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NEAR QUANTITATIVE PRODUCTION OF MOLECULAR NITROGEN FROM METABOLISM OF DIMETHYLNITROSAMINE

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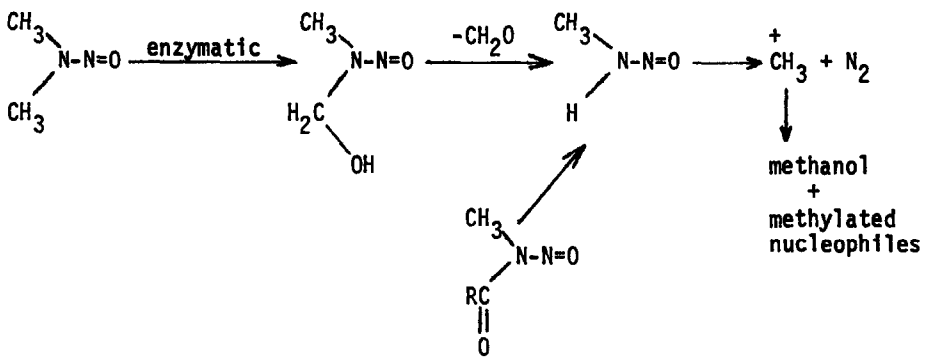
SUMMARY: Molecular nitrogen is produced stoichiometrically from the spontaneous decomposition of N-methyl-N-nitrosourea in phosphate buffer and is readily detected by gas chromatography. It is also generated in high yield from the oxidation of dimethylnitrosamine by mouse or rat liver microsomes. Gas chromatographic analysis for nitrogen generated from dimethylnitrosamine was more difficult because the amount of nitrogen generated was similar to that produced in control tubes which contained no dimethylnitrosamine.

Dimethylnitrosamine (DMN)[1] is a carcinogen, mutagen and hepatotoxin whose actions are very potent in a number of experimental systems (1,2,3). DMN is believed to be metabolized to methyldiazonium ion which rapidly decomposes yielding methyl carbonium ion and molecular nitrogen. The carbonium ion, being an S_N1 type alkylating agent, may react with water producing methanol (4,5) or with cellular nucleophiles leading to toxicity and genetic alterations (3,6). This pathway is depicted below.



Evidence supporting this pathway includes the detection of formaldehyde (7) and methanol (4,5) from the *in vitro* and *in vivo* metabolism of DMN, a

1. The abbreviations used are: DMN, dimethylnitrosamine; NMU, N-methyl-N-nitrosourea; DNA, deoxyribonucleic acid.

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deuterium isotope effect on the generation of formaldehyde when deuterated DMN is metabolized *in vitro* (8), the transfer of intact CD_3 to DNA (9), and the *in vivo* methylation of nucleic acids and proteins by DMN (3). The initial enzymatic oxidation of DMN is believed to be cytochrome P-450 dependent as evidenced by the 450 nm maximum in the photoreactivation spectra for DMN demethylation and DMN induced mutagenesis catalysed by CO-treated mouse liver microsomes (10). However, the one major product of DMN metabolism which has not been detected in high yield is molecular nitrogen. In fact it has been reported that generation of N_2 from DMN metabolized *in vitro* is only 5% of the rate of formaldehyde production (11). This finding and others have led to the suggestion that alternate enzymatic pathways for DMN metabolism may exist (4,5,11). In view of the frequent use of DMN as a prototype alkylating agent and carcinogen, we have investigated by a different technique its metabolism to N_2 and find a production of N_2 which is about 2/3 that of formaldehyde. Also, as a positive control we find that NMU decomposes to yield a stoichiometric amount of nitrogen. Since NMU and DMN are believed to generate the common intermediate, monomethylnitrosamine, this result is also consistent with the pathway outlined above.

MATERIALS AND METHODS

Metabolism of DMN: Hepatic microsomes were isolated from Aroclor 1254 (Monsanto, St. Louis, Mo.) pretreated rats or mice (12) by centrifugation of 9000 x g supernatents of liver homogenates at 100,000 x g for 60 min. The microsomes were resuspended in an NADPH generating system (13) immediately before use. 5 ml of suspension were transferred to a 10 ml "Reactiflask" (Pierce, Rockford, Ill.) and sealed with a screw cap septum. Higher concentrations of rat liver microsomes (6 mg/ml protein) than mouse liver microsomes (3 mg/ml protein) were used as rat liver microsomes were less active in metabolizing DMN in preliminary experiments carried out with lower DMN concentrations. However, at higher DMN concentrations mouse and rat liver microsomes had similar specific DMN demethylase activities (Table 2). The suspension was then bubbled with helium containing 1.3% isobutane (as an internal standard) for 15 min. The inlet and outlet needles were removed and a volume of oxygen equivalent to 20% of the head space was injected into the reaction flask, and with the syringe still in place allowed to mix with the gases in the head space for 30 sec. An equivalent volume of the head space gas mixture was then removed to avoid any possible gas leakage due to the increased pressure from the added oxygen. All procedures were carried out in rapid succession with the reaction flask kept on ice. The reaction

was then initiated with the injection of DMN (Aldrich, Milwaukee, Wis.) to a final concentration of 200 mM and run at 37° in a Dubnoff shaker. Control tubes containing all components except either DMN or microsomes were simultaneously purged on the same gas line as the experimental tubes so that residual nitrogen would be similar in all tubes. When the head space in any tube was repetitively sampled, the average variation between nitrogen values obtained from the tube was within 3% (after correction for the gas volume loss due to the previous sample). The control tube containing no microsomes did not liberate any nitrogen and was therefore discontinued. Immediately after incubation 1 ml of the head space from each tube was injected into an F & M Model 400 gas chromatograph, fitted with a 5 ft., 1/8" MS 5A column and a thermal conductivity detector and analysed for head space gases. Analysis conditions were: column T, 22°; injector T, 175°; detector T, 220°; and flow rate, 30 ml/min. The percentage of oxygen consumed was not significant as judged by the oxygen peak area. A sample of air was used as a nitrogen standard. Immediately after injection, 2.5 ml of 10% trichloracetic acid was added to each tube and formaldehyde was measured colorimetrically by the Nash method (14).

Decomposition of NMU: Disappearance of NMU (ICN, Plainview, NY) was monitored by its absorbance at 390 nm. NMU decomposed spontaneously in chilled, pH 7.4 phosphate buffer but was stable for at least 20 min in cold water. Freshly prepared 100 mM solutions of NMU in water were then used as stock solutions and diluted as needed. Volumes of 0.9 ml of phosphate buffer were transferred to 16 x 125 mm tubes, sealed with serum stoppers and the head space purged with helium for 15 min. The decomposition was initiated with the injection of 0.1 ml 100 mM NMU. The tubes were incubated at 37°. Control tubes contained NMU in water instead of buffer. Determination of nitrogen was carried out as described above.

RESULTS AND DISCUSSION

Molecular nitrogen was generated nearly stoichiometrically from NMU in phosphate buffer (Table 1). The nitrogen generated was readily determined because of its high concentration in the head space relative to the concentration of nitrogen detected in the control tube. The stoichiometric production of N_2 supports the mechanism for decomposition of N-nitroso compounds outlined above.

Determination of N_2 generated from DMN was more difficult because the amount of N_2 present in the control tube containing only microsomes was nearly as great as that in the tubes containing the full incubation mixture. Presumably the relatively high concentration of N_2 in the head space of the control tubes resulted from residual nitrogen in the microsomal lipid which was not removed by bubbling with helium. In addition, the concentration of DMN metabolized was nearly a factor of ten less than the concentration

TABLE 1

GENERATION OF N_2 FROM NMU

incubation time (min)	NMU decomposed[a] (umoles)	N_2 experimental (umoles)	N_2 control (umoles)	% yield[b]
5	4.4	6.0	1.6	100
10	6.1	7.6	1.6	98

a. An incubation volume of 1 ml was used. Other details are described in Materials and Methods
b. N_2 experimental - N_2 control/NMU decomposed

TABLE 2

GENERATION OF N_2 AND FORMALDEHYDE FROM DMN

microsomes[a]	incubation time (min)	Formaldehyde (umoles)	N_2 experimental (umoles)	N_2 control (umoles)	% yield[b]
mouse	40	0.91	0.86	0.29	63
mouse	40	0.99	1.44	0.78	68
rat	40	1.94	1.60	0.30	67
rat	40	1.80	2.40	1.09	72
rat	20	1.05	1.68	0.84	80

a. The concentrations of mouse and rat microsomes were 3 and 6 mg/ml resp. The incubation mix contained 5 ml and all values in the table are expressed per ml of mix. Other details are described in Materials and Methods. Each line in the table represents an experiment performed on a separate day with the exception of the last 2 lines which represent experiments performed on the same day
b. N_2 experimental - N_2 control/formaldehyde

of NMU which decomposed in the previous experiments. In order to facilitate DMN metabolism relatively high concentrations of microsomes from induced animals and high concentrations of DMN were employed (10,15). As seen in Table 2, production of N_2 was about 70% that of formaldehyde when either mouse or rat liver microsomes were employed. The production of nitrogen and formaldehyde was greater after 40 min incubation than after 20 min. There was considerable daily variation in the nitrogen evolution from the control tubes perhaps due to the difficulties involved in reproducibly bubbling the

microsomes. However, each control tube in Table 2 corresponds to an experimental tube incubated on the same day at the same time and precision in nitrogen generation from duplicate experiments performed on separate days was about 10% after subtraction of control values. Similarly the precision in ratio of nitrogen produced to formaldehyde produced was about 10%. Since formaldehyde is generated about 1.3 x as rapidly as DMN is degraded (4,5,11) due to oxidation of methanol to formaldehyde, the production of N_2 from DMN is nearly quantitative.

Our results are at variance with those of Cottrell et al who report an N_2 generation which is 5% that of formaldehyde (11). In their report, the nitrogen generated was produced from ^{15}N double labelled DMN, and detected by mass spectroscopy. Although the mixtures containing DMN were incubated under an oxygen atmosphere they were not bubbled and could have contained dissolved nitrogen which may have had an effect on their nitrogen assay. However, their experiments did not employ microsomes from Aroclor induced animals, they used lower concentration of DMN and differences in pathways of DMN metabolism between the two groups of experiments are possible since DMN is metabolized by different forms of cytochrome P-450 with different values of K_m for DMN demethylase (16). The same workers did however report quantitative production of N_2 from the hydrolysis of α-acetoxydimethylnitrosamine, a result which supports the decomposition pathway given above. Our results are consistent with the pathway of DMN decomposition outlined above and also with experiments performed on DMN metabolism in rats in vivo where N_2 generation from metabolized DMN was near stoichiometric (17).

The results presented here may have a practical application as an alternative assay for DMN metabolism which is unaffected by any methanol-formaldehyde interconversions. Although the assay as described here is relatively insensitive, the use of more sensitive nitrogen detectors in the gas chromatograph and improved purging techniques could substantially improve sensitivity.

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