Biochemical Pharmacology, Vol. 33, No. 9, pp. 1509–1513, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00
Pergamon Press Ltd.

METABOLISM OF ^{15}N-LABELLED *N*-NITROSODIMETHYLAMINE AND *N*-NITROSO-*N*-METHYLANILINE BY ISOLATED RAT HEPATOCYTES

STEVEN R. KOEPKE,* YVES TONDEUR, JAMES G. FARRELLY, MARY L. STEWART, CHRISTOPHER J. MICHEJDA and MARILYN B. KROEGER-KOEPKE

LBI-Basic Research Program, Chemical Carcinogenesis Program, NCI-Frederick Cancer Research Facility, Frederick, MD 21701, U.S.A.

(*Received* 2 *June* 1983; *accepted* 25 *October* 1983)

Abstract—The N-demethylation of ^{15}N-labeled *N*-nitrosodimethylamine (DMN) and *N*-nitroso-*N*-methylaniline (NMA) by isolated rat hepatic cells has been investigated. The values obtained in this system for molecular nitrogen formed during metabolism, compared with substrate consumed, were DMN 47%, NMA 23%, and *N*-nitroso-*N*-methylurea (NMU) 105%. The results for DMN are roughly halfway between those previously determined with rat liver S-9 fraction *in vitro* (33%) and *in vivo* (67%). For NMA, the hepatocyte data are closer to those obtained from S-9 *in vitro* (19%), rather than the *in vivo* (52%). No mixed nitrogen (^{15}N^{14}N) or labeled nitrogen oxides were found.

In recent years, the isolated hepatocyte has been utilized increasingly as a tool in the study of various aspects of drug metabolism, especially the modifications of metabolism due to changes in cellular integrity and membrane permeability [1]. The attractiveness of the hepatocyte for drug research rests partly on its ability to perform sequential metabolic reactions, rather than just the primary metabolic event [2], as is the case with most subcellular fractions. For this reason, the metabolism of drugs in isolated hepatocytes has been reported to correlate better with *in vivo* drug metabolism than with metabolism in the 9000 *g* supernatant fraction (S-9) or microsomes [3]. However, this correlation appears to depend on the substrate being analyzed.

We have determined previously the release of labeled nitrogen during the metabolism of ^{15}N-labeled *N*-nitrosodimethylamine (DMN†) and *N*-nitroso-*N*-methylaniline (NMA) both *in vitro* using S-9 rat liver preparations and *in vivo* [4, 5]. These data, which provide a measure of the α-hydroxylation pathway, revealed that, relative to total metabolism, the *in vivo* metabolism resulted in a significantly higher yield of $^{15}N_2$ for both compounds.

To investigate whether the isolated hepatocyte would be a better model for the intact animal than are subcellular preparations, we carried out a study of the metabolism of ^{15}N-labeled DMN and NMA by isolated hepatocytes. This system has been shown previously to metabolize DMN to an alkylating agent [6], and *N*-nitrosopyrrolidine to CO_2 [7]. The results of the metabolism studies as well as a correlation with the *in vivo* and *in vitro* data are reported in this paper.

* Author to whom correspondence should be addressed.

† Abbreviations: DMN, *N*-nitrosodimethylamine; NMA, *N*-nitroso-*N*-methylaniline; NMU, *N*-nitroso-*N*-methylurea; DEN, *N*-nitrosodiethylamine; and HPLC, high pressure liquid chromatography.

MATERIALS AND METHODS

Materials. Chemicals were obtained from the following sources: ^{15}N-labeled aniline, sodium nitrite and dimethylamine hydrochloride from Stohler Isotope Chemicals (Waltham, MA); trypan blue and Hanks' balanced salt solution from Flow Laboratories (McLean, VA); the primary standard gas mixture (analyzed) of 0.5% (wt/wt) neon and 5% CO_2 in O_2 from Matheson (Dorsey, MD); the primary standard gas mixture of 0.5% $^{15}N_2$ and 0.5% Ne in O_2 from Scott Speciality Gases (Plumsteadville, PA); and diethylnitrosamine and other general reagents from the Sigma Chemical Co. (St. Louis, MO). ^{15}N-Labeled *N*-nitrosomethylurea (NMU) was a gift of Dr. Peter N. Magee (Fels Research Institute, Philadelphia, PA).

Preparations of labeled nitrosamines. The ^{15}N-labeled nitrosamines were synthesized and purified as described previously [4]. Both products showed 99% enrichment in each nitrogen.

Animals. All animals used in these experiments were 10–12-week-old male Fisher F-344 rats obtained from the NCI-FCRF rodent colony. They were given free access to food and water until they were killed.

Preparation and incubation of isolated hepatocytes. Hepatocytes were prepared as previously described [7]. Yields of 1–3 × 10^8 cells/liver with viabilities of 84–90% as determined by trypan blue exclusion were obtained from different preparations. In a typical experiment, 6 ml of hepatocytes (5 × 10^6 viable cells/ml) and 1.5 ml of 5 mM ^{15}N-labeled DMN, NMA, or NMU (final concentration 1 mM) were incubated in 25 ml bulbs equipped with Teflon high-vacuum stopcocks. Bulbs containing the reaction mixtures were cooled to 0° and attached to a high-vacuum line (described previously [4]). Control experiments, in which the bulbs contained media and nitrosamine but no hepatocytes, were carried out in parallel. The control samples were treated in the same way as

the experimental samples. Preliminary experiments were also carried out using unlabeled substrate to ensure that the effects of placing the bulbs under vacuum did not alter the hepatocyte viability.

Each sample bulb was placed under reduced pressure, and the standard gas mixture of 0.5% (wt/wt) neon and 5% carbon dioxide in O_2 was backfilled into the bulb. This procedure was repeated twice to remove residual nitrogen, and the bulb was sealed. After incubation at 37° for 2 hr in a water-bath shaker, the sample bulb was cooled to 0° and was attached to the vacuum line. The gaseous contents were transferred to a 25 ml gas bulb with the aid of a Toepler pump, and the pressure was adjusted to atmospheric in order that equal total amounts of gas for each determination were used in the analysis.

Gaseous nitrogen determinations. The quantification of labeled nitrogen production was accomplished with the aid of an internal standard. Neon was chosen for this purpose because of its low natural abundance and its close proximity in mass to $^{15}N_2$ (m/z, 30). The absolute quantity of neon added to each reaction was known accurately, since the volume of the reaction vessel had been determined previously.

The gas samples were expanded into the 75 ml chamber of a gas inlet assembly connected to a VG-Micromass ZAB-2F mass spectrometer. The gas inlet was a modified version of the VG-Micromass design and was built inhouse. The Peak Matching Unit was used to monitor the two masses (^{20}Ne at m/z 19.9924 and $^{15}N_2$ at m/z 30.0002) by voltage sweep across a mass window size equal to three peak widths (i.e. 200 ppm) and a dwell time of 6 sec. The instrument was operated in the electron impact mode at a resolution of 15,000 using a 70 eV electron energy beam, 100 μA trap current, and a source temperature of 200°. The energy of the ^{20}Ne ions was 8 keV. The response factors were obtained by measuring the relative intensities of the Ne and $^{15}N_2$ signals output on a chart recorder using 100 mV full scale deflection (FSD) sensitivity range. The pen was adjusted so that the most intense signal was at least 50% FSD.

Known volumes of primary standards of 0.5% Ne and 0.5% $^{15}N_2$ in O_2 and 0.5% Ne in O_2 were mixed to provide secondary standards used to calibrate the mass spectrometer in order to eliminate differences in mass discrimination through the molecular leak and ionization efficiencies. The same gas volume and pressure were used for all the standards as well as the reaction gas mixtures. In all experiments, the $^{14}N_2$ peak was measured to ensure that there was not an unexpectedly large amount of naturally occurring $^{15}N_2$ resulting from a leak during incubation or gas transfer.

A bulb of the primary standard of 0.5% $^{15}N_2$ and 0.5% Ne in O_2 was examined just prior to each secondary standard or sample. Ten measurements of each ion intensity ($^{15}N_2$ and Ne) were averaged, and the ratios of $^{15}N_2$ to Ne were determined. These ratios were then normalized to the primary standard ($^{15}N_2$/Ne) ratio. These normalized ratios were plotted versus the percentage of $^{15}N_2$ in the secondary standards to form the calibration curve.

Determination of nitrosamine loss. Following the transfer of gas from the reaction bulbs, 3.75 ml of a saturated solution of $Ba(OH)_2$ was added. Hepatocytes were disrupted by sonication for 15 sec, after which 375 μl of 2.7 mM *N*-nitrosodiethylamine (DEN) (used as an internal standard in the HPLC analysis) and 3.75 ml $ZnSO_4$ were added [7, 8] to precipitate the protein. After cooling to 0°, the precipitate was removed by centrifugation at 8000 *g* for 10 min.

The filtrates were analyzed immediately for loss of substrate by HPLC using a Whatman Partisil PXS 10/25 ODS 10 μm column and a Laboratory Data Control (LDC) pumping system; the column elution conditions depended on the substrate being analyzed. For DMN, the column was developed at 1 ml/min with H_2O/CH_3CN, 85:15 (v/v). Under these conditions, the retention time for DMN was 4.6 min and 6.9 min for DEN. When NMA was the substrate, the column was eluted at 1 ml/min with a 12 min linear gradient from 100% H_2O to 100% CH_3CN. In this case, the retention time for DEN was 8.6 min and 12.5 min for NMA. Samples were introduced through a 100 μl loop, and compounds were detected by u.v. absorbance at 254 nm on an LDC UVII detector. Analytical data were processed with a Hewlett–Packard 3354 data system interfaced to the instrument. For each sample, two or three injections were used to determine the area of the nitrosamine peak. The ratio of the areas of DEN standard of the control and hepatocyte reactions was determined. This was the normalization factor. This ratio was multiplied by the areas of DMN (or NMA) after the 2-hr reaction. The fraction of metabolism was then determined by subtracting the hepatocyte reaction area after 2 hr from the control reaction area and dividing by the latter.

RESULTS

Determination of the rate of metabolism of DMN and NMA. The metabolism of DMN and NMA by hepatocytes was monitored by high-pressure liquid chromatography on a standard reverse-phase column. The largest error in the precision in this measurement was found to be 11%. Shown in Table 1 are the values for the nitrosamine loss of 1 mM DMN and NMA during a 2-hr incubation with hepatocytes. Preliminary experiments to test the effect of vacuum on the isolated hepatocytes indicated that the viability of the hepatocytes was not affected. It must be pointed out that the hepatocytes themselves were never directly exposed to the vacuum since they were covered by the medium.

Determination of N_2. The measurement of $^{15}N_2$ was performed on a mass spectrometer using neon gas as an internal standard. Using known standard gas mixtures, it was found that neon was less efficiently ionized than $^{15}N_2$, but that the ratio of nitrogen to neon was directly proportional to the concentration (Fig. 1).

The quantity of $^{15}N_2$ produced in the isolated hepatocyte experiments is shown in Table 2. For DMN, the production of $^{15}N_2$ was 47% of the substrate lost; for NMA the value was 23%. The evolution of $^{15}N_2$ from labeled NMU was used as a positive control. Incubation of 1 mM NMU with the hepatocytes indicated that 105% of the theoretical $^{15}N_2$ was evolved.

Table 1. Analysis of nitrosamine metabolism by isolated hepatocytes

Substrate	Viability* (%)	Substrate loss (μmoles)	% Metabolism	% Metab. (average ± S.D.)
DMN	85	1.74	23.1	
	85	1.79	23.8	
	92	2.00	26.7	
	92	1.59	21.2	
	91	1.31	17.4	19.70 ± 4.23
	88	1.14	15.3	
	86	1.06	14.1	
	87	1.37	18.3	
	86	1.30	17.4	
NMA	89	4.33†	27.11	
	89	3.79†	23.68	
	84	2.17	28.96	26.13 ± 4.98
	84	2.18	29.02	
	86	2.52	33.61	
	85	2.58‡	20.60	
	85	2.49‡	19.90	

* Determined by trypan blue exclusion.
†,‡ The incubation volume of these reaction mixtures was 16.0 and 12.5 ml respectively; all other volumes were 7.5 ml.

Incubation was carried out for 2 hr, which was equivalent to about 10 half-lives [9]. In the control experiments containing only media and labeled nitrosamine, the amount of $^{15}N_2$ detected was negligible.

The gas mixtures from reactions using $^{15}N_2$-labeled nitrosamines were also examined mass spectrometrically for the presence of ^{15}N-labeled nitrogen oxides.

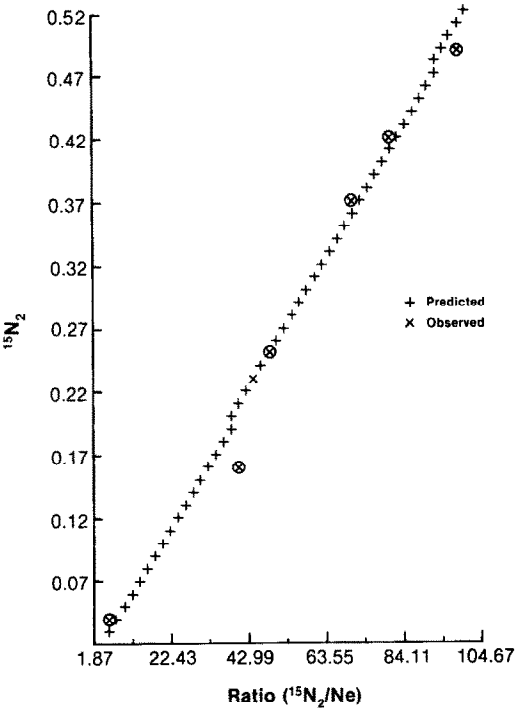

Fig. 1. Mass spectrometric correlation of the ratio of $^{15}N_2$/Ne to concentration, using known standard gas mixtures.

However, no other ^{15}N-labeled gaseous products were present in levels above background. Significantly, no isotopically mixed nitrogen (i.e. $^{15}N^{14}N$) was detected in levels above background.

DISCUSSION

The metabolism of carcinogenic nitrosamines has been studied extensively both *in vitro*, using tissue slices or cell-free preparations, and *in vivo*. However, only a few investigations have been carried out using the isolated hepatocyte to study the metabolic fate of nitrosamines [6, 7, 10], and these have focused on either the alkylation of DNA by DMN [6, 10] or the production of CO_2 and the identification of metabolites from *N*-nitrosopyrrolidine [7].

The present results clearly show that isolated hepatocytes optimally metabolize both DMN and NMA to the extent of 20 and 26% at 1 mM, respectively, as detected by loss of substrate. The apparent error in these measurements can be attributed to two causes: first, there is a variation from preparation to preparation in the ability of hepatocytes to metabolize the nitrosamines; and second, there is an inherent lack of precision in the HPLC method of analysis (in the worst case, the precision in the actual measurements was determined to be ± 11%). The values mentioned above, however, show an approximate 2-fold increase in the amount of metabolism as compared with the S9 reaction [4], when the values are compared on the basis of μmoles/g liver metabolized during 1 hr, applying a correction factor for the hepatocytes of 2×10^8 cells per 5 g average liver per animal. The number of hepatocytes is based on the average value obtained from the liver by our isolation procedure. It thus appears that isolated hepatocytes are an excellent system for inquiry into the metabolism of nitrosamines.

In addition, the positive control substrate, [$^{15}N_2$]-NMU, was cleanly decomposed to $^{15}N_2$, when the reaction was measured under the same conditions as

Table 2. Analysis of $^{15}N_2$ in the hepatocyte metabolism of labeled nitrosamines

Substrate	$^{15}N_2$ produced (μmoles)	% of Theory*	% of Theory (average ± S.D.)
DMN	0.900	51.9	
	0.917	51.3	
	0.652	32.6	
	0.647	40.8	
	0.629	48.1	47.41 ± 10.21
	0.711	62.0	
	0.648	61.2	
	0.524	38.2	
	0.529	40.6	
NMA	1.026†	23.64	
	1.261†	33.29	
	0.56	25.81	
	0.49	22.48	22.54 ± 6.55
	0.29	11.51	
	0.52‡	20.16	
	0.52‡	20.88	
NMU	8.58	104.03	104.77 ± 1.04
	8.70	105.50	

* Molecular nitrogen formed as compared to total metabolism.
†‡ The incubation volume of these reaction mixtures was 16.0 and 12.5 ml respectively; all other volumes were 7.5 ml.

those used for the nitrosamines. Our results, presented in Table 2, reveal that isolated hepatocytes are also able to metabolize ^{15}N-labeled DMN and NMA to $^{15}N_2$ as well, to the extent of 47 and 23% of theoretical, respectively, with no other discernable gaseous ^{15}N-containing products being detected. While such comparisons are somewhat risky, these values can be compared with our earlier reported findings on the production of $^{15}N_2$ from these same substances both *in vitro* using the S9 fraction [4] and *in vivo* [5]. In the former case, the values for 5 mM DMN and NMA were determined to be 33 and 19% of theory. For the latter, 67 and 52% of the injected dose of DMN or NMA was found to be metabolized to $^{15}N_2$. It can be readily seen that the results for hepatocyte metabolism of DMN are roughly halfway between those determined *in vitro* and *in vivo*, while for NMA the hepatocyte data closely parallel the *in vitro* situation. However, it must be pointed out that on a statistical basis the differences between the nitrogen formation from DMN in S9 and in hepatocytes may be very small. The reason for this is that each of the cited numbers is an average of multiple determinations which were subject to considerable individual variation. Nevertheless, the N_2 production during metabolism by hepatocytes was consistently higher than the N_2 production in the S9 fraction. This difference, however, could be a function of substrate concentration (5 mM for S9 vs 1 mM for the hepatocytes). The question of the influence of substrate concentration on nitrogen formation is an important one which requires additional experimental investigation.

In spite of these difficulties, there is precedent for substrate-dependent differences in metabolism by isolated hepatocytes, S9 and *in vivo*. Although it has been suggested that the metabolism of drugs in isolated hepatocytes correlates with *in vivo* drug metabolism better than does metabolism in the S-9 fraction [3], there are many exceptions. In fact, the relative rates of metabolism in hepatocytes vs S9 seem to be substrate specific. For example, the rates of metabolism of α-*l*-acetylmethadol, *d*-propoxyphene, and *N*,*N*-dimethylphenoxyethylamine are the same in the two systems, while the rates for *N*,*N*-dimethyl-*p*-chlorophenoxyethylamine, ethinimate, butamoxane, and 8-methoxybutamoxane are slower in hepatocytes [3]. The type of enzyme activity assayed also seems to play a role in the correlation. For example, for the isolated hepatocytes, N-demethylating activity is about 100%, hydroxylating activity 25%, and glucuronidating activity 50% of that of the crude homogenate [11]. For nitrosamines, the data are more limited. In the case when the ability of DMN to alkylate exogenous DNA was examined using isolated hepatocytes, it was found that alkylation occurred 7–10-fold less *in vitro* than in the intact animal, and this difference was not due to the ability of the hepatocytes to metabolize the added DMN [6].

It is apparent from our *in vitro* as well as *in vivo* data that a significant fraction of the metabolism of both DMN and NMA does not proceed through the generally accepted α-hydroxylation pathway (i.e. the diazonium ion is not the sole intermediate). Other metabolic reactions such as denitrosation have been suggested [12]. However, in our case, lack of detection of ^{15}N-labeled nitrogen oxides suggests that, if these are formed, they must undergo further reactions immediately. On the basis of our data, however, we cannot rule out the possible reduction of the NO group to the unsymmetrical hydrazine [13], although it is likely that N_2 would also be produced from the hydrazine. In the case of NMA, the phenyldiazonium ion could react by azo coupling with aromatic substrates (e.g. guanine residues [14]),

which may account for the low yields of nitrogen. The lack of detection of isotopically mixed nitrogen ($^{15}N^{14}N$) indicates that the phenyldiazonium ion does not react with free amino groups or proteins (e.g. lysyl residues). Were that to happen, the initial product of the coupling reaction would be a triazene, which would decompose to nitrogen, aniline, and other hydrolysis products. In the case of a ^{15}N-labeled NMA substrate, this nitrogen would be isotopically mixed ($^{15}N^{14}N$). However, this product was not detected in our experiments, so consequently that type of coupling cannot be important. A number of studies have indicated that multiple enzyme pathways may be involved in the metabolism of DMN [15–18], not all of them necessarily P-450-dependent processes. Some of these pathways also appear not to be compatible with the α-hydroxylation pathway [4, 19]. To this end, it is likely that at least part of the metabolism of DMN and NMA which does not proceed via the α-hydroxylation route is mediated by non-membrane bound enzymes, probably in the cytosolic fraction [4, 20]. However, the nature and function of these alternative enzymic pathways remain to be elucidated.

Acknowledgements—Research was sponsored by the National Cancer Institute, DHHS, under Contract No. NO1-CO-23909 with Litton Bionetics, Inc. The contents of this publication do not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. government.

REFERENCES

1. P. Th. Henderson and J. H. Dedwaide, *Biochem. Pharmac.* **18**, 2087 (1969).
2. R. E. McMahon, *Ann. N.Y. Acad. Sci.* **394**, 46 (1980).
3. R. E. Billings, R. E. McMahon, I. Ashmore and S. R. Wagle, *Drug Metab. Dispos.* **5**, 518 (1977).
4. M. B. Kroeger-Koepke, S. R. Koepke, G. A. McClusky, P. N. Magee and C. J. Michejda, *Proc. natn. Acad. Sci. U.S.A.* **78**, 6489 (1981).
5. C. J. Michejda, M. B. Kroeger-Koepke, S. R. Koepke, P. N. Magee and C. Chu, in *Banbury Report No. 12: Nitrosamines and Human Cancer* (Ed. P. N. Magee), p. 69. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982).
6. D. R. Umbenhauer and A. E. Pegg, *Chem. Biol. Interact.* **33**, 229 (1981).
7. J. G. Farrelly, M. L. Stewart and L. I. Hecker, *Chem. Biol. Interact.* **41**, 341 (1982).
8. M. Somogyi, *J. biol. Chem.* **160**, 69 (1945).
9. E. R. Garrett, S. Goto and J. F. Stubbins, *J. pharm. Sci.* **54**, 119 (1965).
10. D. R. Umbenhauer and A. E. Pegg, *Cancer Res.* **41**, 3471 (1981).
11. R. E. Billings, P. J. Murphy, R. E. McMahon and J. Ashmore, *Biochem. Pharmac.* **27**, 2525 (1978).
12. K. E. Appel, D. Shrenk, M. Schwartz, B. Mahr and W. Kunz, *Cancer Lett.* **9**, 13 (1980).
13. S. Grilli and J. Prodi, *Gann* **66**, 473 (1975).
14. N. K. Kochetkov and E. I. Budovskii (Eds.), *Organic Chemistry of Nucleic Acids*. Plenum Press, New York (1972).
15. B. G. Lake, J. C. Phillips, C. E. Heading and S. D. Gangolli, *Toxicology* **5**, 297 (1976).
16. H. M. Godoy, M. I. Diaz Gomez and J. A. Castro, *J. natn. Cancer Inst.* **61**, 1285 (1978).
17. M. B. Kroeger-Koepke and C. J. Michejda, *Cancer Res.* **39**, 1587 (1979).
18. B. G. Lake, M. A. Collins, R. A. Harris, J. C. Phillips, R. C. Cottrell and S. D. Gangolli, *Xenobiotica* **12**, 435 (1982).
19. R. C. Cottrell, B. G. Lake, J. C. Phillips and S. D. Gangolli, *Biochem. Pharmac.* **26**, 809 (1977).
20. P. L. Skipper, J. F. Tomera, J. S. Wishnok, H. Brunengraber and S. R. Tannenbaum, *Cancer Res.* **43**, 4786 (1983).