Biochemical Pharmacology, Vol. 16 pp. 1741–1746. Pergamon Press Ltd. 1967. Printed in Great Britain

METHANE FORMATION *IN VIVO* FROM *N*-ISOPROPYL α(2-METHYLHYDRAZINO)-*p*-TOLUAMIDE HYDROCHLORIDE, A TUMOR-INHIBITING METHYLHYDRAZINE DERIVATIVE

F. N. DOST and D. J. REED

Science Research Institute and Radiation Center, Oregon State University, Corvallis, Oregon, U.S.A.

(*Received* 9 *December* 1966; *accepted* 28 *February* 1967)

Abstract—*N*-isopropyl-α(2-methylhydrazino)-*p*-toluamide hydrochloride (MIH), a cytotoxic agent with both antitumor and carcinogenic properties, was found to be rapidly degraded *in vivo*. ^{14}C and ^{3}H *N*-methyl labeled MIH were given intraperitoneally to rats (20 and 200 mg/kg) and, in 8 hr, 7–10 per cent of the methyl group was converted to respired methane and 11–22 per cent to respiratory CO_2. A comparison of the rate of methane and CO_2 formation from MIH and monomethylhydrazine (MMH) suggests that MMH may be an intermediate in MIH metabolism.

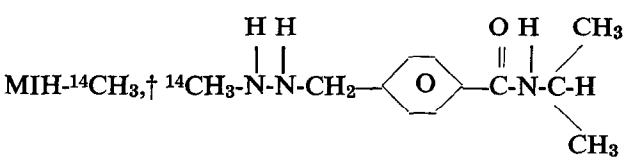
A GROUP of methylhydrazine derivatives, of which *N*-isopropyl-α(2-methylhydrazino)-*p*-toluamide hydrochloride (MIH)* is the most widely known representative, constitutes a new class of cytotoxic agents which have both antitumor and carcinogenic properties.[1–5]. Recent work in our laboratory[6, 7] has shown that the parent compound, monomethylhydrazine (MMH), is converted by rats to products which include methane and CO_2. Although MMH has been postulated as a metabolic product of MIH[8–10] these workers did not detect its presence after administration of MIH. Baggiolini *et al.*[11] reported the formation of $^{14}CO_2$ from MIH-$^{14}CH_3$, but concluded that MMH was not an intermediate in the demethylation (CO_2 formation) of MIH.

Schwartz[12] has reported methylamine as a product of both MMH and MIH metabolism. Methylamine was characterized as a 2,4-dinitrophenyl derivative. Baggiolini and Bickel[13] concluded that methylamine could not be an intermediate in the demethylation (CO_2 formation) of MIH or MMH, since they found that both of these compounds could inhibit the conversion *in vivo* of methylamine to respiratory CO_2.

In this communication, we report the formation of methane as well as of CO_2 from the hydrazine methyl group of MIH during the metabolism of this compound by rats, which we believe implicates MMH as an intermediate of MIH metabolism.

MATERIALS AND METHODS

Labeled MIH



(sp. act. 11 μc/mg), and MIH-C^3H_3 (sp. act. 57 μc/mg)

* Known as MIH or RO 4-6467 and by trade names Natulan and Ibenzmethyzin.

† MIH and labeled MIH were kindly provided by Dr. W. E. Scott of Hoffman LaRoche, Inc., Nutley, N.J.

were examined for radiochemical purity by ascending paper chromatography and radiochromatography. The paper chromatograms were developed for 12 hr in *n*-butanol saturated with an aqueous solution of oxalic acid (0·5 M). After air drying, the chromatograms were radioassayed with a Packard model 7200 radiochromatogram scanner.

R_f values were established for several known compounds by using the solvent system described: MIH, 0·50; MMH, 0·06; *N*-isopropyl-(*p*-methyl-azo-methyl)-benzoic acid-amide, 0·94. Ninhydrin (0·2% in acetone) and u.v. absorption were used to detect these compounds on the chromatograms. Chromatography of freshly prepared solutions of MIH-$^{14}CH_3$ and MIH-C^3H_3 indicated that the majority of the radioactivity had an R_f value identical with that of MIH. Less than 3 per cent of the radioactivity had an R_f value identical with that of MMH. Approximately 3 per cent of the total radioactivity had an R_f value of 0·78, whereas the remaining radioactivity had an R_f value identical with that of the azo derivative of MIH. Solutions of labeled MIH were radioassayed by liquid scintillation counting with a Packard liquid scintillation spectrometer, model 314 EX-2. The fluor solution was 5 ml of ethanolamine–ethanol (1:3 v/v) and 10 ml of toluene containing 3 g/l. of terphenyl and 30 mg/l. of 1,4-bis-2(phenyloxazolyl)-benzene (POPOP).

Radiorespirometry

Sprague–Dawley male rats weighing 250 g were given, i.p., either 19·5 or 195 μmole (20 or 200 mg/kg body wt.) of labeled MIH. The rats were placed in metabolism chambers and the ^3H and ^{14}C radioactivity in the respiratory gases was measured continuously by a radiorespirometer consisting of four flow ion chambers and vibrating reed electrometers. The output voltage of each electrometer is converted to digital form, integrated over a preset time interval, and printed out by a digital recorder. In experiments with MIH-$^{14}CH_3$, the radiorespirometer was modified to distinguish between radioactivity in CO_2 and that in methane in the respiratory gases. Total radioactivity in dried and acid-scrubbed respiratory gases was measured during passage through a flow ion chamber, and radioactivity from methane was measured in a second flow ion chamber after removal of CO_2 by a soda–lime column. In experiments with MIH-C^3H_3, the respiratory gases were dried by Drierite and soda-lime and their radioactivity was measured with a flow ion chamber as described above.

Gas-chromatography

An F & M model 810 gas-chromatograph, equipped with a hydrogen flame detector and a Porapak Q column, and a Barber Colman model 5190 radioactivity monitor system were used to identify ^{14}C-labeled methane in respiratory gases. The respiratory gases were recycled in a closed metabolism system through an absorber column packed with Drierite, soda–lime, and oxalic acid crystals. The scrubbed gases were circulated through a 5–ml loop of a gas sampling valve for gas-chromatography. Oxygen was admitted to maintain atmospheric pressure within the closed system.

RESULTS

The radioactivity in solutions of labeled MIH that had an R_f value identical to the corresponding azo derivative when chromatographed was assumed to be this

intermediate. Other workers[9] have demonstrated both a slow oxidation of MIH in aqueous solution to the azo derivative and a rapid conversion rate *in vivo*.

Gas-chromatography of the respiratory gases gave evidence that methane is the only neutral and volatile product released from rats metabolizing MIH-$^{14}CH_3$ (Table 1). The gas-chromatographic column employed gave excellent separation of

TABLE 1. GAS-RADIOCHROMATOGRAPHY OF A VOLATILE PRODUCT OF MIH-$^{14}CH_3$ METABOLISM BY RATS*

Sample	Retention time (sec)	Radioactivity (cpm)
Methane	38–42	40–50 (background)
Volatile product formed in 2 hr	38–42	250–300

* A gas chromatograph column, 1/8 in. × 6 ft, packed with 150–200 mesh Porapak Q was maintained at 30°. Argon flow rate through the column was 50 ml/min with approximately 25 per cent of the effluent diverted into the radioactivity detector system. MIH-$^{14}CH_3$, 5 mg containing 5 μc of ^{14}C, was administered to a 250-g rat by i.p. injection. The total volume of the animal chamber was approximately 2 l, including the atmosphere recycling system. Five-ml samples of the respiratory gases were injected into the gas chromatograph by a gas sampling system.

hydrocarbons through C_4 and only methane was observed in the recycled respiratory gases up to 4 hr after MIH administration to rats. No attempt was made to quantitate by gas-radiochromatography the release of radioactivity by rats. This procedure was used only to show that methane was the only hydrocarbon released and that it contained radioactivity.

The conversion of the ^{14}C-labeled methyl group of MIH to $^{14}CO_2$ and $^{14}CH_4$ by rats during 8-hr radiorespirometric experiments is shown in Fig. 1 and Table 2. Although the micromoles of CO_2 formed from MIH exceeded that of methane at both the 19·5 and 195 μmole dose levels, respired methane clearly constitutes much of the respired radioactivity at both dose levels. Of interest also is the similar percentage of MIH-$^{14}CH_3$ converted to CO_2 and methane at the two dose levels, even though the dose levels differed tenfold. In longer experiments, radioactivity in the respiratory gases from rats that were given labeled MIH was observed for periods up to 24 hr. The ^3H-labeled methyl group of MIH was converted to C^3H_4 at a rate which essentially duplicated the rate of formation of $^{14}CH_4$ from MIH-$^{14}CH_3$ (Fig. 2, Table 2). A comparison of the conversion of ^3H- and ^{14}C-labeled MIH to methane at the two dose levels indicates that the methyl group is converted to methane as an intact entity.

DISCUSSION

The unique metabolic conversion of hydrazine methyl groups to methane has been observed previously in studies of the metabolic fate of MMH, but was not found to occur during the metabolism of 1,1 dimethylhydrazine by rats.[7] The rapid conversion *in vivo* of MIH to the corresponding azo derivative[9] suggests that MIH may not be the immediate precursor of methane. Since MMH yielded methane in the rat at a more rapid rate than did MIH,[7] the conversion *in vivo* of MIH to MMH via the proposed

metabolic scheme shown by Raaflaub and Schwartz[10] and by Oliverio *et al.*,[9] based upon experiments *in vitro* by Berneis *et al.*,[8] may be the pathway of methane formation from MIH. The differences in the ratio between CH_4/CO_2 formation from MMH and MIH may only reflect the distribution and difference in the rate of metabolism of these two compounds.

The relatively constant ratio between methane and CO_2 production at the 20 and 200 mg/kg dose levels is not entirely understood even though similar dose responses were observed during MMH metabolism.[7] Baggiolini *et al.*[11] treated rats with phenobarbital at 100 mg/kg body wt./day for 7 days prior to MIH metabolism studies

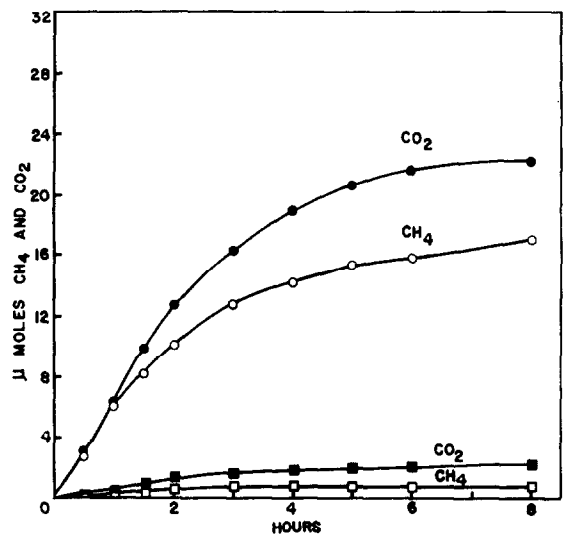


FIG. 1. Time course of $^{14}CH_4$ and $^{14}CO_2$ formation from MIH-$^{14}CH_3$ administered to rats. Methane and CO_2 formation, expressed as μmole, were calculated on the basis of specific activity and total radioactivity administered as MIH-$^{14}CH_3$. The symbols ■ and □ denote accumulative yields of CO_2 and CH_4, respectively, from 19·5 μmole of MIH; ● and ○ denote yields of CO_2 and CH_4, respectively, from 195 μmole of MIH. Total radioactivity administered to each rat was 2·5 μc.

TABLE 2. PER CENT RECOVERY OF RADIOACTIVITY FROM RATS AFTER ADMINISTRATION OF LABELED MIH*

	MIH-$^{14}CH_3$	
Respired radiochemical	(20 mg/kg)	(200 mg/kg)
$^{14}CO_2$	21·3 ± 1·4 (4)	11·6 ± 0·1 (2)
$^{14}CH_4$	8·3 ± 1·7 (4)	8·7 ± 0·2 (2)
	MIH-C $^3H_3$	
C $^3H_4$	8·6 ± 1·3 (3)	9·4 ± 0·7 (4)

* Per cent accumulative recovery 8 hr after i.p. injection of labeled MIH. Unlabeled MIH was used to adjust the specific activity of labeled MIH. Total radioactivity administered to each rat was 2·5 μc of MIH-$^{14}CH_3$ or 7·75 μc of MIH-C $^3H_3$. Number of experiments is shown in parentheses. Single experiment deviation of per cent recovery from the mean value presented was within the ± per cent values shown.

and showed that the rate and extent of CO_2 formation could be increased. Preliminary experiments in our laboratory with phenobarbital-treated rats given MIH-$^{14}CH_3$ have provided similar results. The rate of ^{14}C-methane production in these experiments differed little from that of rats not treated with phenobarbital.

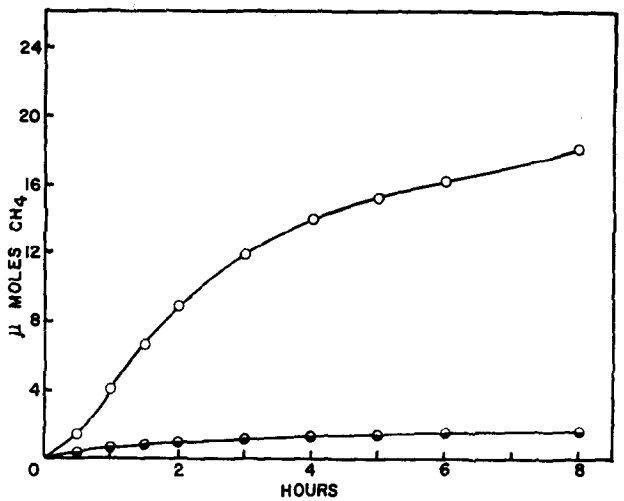


FIG. 2. Time course of C^3H_4 formation from MIH-C^3H_3 administered to rats. Methane formation, expressed as μmole, was calculated on the basis of sp. act. and total radioactivity administered as MIH-C^3H_3. The symbols ◒ and ○ denote accumulative yields of methane from doses of MIH of 19·5 and 195 μmole, respectively. Total radioactivity administered to each rat was 7·75 μc.

Since the *N*-methyl group is required for these hydrazine derivatives to be biologically active as tumor inhibitors,[14] the methyl group must play an important role in their biological activity. The formation of a free radical intermediate has been suggested as a mechanism of oxidation of alkyl and aryl hydrazines.[15–17] Thus, the importance of the methyl group in methylhydrazine antitumor agents and their carcinogenic activity may be related in its possible transitory existence as a methyl free radical. If so, relative effectiveness of these derivatives could depend on the nature of their body distribution prior to their conversion *in vivo* to MMH. The lack of carcinogenic activity of MMH[18] may suggest the need for cellular distribution of MIH before the formation of MMH and subsequent methyl free radical formation. A synergism between ionizing radiation and MIH has been observed by Berneis *et al.*[19] which supports the possible formation of some type of free radical from MIH.

Acknowledgements—The support of this work by USPHS Grant CA 08348 is gratefully acknowledged. The use of equipment procured under Contract AF 33 (657-11757) is also acknowledged.

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