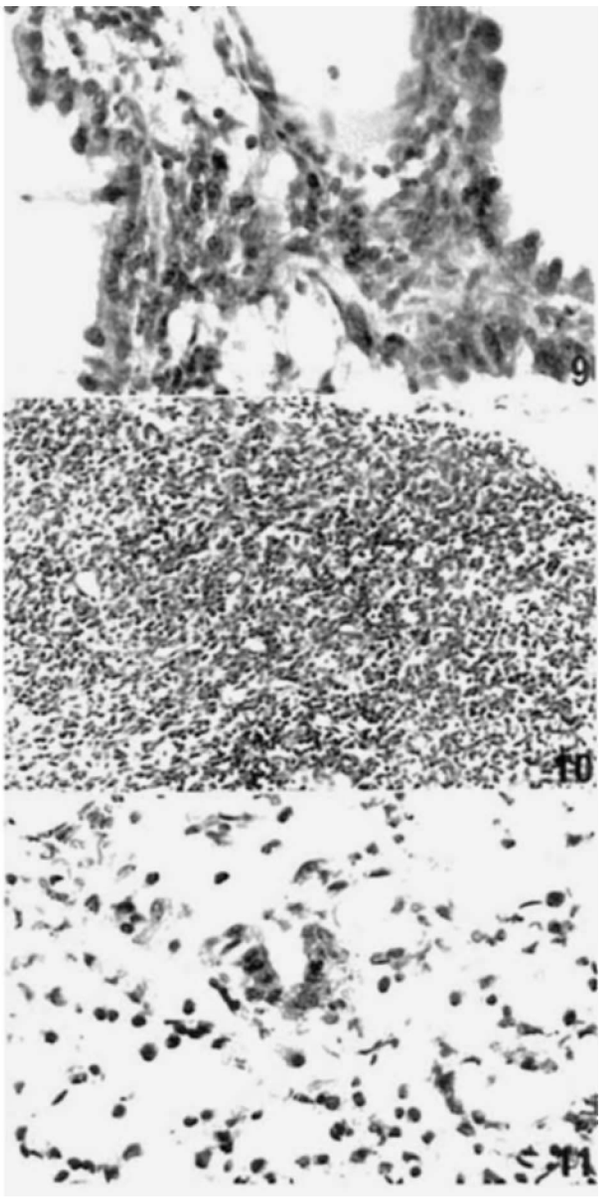


FIG. 9.—Epithelial hyperplasia and interstitial fibrosis in the lung at 7 days after intravenous MNNG. H&E. ×460.

FIG. 10.—Cell depletion and loss of the cortico-medullary boundary in the thymus 3 days after oral MNUt. H&E. ×100.

FIG. 11.—Focal proliferation of type II alveolar cells in the lung 7 days after intravenous MNUt. H&E. ×460.

vation after nitrosocimetidine administration by intragastric or intravenous routes involved slight depression of hematopoiesis in the spleen and slight thickening of the alveolar wall in the lungs in some but not all of the animals. The same type and degree of change was also observed in the vehicle-treated control rats as well as in rats used in the median lethality assay for cimetidine; there were therefore no conspicuous or specific lesions induced exclusively by nitrosocimetidine or cimetidine.

In contrast, severe degenerative changes were produced in various organs by the three carcinogenic nitroso compounds by all routes of administration. The salient histopathological findings are seen in Tables III–IV. The route of administration influenced some of the organ distribution of toxic cellular injury. All three carcinogens produced effects in the stomach which were not present after systemic injection. Apart from involving both compartments

TABLE III.—Summary of sequential tissue alterations induced by the test compounds perorally. (The organs are listed in order of lesion severity.)

Compound	Organ	Histopathology
Nitrosocimetidine	All	No specific tissue alterations.
MNNG	Forestomach	Hyperkeratosis, epithelial necrosis, submucosal edema at 1 day, becoming severe by 2 days; ulceration, submucosal inflammation by 3 days; epithelial hyperplasia, submucosal fibrosis at 7–14 days.
	Glandular stomach	Epithelial necrosis, intramucosal hemorrhage at 1 day, becoming severe by 2 days; ulceration, submucosal inflammation by 3 days; mucosal regeneration by 7–14 days.
	Liver	Focal periportal necrosis by 1 day, becoming severe by 3 days; lymphocyte infiltration at 7 days; slight fibrosis at 14 days.
	Spleen	Depression of hematopoiesis with recovery by 14 days.
	Bone marrow	Depression of hematopoiesis with recovery by 7 days.
	Thymus	Slight cortical atrophy at 2–3 days with recovery thereafter.
MNU	Glandular stomach	Pyknosis affecting middle to upper glandular epithelium by 6 hours, and basal layer by 1 day; intramucosal hemorrhage, inflammation, partial mucosal necrosis, submucosal edema at 1 day, persisting along with submucosal fibrosis until 14 days.
	Thymus	Lymphoblast necrosis in subcapsular zone from 6 hours to 1 day; severe cortical atrophy commencing by 1–3 days, persisting until 14 days.
	Spleen—red pulp	Pyknosis of hematopoietic cells at 6 hours; aplasia, atrophy until 3 days; multiple hematopoietic colony formation at 7–14 days.
	—white pulp	Increased lymphocyte pyknosis at 6 hours; varying degrees of atrophy until day 14.
	Bone marrow	Hypoplasia by 1 day, becoming severe by 2–3 days with recovery from 7–14 days.
	Lymph nodes	Identical changes to those seen in splenic white pulp.
	Forestomach	Epithelial hyperplasia at 2 days persisting until 14 days.
	Intestine	Pyknosis at base of crypts by 3–6 hours, decreasing by 2–3 days; no lesions at 7–14 days.
MNUt	Glandular stomach	Partial necrosis of mucosa, submucosal hemorrhage at 1 day with increasing severity over the next few days and persisting until 14 days.
	Forestomach	Epithelial cell necrosis, submucosal edema at 1 day; severe ulceration of mucosa and inflammation persisting until day 14.
	Thymus	Pyknosis in subcapsular zone at 1 day; cortical atrophy by 2 days, becoming severe at 3 days, and involving whole thymus from 3–7 days; recovery by 14 days.
	Liver	Focal periportal necrosis at 1 day; fibrosis at 7 days, decreasing at 14 days.
	Spleen—red pulp	Slight hypoplasia from 2–7 days; recovery by 14 days.

of the stomach (Figs. 4, 6), the most prominent lesions induced by MNNG affected the liver by the intragastric route (Fig. 7), and the lung (Figs. 8 and 9), thymus, and thyroid after intravenous administration. Lesions induced by MNU affected predominantly the hematopoietic and lymphoid tissues by both routes. MNUt caused acute toxicity in both stomach compartments, thymus (Fig. 10), liver, and spleen following intragastric intubation, but mainly the lung (Fig. 11), spleen, and bone marrow by intravenous injection. The lung changes in particular, involving injury to the alveolar epithelium followed

TABLE IV.—Summary of sequential tissue alterations induced by the test compounds intravenously. (The organs are listed in order of lesion severity.)

Compound	Organ	Histopathology
Nitrosocimetidine	All	No specific tissue alterations.
MNNG	Lung	Interstitial edema and scattered epithelial necrosis within 3–6 hours; thickening of alveolar wall at 2–3 days; peribronchial and perivascular lymphocyte accumulation, epithelial hyperplasia, fibrosis, and serosal thickening at 7–14 days.
	Thymus	Lymphoblast necrosis in subcapsular zone at 6 hours; cortical atrophy at 1–3 days; repopulation with large lymphoblasts at 7–14 days.
	Thyroid	Follicular atrophy with disappearance of colloid by 2 days, continuing up to 14 days.
	Spleen	Slight atrophy of white pulp at 2–3 days followed by gradual recovery; slight depression of hematopoiesis at 7–14 days.
	Liver	Slight focal necrosis in 2 rats at 14 days.
MNU	Spleen—white pulp	Increased lymphocyte pyknosis at 6 hours and later; severe atrophy at 7 days; some recovery by 14 days.
	—red pulp	Depressed hematopoiesis and aplasia becoming severe between 2–7 days; hematopoietic colony formation by 14 days.
	Lymph nodes	Identical changes to those seen in splenic white pulp.
	Thymus	Pyknosis in subcapsular zone from 3 hours to 1 day progressing to cortical atrophy at 7 days; recovery by 14 days.
	Bone marrow	Hyperplasia at 1 day, becoming severe by 3 days; recovery by 7 days.
MNUt	Lung	Increased alveolar macrophages, rounding of alveolar epithelial cells at 1 day; thickening of alveolar wall at 3 days; serosal thickening, occasional foci of hyperplastic type II alveolar epithelial cells between 7–14 days.
	Spleen	Slight depression of hematopoiesis at 2 days, becoming moderate at 3–7 days; recovery by 14 days.
	Bone marrow	Similar changes to those seen in spleen.
	Thymus	Slightly increased pyknosis in cortex at 1 day; some cortical atrophy at 2–3 days; rapid recovery by 7 days.

by occasional focal proliferation of the type II cells (Fig. 11), appeared to be a rather specific response to intravenous MNUt.

DISCUSSION

The high median lethal values and the absence of any specific histopathological alterations indicate that, in the rat, nitrosocimetidine has an extremely low acute toxicity, irrespective of the route of administration. This stands in distinct contrast to the acute lethal toxicity and severe tissue alterations induced by three strongly carcinogenic nitroso compounds which are structurally related to nitrosocimetidine. On the other hand, the acute toxicity levels obtained at each route of administration for nitrosocimetidine and cimetidine are clearly comparable. Most significantly, no obvious pathological change was seen in the small intestine of rats treated orally with nitrosocimetidine even though DNA methylation subsequent to oral dosing with the compound has been found to be highest in this organ (10). However, it has been shown that the levels of DNA alkylation produced by orally or intravenously administered nitrosocimetidine in various organs of rats are very low, some 3 to 50 times less than those produced by MNNG (10, 18). Intravenous or intraperitoneal injection of high doses of both nitrosocimetidine and the parent compound proved to be rapidly lethal to some animals (these dying within minutes of injection). Hemolysis, coagulation, or

hemorrhage were not observed in the affected rats; nor were any histopathological changes seen in the brains. Nevertheless, all rats in these high dose groups showed clonic muscle spasms shortly after treatment suggesting that rapid death may have been attributable to an effect on the nervous system.

It can be concluded that although a number of short-term *in vitro* assays indicate nitrosocimetidine to be genotoxic, the acute *in vivo* activity of this compound, at least in the rat, is consimilar with cimetidine. It does not parallel the severe toxic effects of three structurally related N-nitroso compounds known to be potent carcinogens. In view of the fact that nitrosocimetidine is rapidly converted to cimetidine when incubated in rat blood *in vitro* (18, 19), the present results, showing a coincidence of the biological activity of each compound *in vivo,* are consistent with the notion that nitrosocimetidine may indeed be rapidly denitrosated in the intact rat upon reaching the bloodstream. Therefore it might be anticipated that nitrosocimeditine would be noncarcinogenic in this species, a presumption supported to date by the animal bioassays already conducted (12, 23, 25).

With respect to the acute histopathology induced by orally administered MNU and MNUt, our results agree with earlier reports (22, 30) but provide some evidence of additional organ damage not previously recorded, for example, the severe injury to the thymus by MNUt. There is little documentation however, on the spectrum of acute tissue injury associated with oral MNNG or intravenously administered MNU, MNUt, or MNNG. Comparative study of these acute tissue changes induced by the three carcinogenic nitroso compounds by two different routes of administration indicates that there is some basis for correlation between the cellular targets for acute toxicity and the organ specificity for later carcinogenic action. Most organs which are known targets for cancer induction by these compounds showed unequivocal evidence of severe injury with the relatively high doses used here.

Thus the acute injury seen in both stomach compartments and the liver damage following oral MNNG correlate with the induction in rats of squamous carcinoma in the forestomach (32) and gastric adenocarcinoma in the glandular stomach (9, 35) even after a single dose (11), and liver tumors (9, 35). The severe lung injury with intravenous dosing accords with the development of lung tumors (34). Orally administered MNU is known to produce squamous cell carcinoma of the forestomach (22, 27), thymic lymphoma (21), and neurogenic tumors (7, 27) in rats, whereas intravenous MNU injection has caused predominantly neurogenic neoplasms (38). Although complete histological examination of the spinal cord and peripheral nerves was not possible in the context of this study for the purpose of pursuing an association between early nerve damage and neurogenic tumors, intragastric MNU was observed to produce severe toxicity in the forestomach and thymus. Again, the ability of oral MNUt to induce squamous cell carcinoma of the stomach (7, 24, 31) can be associated with early damage to this organ, while induction of lung tumors by intravenous injection (7) correlates with the acute injury affecting the alveolar lining. These various findings all support the concept that chemical carcinogens induce early cellular damage in the organ specific for tumor induction which can be visualized histologically when sufficiently high doses are administered.

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