ABSTRACT

The distribution of N-nitrosomethylbenzylamine (MBN) was studied using whole-body radioautography and densitometry. Male Sprague-Dawley rats were given N-nitroso-[methyl-14C]benzylamine ([methyl-14C]MBN) (3.3 mg, 1 mCi/kg body weight i.p.) or N-nitrosomethyl[benzyl-7-14C]amine (2.1 mg, 1 mCi/kg body weight i.p.), and sagittal sections were taken at 15 min, 60 min, 3.5 hr, 7.25 hr, 24 hr, and 3 days after injection. Very high levels of [methyl-14C]MBN-derived radioactivity were present at all time periods (15 min to 3 days) in the three target tissues for MBN-induced tumorigenesis (nasal cavity, lung, and esophagus). The liver contained high and the kidney contained moderately high levels of radioactivity at all time periods. A considerable amount of radiolabel was present in a number of tissues at 24 hr. After 3 days, very high levels were observed only in the tissues in which MBN-induced tumors eventually developed.

Following administration of N-nitrosomethyl[benzyl-7-14C]amine, levels of radioactivity in most tissues were lower than following injection of [methyl-14C]MBN; however, very high levels were present in the nasal cavity, liver, and kidney. Considerably less radiolabel remained at 24 hr and 3 days following injection of N-nitrosomethyl[benzyl-7-14C]amine than following [methyl-14C]MBN administration. At 24 hr, benzyl moiety-derived radiolabel was present in either the enterohepatic circulation or the tissues in which MBN-induced tumors arose. At 3 days, the highest level of radioactivity was contained by the liver, although detectable levels remained in the nasal cavity and lung, two tissues in which MBN induced carcinomas.

The findings of this study indicate that the carcinogenicity of MBN may be the result of the inability of the target organs (esophagus, nasal cavity, and lungs) to readily clear methylated macromolecules, benzylated macromolecules, or the oxidized metabolites which arise during nitrosamine metabolism.

INTRODUCTION

MBN is an unsymmetrically substituted nitrosamine which is mutagenic (13, 19, 21–24), toxic (5, 9), and carcinogenic (1, 5, 6, 14, 17). The compound has been shown to selectively induce esophageal tumors in rats (5), and the 2 α-acetoxy derivatives of MBN display striking differences in mutagenicity (19). Of interest because of its structure, toxicity, organ-specific carcinogenicity, and mutagenicity of oxidized derivatives, we undertook an extensive investigation of MBN which encompassed toxicity and carcinogenicity testing, pharmacokinetic and metabolic studies, and tissue distribution and persistence evaluations. The latter topic is the focus of the present report.

Whole-body radioautography was performed at 6 time points after injection of [14C]MBN. The 4 earlier time points were chosen to reflect tissue distribution and uptake. Tissue persistence of radiolabel was determined at the 2 later time periods. Densitometric analysis of the radioautograms permitted the calculation of half-lives of dpm equivalents in selected tissues. Whole-body radioautography has recently been used to investigate the distribution of DMN and MBN (9, 10). In the latter study, a dose of N-methyl[U-3H]-N-nitrosobenzylamine producing acute toxicity (100 mg, 10 mCi/kg body weight i.v.) was administered to male Donryu rats. In the present study, a fraction of the 50% lethal dose of MBN was administered to male Sprague-Dawley rats, and the use of [14C]MBN circumvented the problem of loss of tritium due to hydrogen exchange and metabolism of the nitrosamine.

MATERIALS AND METHODS

Animals and Diet. Male Sprague-Dawley rats (Charles River Breeding Laboratories, North Wilmington, Mass.) weighing 120 to 160 g were permitted food (Charles River Rat/Mouse/ Hamster Formula, Country Foods, Syracusc, N. Y.) and tap water ad libitum. The animals were housed in fume hoods in plastic tubs 13 x 11 x 5 inches (Superior Chemical Company, Somerville, Mass.) containing Ab-Sorb-Dri bedding.

Chemicals. [methyl-14C]MBN (specific activity, 6 mCi/mmol) and [benzyl-7-14C]MBN (specific activity, 5 mCi/mmol) were synthesized by Paul L. Skipper (15). The radiochemical purity of both compounds, determined by thin-layer chromatography on silica gel using acetone, was greater than 99%.

Whole-Body Radioautography. Twelve rats were given i.p. injections of [methyl-14C]MBN (3.3 mg, 1 mCi/kg body weight) or [benzyl-7-14C]MBN (2.1 mg, 1 mCi/kg body weight) and then frozen by immersion in a bath of dry ice and hexane at 6 intervals postinjection: 15 min, 60 min, 3.5 hr, 7.25 hr, 24 hr, and 3 days (1 rat/time period for each of the 2 compounds). The 4 earlier time points were selected based on whole-blood clearance of MBN. In approximately 10 min, 10% of an i.p. dose of MBN was cleared from whole blood. Fifty % was cleared in 65 min, 90% in 217 min, and 99% in 434 min.5

Following immersion in dry ice-hexane, the animals were embedded in a mold containing methylcellulose which was

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3 To whom requests for reprints should be addressed.
4 The abbreviations used are: MBN, N-nitrosomethylbenzylamine; DMN, di-methylhydroxylamine; [methyl-14C]MBN, N-nitrosomethyl[14C]benzylamine; [benzyl-7-14C]MBN, N-nitrosomethyl[14C]benzylamine.
placed in a −25° freezer until frozen. The frozen methylcellulose block was mounted on the stage of a Jung microtome (Kenneth A. Dawson, Inc., Belmont, Mass.) housed in a Harris cryostat (Harris Manufacturing Company, Cambridge, Mass.). Sagittal sections (60 μm thick), 60 to 80 at the first 4 time points and 30 to 40 at the latter 2 intervals, were picked up on Scotch 810 transparent tape (L. E. Muran, Boston, Mass.), transferred over dry ice, and stored in a low-humidity cold cryostat (Harris Manufacturing Company, Cambridge, Mass.) clamped between aluminum sheets and exposed at room temperature. Amounts of radioactivity (dpm = 81, 143, 243, 538, 905, 1619, 3344 ± 10% or dpm = 137, 215, 421, 758, 1458, 2965, 5512 ± 10%) were taped to Kodak No-Screen Medical X-ray film (E. M. Parker, Brookline, Mass.). The films were clamped between aluminum sheets and exposed at room temperature. Film exposure resulting from the standardized discs provided a scale for evaluating densities in the whole-body section radioautogram. After suitable exposure, sections were removed, and the X-ray films were processed in Kodak X-ray developer and fixer (E. M. Parker). Exposure ranged from 2 to 6 weeks, depending upon the densitometric quality of the radioautograms.

**Densitometry.** Radioautographic densities of the standard spots and selected tissues (the nasal cavity, the blood contained within the heart, the liver, and the musculature) were determined using a Macbeth densitometer, Model TD-502 LB (Macbeth Instrument Company, Newburgh, N. Y.). A linear-standard curve relating the dpm equivalents of the standardized discs and the corresponding densitometer readings enabled the calculation of dpm equivalents of tissues, including those lighter or darker than the standards, based on densitometer readings. Densitometric analysis required an area of uniformly darkened film 1 mm in diameter which precluded examination of a number of tissues including the esophagus, lung, and regions of the kidney at certain time points.

**RESULTS**

**Whole-Body Radioautography.** Semiquantitative data for the distribution of radioactivity following injection of \([\text{methyl-}^{14}\text{C}]\text{MBN}\) and \([\text{benzyl-7-}^{14}\text{C}]\text{MBN}\) is presented in Tables 1 and 2, respectively. Tissues were assigned values from 1 to 10 on the darkening of the X-ray film. Numbers were designated on the basis of dpm equivalents, determined by densitometry and by visual comparison to the 7 standard spots. Several tissues were either lighter or darker than were the standard discs which required expansion of the scale from 1 to 7 to 1 to 10. Radioautographs are shown in Figs. 1 to 3.

Following \([\text{methyl-}^{14}\text{C}]\text{MBN}\) administration, very high levels of radioactivity were present at all time points in the 3 tissues in which tumors developed following i.p. administration of MBN (nasal cavity, lung, and esophagus²). Radioactivity distribution was not uniform throughout the lung; areas of radiolabel concentration were observed at all time periods. Label localization also occurred in the salivary glands at 3 days. The liver contained high and the kidney moderately high levels of radioactivity at all time periods. The radioactivity distribution pattern of the liver at 24 hr and 3 days indicated that intestinal radiolabel entered the enterohepatic circulation. A considerable amount of radioactivity was present in a number of tissues at 24 hr: the esophagus, nose, and lung, as well as the thymus, liver, kidney, salivary glands, and epithelium of the gastrointestinal tract. By 3 days, very high levels were observed only in tissues in which MBM-induced tumors ultimately arose.

In general, tissue levels of radioactivity following injection of \([\text{benzyl-7-}^{14}\text{C}]\text{MBN}\) were lower than following administration of \([\text{methyl-}^{14}\text{C}]\text{MBN}\). Notable exceptions were the nasal cavity, liver, and kidney. The nasal cavity contained very high levels of \([\text{benzyl-7-}^{14}\text{C}]\text{MBN}\)-derived radioactivity at the 4 earlier time points. Very high levels of radiolabel were present in the kidney and urinary tract or bladder at 15 and 60 min. Radioactivity uptake by the tongue, pharynx, trachea, and esophageal epithelium was much less pronounced than that observed with \([\text{methyl-}^{14}\text{C}]\text{MBN}\) and had only begun to exceed tissue (tongue)

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² P. L. Kraft, J. C. Murphy, and S. R. Tannenbaum. Toxicity and carcinogenicity of N-nitrosomethylbenzylamine, submitted for publication.
Table 2

<table>
<thead>
<tr>
<th>Region of corresponding film</th>
<th>Film density*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15 min</td>
</tr>
<tr>
<td>Nasal cavity</td>
<td>10 10 9 6,10</td>
</tr>
<tr>
<td>Tongue</td>
<td>4 5 1</td>
</tr>
<tr>
<td>Tongue, pharynx, trachea, esophagus (epithelium)</td>
<td>5 5–6 1–2 3 2 0</td>
</tr>
<tr>
<td>Blood in heart</td>
<td>6 6 3 3 2 0</td>
</tr>
<tr>
<td>Lung</td>
<td>6 6 4 4 2 1</td>
</tr>
<tr>
<td>Localization</td>
<td>6 6 5 3 2 2</td>
</tr>
<tr>
<td>Background</td>
<td>7 9 6 5 4 3</td>
</tr>
<tr>
<td>Kidney</td>
<td>7 8 2</td>
</tr>
<tr>
<td>Cortex</td>
<td>7 8 2</td>
</tr>
<tr>
<td>Corticomedulla</td>
<td>9 10</td>
</tr>
<tr>
<td>Liver</td>
<td>10 10</td>
</tr>
<tr>
<td>Urinary tract (contents)</td>
<td>10 10</td>
</tr>
<tr>
<td>Esophageal entry into stomach (epithelium)</td>
<td>1 1 2 1</td>
</tr>
<tr>
<td>Small intestine, colon</td>
<td>10 10 8 3</td>
</tr>
<tr>
<td>Epithelium</td>
<td>10 10 8 3</td>
</tr>
<tr>
<td>Proximal contents</td>
<td>3 2 1</td>
</tr>
<tr>
<td>Distal contents</td>
<td>5 6 3 2 2</td>
</tr>
<tr>
<td>Spleen (red pulp)</td>
<td>4 5 1</td>
</tr>
<tr>
<td>Salivary glands</td>
<td>3 2 1</td>
</tr>
<tr>
<td>Musculature</td>
<td>4 5 1</td>
</tr>
</tbody>
</table>

* Dose was 2.1 mg, 1 mCi/kg body weight i.p.

Corresponding film blackened.

Chart 1. Tissue dpm equivalents obtained from whole-body radioautographs of male Sprague-Dawley rats given injections of [benzyl-7-14C]MBN (2.1 mg, 1 mCi/kg body weight i.p.). Radioautographic densities produced by selected tissues and standard spots were compared using a densitometer. Tissue levels were expressed as dpm equivalents (thousands). ○, nasal cavity; ▲, kidney (medulla); x, liver; Δ, kidney (cortex); ●, heart (blood); □, musculature.

Chart 2. Tissue dpm equivalents obtained from whole-body radioautographs of male Sprague-Dawley rats given injections of [methyl-14C]MBN (3.3 mg, 1 mCi/kg body weight i.p.). Radioautographic densities produced by selected tissues and standard spots were compared using a densitometer. Tissue levels were expressed as dpm equivalents (thousands). ○, nasal cavity; x, liver; Δ, kidney (medulla); ●, heart (blood); □, musculature.

Background at 3.5 hr. Areas of radiolabel localization also occurred in the lung following [benzyl-7-14C]MBN injection, but only from 3.5 hr onward. The distribution of radioactivity in the liver at 3.5 hr and beyond indicated that [benzyl-7-14C]MBN-derived radioactivity also entered the enterohepatic circulation. Considerably less radioactivity persisted (present at 24 hr and 3 days) following administration of [benzyl-7-14C]MBN than following injection of [methyl-14C]MBN. At 3 days, the highest level of radioactivity was contained by the liver. Barely detectable levels remained in the nasal cavity and lung.

**Densitometry.** Tissue dpm equivalents rose and fell more sharply following injection of [benzyl-7-14C]MBN (Chart 1) than following administration of [methyl-14C]MBN (Chart 2). Following administration of [methyl-14C]MBN, peak dpm equivalents were observed at 60 min in the blood (in the heart) and at 3.5 hr in the nasal cavity and liver. Musculature dpm equivalents probably reached a peak between 60 min and 3.5 hr (Chart 2). In contrast, following [benzyl-7-14C]MBN injection, peak dpm equivalents were observed at 60 min in all 4 tissues analyzed (Chart 1).

Equations for clearance of dpm equivalents could only be calculated using data at time points at and after the observed peak, namely, 3.5, 7.25, and 24 hr for [methyl-14C]MBN, and 60 min, 3.5 hr, and 7.25 hr for [benzyl-7-14C]MBN. Clearance equations calculated for [benzyl-7-14C]MBN using data at 3.5, 7.25, and 24 hr had poorer correlation coefficients. Estimated tissue half-lives of 14C equivalents were calculated from clearance equation rate constants. The 14C equivalent half-lives were considerably shorter following administration of [benzyl-7-14C]MBN than following injection of [methyl-14C]MBN: 2 to 4 hr versus 18 to 34 hr, respectively (Tables 3 and 4). For a given tissue, there was favorable agreement between the clearance equation intercepts calculated for methyl and benzyl dpm equivalents. For example, intercepts for nasal cavity clearance equations were 9.9 and 9.8.

**DISCUSSION**

Whole-body radioautography was utilized to demonstrate the...
cule. Thereafter, benzyl dpm equivalents rose faster than shown by the radioautograph at 15 mm (Fig. 2a), benzyl
dized benzyl moiety-derived metabolites into the blood. As
rate, reflecting the initial distribution of the intact parent mole
was calculated from densitometer readings. Methyl and benzyl
calculated using the equation for whole-blood clearance of
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MBN alone. In the first 5 to 10 mm after injection, methyl and
compound and metabolites, were cleared more slowly than
methyl dpm equivalents, probably due to entrance of the oxi
biosynthetic pathways (6). Following iv. injection of mice with
activity, but detectable amounts of [benzyl-7-14C]MBN-derived
radioactivity were taken up by tissues with high rates or protein
metabolites would be generated within the cell and all are
the nasal cavity, lung, and esophagus, the 3 tissues in which
MBN-induced tumors arose.6 Iizuka et al. (9) also reported that
considerable radioactivity remained in the esophagus at 24 hr
following a lethal dose of MBN. After 3 days, all 3 target tissues
contained very high levels of [methyl-14C]MBN derived radio-
activity, but detectable amounts of [benzyl-7-14C]MBN-derived
radioactivity remained in only the nasal cavity and lung (the 2
tissues in which carcinomas developed). At both 24 hr and 3
days, there was more methyl label than benzyl label.
The dpm equivalents of selected tissues were determined by
comparing densitometer readings of regions of X-ray film cor-
responding to tissues and standard discs. In the nasal cavity,
liver, and musculature, methyl and benzyl dpm equivalents
peaked at 3.5 hr and 60 mm, respectively. Methyl dpm equiv-
als remained in these tissues approximately 10 times longer
than did benzyl dpm equivalents. The methyl moiety may have
been incorporated into blood and tissue constituents via normal
biosynthetic pathways (6). Following i.v. injection of mice with
[14C]DMN and [14C]formaldehyde, considerable amounts of ra-
dioactivity were taken up by tissues with high rates or protein
synthesis or cell turnover, including the epithelia of the nasal
cavity and tongue (10). Incorporation of the methyl group into
biomolecules would lengthen the half-life of clearance.
The appearance and clearance of MBN and [methyl-14C]-
MBN and [benzyl-7-14C]MBN-derived radioactivity from the
blood were compared graphically (Chart 3). The percentage of
unmetabolized parent compound remaining in the blood was
calculated using the equation for whole-blood clearance of
MBN.3 The percentage of radioactivity present in the blood
was calculated from densitometer readings. Methyl and benzyl
moiety-derived dpm equivalents, representing both parent
compound and metabolites, were cleared more slowly than
MBN alone. In the first 5 to 10 min after injection, methyl and
benzyl dpm equivalents increased at approximately the same
rate, reflecting the initial distribution of the intact parent mole-
cule. Thereafter, benzyl dpm equivalents rose faster than
methyl dpm equivalents, probably due to entrance of the ox-
idized benzyl moiety-derived metabolites into the blood. As
shown by the radioautograph at 15 min (Fig. 2a), benzyl
metabolites had been rapidly removed by the kidney and
accumulated in the urinary tract. As a consequence, benzyl
dpm equivalents in the blood fell sharply after peaking at 60
min. Methyl dpm equivalents also peaked at 60 min but cleared
more slowly (t1/2 = 18 hr) than did the benzyl dpm equivalents
(t1/2 = 2 hr).
Nitrosamines require metabolic activation to alkylation
agents in order to exert their biological effects (8, 11). MBN
can be oxidized to either a methylating or a benzylating species
(7, 18) which could react with cellular nucleophiles yielding
methylated or benzylated macromolecules or with water to
produce methanol or benzyl alcohol. The alcohols can be
oxidized further to the corresponding aldehydes and acids by
alcohol and aldehyde dehydrogenases (4, 12). The oxidized
metabolites would be generated within the cell and all are
either irritants or capable of inflicting tissue damage (16).
During ensuing regenerative processes, cell division may pro-
vide an opportunity for incorporation of the carcinogenic lesion
into the genetic code. The oxidized metabolites of MBN may
be acting as cocarcinogens; noncarcinogens which enhance
the carcinogenicity of MBN, in this case by necessitating cell
repair and division which may increase the probability of lesion
fixation and eventual tumor development. Stimulation of liver
cell proliferation via partial hepatectomy has been used to
increase the yield of tumors following administration of DMN
(2, 3) and aromatic hydrocarbons, nitrosamines and nitrosa-
mides, aromatic amines, and miscellaneous carcinogens (20).
Liver cell necrosis and the ensuing compensatory cell prolif-
eration following administration of N-nitrosodiethylamine have
been shown to play an important role in the induction of
preneoplastic lesions (25). The carcinogenic effect of MBN
may be enhanced by the inability of the esophagus, nasal
cavity, and lung to readily clear the methylated or benzylated
macromolecules or oxidized metabolites which arise during
nitrosamine metabolism.
ACKNOWLEDGMENTS

We wish to thank Dr. Robert Liss for use of the radioautography facilities at Arthur D. Little, Inc., Cambridge, Mass. We also wish to thank Bruce McPherson, Phil Schepis, and Dick Lauterno for instruction and advice.

REFERENCES

Fig. 1. Radioautographs of male Sprague-Dawley rats 15 min (a), 7.25 hr (b), and 24 hr (c) after i.p. injections of \( \text{[methyl-}^{14}\text{C]MBN} \) (3.3 mg, 1 mCi/kg body weight).

Fig. 2. Radioautographs of male Sprague-Dawley rats 15 min (a), 7.25 hr (b), and 24 hr (c) after i.p. injections of \( \text{[benzyl-}^{14}\text{C]MBN} \) (2.1 mg, 1 mCi/kg body weight).
Fig. 3. Radioautographs of male Sprague-Dawley rats 3 days after i.p. injection of [methyl-14C]MBN (3.3 mg, 1 mCi/kg body weight) (a, b) and [benzy1-7-14C]MBN (2.1 mg, 1 mCi/kg body weight) (c).
Distribution of \textit{N}-Nitrosomethylbenzylamine Evaluated by Whole-Body Radioautography and Densitometry

Patricia L. Kraft and Steven R. Tannenbaum


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