Biochemical Pharmacology, Vol. 25, pp. 1921–1926. Pergamon Press, 1976. Printed in Great Britain.

PRELIMINARY COMMUNICATIONS

MICROSOME-MEDIATED METHYLATION OF DNA BY N,N-DIMETHYLNITROSAMINE *IN VITRO*

Arthur E. Chin and H. Bruce Bosmann

Department of Pharmacology and Toxicology
University of Rochester School of Medicine
and Dentistry
Rochester, New York 14642

(Received 10 April 1976; accepted 10 May 1976)

Efforts to elucidate the enzyme(s) responsible for the demethylation of the carcinogen N,N-dimethylnitrosamine (DMN) have not been conclusive. Although it is generally agreed that the metabolism of DMN appears to occur exclusively in the microsomal fraction (105,000 x g pellet) of the cell, there has been much debate on whether the microsomal enzyme(s) responsible for this demethylation are dependent on cytochrome P-450. Previous investigations into the hepatic microsomal metabolism of DMN *in vitro* showing a requirement for NADPH and molecular oxygen[1] suggested that DMN is N-demethylated by enzymes of the mixed function oxidase system dependent on cytochrome P-450.[2,3] Subsequent studies showed that demethylation of DMN to formaldehyde was inhibited by carbon monoxide,[4] and could be mediated by a reconstituted microsomal cytochrome P-450-dependent enzyme system.[5]

Another approach in elucidating the enzyme(s) responsible for the metabolism of DMN has been to utilize various inducers and inhibitors of the microsomal mixed function oxidases. As the literature has borne out, these modifiers of enzyme activities did not always produce the expected effect on DMN metabolism that one would predict from the actions of these compounds on the metabolism of substrates known to be metabolized by the mixed function oxidases. The use of the classic inducers of the mixed function oxidases, sodium phenobarbital (PB) and 3-methylcholanthrene (3-MC), has yielded either inconsistent or anomalous results. PB, which increases the amounts of NADPH-cytochrome c reductase and cytochrome P-450,[6-8] has been known to increase the rate of drug hydroxylation[6,9] and, therefore, drug metabolism. The results of several researchers,[10,11] showing that the rate of metabolism of DMN to formaldehyde (and methanol, ref. 10) was more than doubled in the hepatic postmitochondrial fraction from PB-pretreated rats, are in agreement with the known inductive effect of PB on the mixed function oxidases. These results tend to support metabolism of DMN by the mixed function oxidases. In contrast, Venkatesan et al.[12] demonstrated that DMN demethylase activity (as measured by formation of formaldehyde) was, in fact, decreased in PB-pretreated animals. In an attempt to correlate their previous finding of an increased demethylation of DMN to formaldehyde and/or methanol by microsomes *in vitro* from PB-pretreated rats[10] with the rate of *in vivo* metabolism of [^{14}C]DMN (as measured by expiration of $^{14}CO_2$) by rats also pretreated with this inducer, Lake et al.[13] found that PB-pretreated animals did not display an increase in the *in vivo* rate of metabolism of DMN to CO_2. Since this inductive effect *in vitro* was not reflected in the metabolic rate of DMN *in vivo*, they questioned the relevance of hepatic DMN demethylase activity, as measured by formaldehyde generation, as an index of the metabolism of DMN *in vivo*.

These inconsistent results obtained with PB are matched by the anomalous result obtained with 3-MC and pregnenolone-16α-carbonitrile (PCN). 3-MC, a well known inducer of the de novo synthesis of N-demethylases,[14-16] would be expected to enhance the metabolism of DMN by increasing the rate of demethylation. On the contrary, the demethylation of DMN to formaldehyde by liver microsomes from 3-MC-pretreated animals was found to be inhibited by several workers.[12,17,18] These results were, in turn, in disagreement with those of Frantz et al.,[11] who demonstrated an increase in rat and mouse liver DMN demethylation to formaldehyde by microsomes isolated from 3-MC-pretreated animals. Furthermore, Somogyi et al.[19] and Arcos et al.[20,21] found that PCN significantly lowered DMN demethylation to formaldehyde without having any corresponding effect on DMN concentration in the liver or disappearance of DMN from the blood.

The inconsistencies in the above studies suggest one of two possibilities. Either DMN metabolism is not mediated solely by the mixed function oxidases or the use of formaldehyde as a measure of DMN metabolism is not completely valid. Since the evidence of the studies cited above favors DMN metabolism by the mixed function oxidases, the second alternative must be considered.

N-demethylation of DMN is postulated to proceed by an enzymatic hydroxylation of one of the methyl groups followed by nonenzymatic cleavage of the "hydroxylated" methyl group giving rise ultimately to diazomethane. Since this "hydroxylated" DMN molecule is postulated to be unstable (hence its nonenzymatic decomposition) and diazomethane is known to be unstable, these postulated intermediates have never been isolated. Thus, it is not known with any certainty whether hydroxylation of DMN does indeed occur, although there are good reasons to believe it does. Formation of formaldehyde, as well as methanol[10,22] from the interaction of DMN with microsomes in vitro has been demonstrated, but how many reaction steps exist between the initial reaction of an enzyme with DMN and the products that are seen (i.e. aldehyde, alcohol, etc.) is not known because of this uncertainty as to the pathway(s) by which DMN is metabolized. Formaldehyde is postulated to be derived directly from the N-demethylation of DMN. However, the finding by Lake et al.[10,23,24] that pyrazole, a competitive inhibitor of alcohol dehydrogenase (EC 1.1.1.1), and 3-amino-1,2,4-triazole, a specific inhibitor of catalase (EC 1.11.1.6), inhibited the formation of both formaldehyde and methanol from DMN led these authors to suggest that a complex sequence of enzymatic steps unrelated to the mixed function oxidase system is involved in the hepatic degradation of DMN to formaldehyde and methanol. This again stresses the inability to conclude what products are derived directly from the N-demethylation of DMN because of the lack of knowledge of how this N-demethylation comes about.

Because of the inconsistencies found in the studies with modifiers of the mixed function oxidases using formaldehyde formation as indicative of DMN metabolism, and the possibility that several enzymes are involved in the formation of formaldehyde from DMN,[10] one is left with a difficult task when trying to utilize the formation of formaldehyde from DMN to provide information on a possible initial "hydroxylation" of DMN. In this study we hoped, therefore, to provide an alternative in vitro system by which to study whether or not metabolism of DMN proceeds by an initial hydroxylation. The present communication reports the development of an in vitro microsome-mediated DNA methylation system in which DMN was the alkylating agent. This reaction was shown to have an absolute requirement for NADPH and to be dependent on intact microsomal enzymes.

Male Sprague-Dawley rats (Holtzman; 280-310 g) were killed by decapitation. The livers were quickly removed, placed in a beaker on ice and weighed separately. Rat liver microsomes and pH 5 enzymes were prepared by the method of Schneider and Kuff[25] as modified by Bosmann and Winston.[26] The purified microsomes were suspended in a 51.9 mM sodium phosphate buffer (pH 7.4) containing 34.7 mM EDTA and 0.694 mM $MgCl_2$ (designated as "Buffer 1"). pH 5 Enzymes, collected by centrifugation, were resuspended to a final concentration of 8 mg protein/ml with Buffer 1. pH 5 Enzymes contain amino acid activating enzymes and other proteins; they were included in the incubation mixture to ensure the presence of a complete microsomal system.

The incubation medium for the *in vitro* alkylation of calf thymus DNA by N,N-dimethylnitrosamine contained 49.8 µmol sodium phosphate buffer (pH 7.4), 33.3 µmol EDTA (Fisher Scientific), 0.808 mg calf thymus DNA (Type 1; Sigma), 0.805 µmol NADPH (Type III; Sigma), 3.3 µmol GSH (Sigma), 0.666 µmol $MgCl_2$, 0.4 ml of microsomal suspension (suspending microsomes from 1 g of liver in 1.4 ml of Buffer 1), and 0.04 ml of pH 5 enzymes. For determination of binding of ^{14}C-label to proteins, the incubation mixture minus exogenously added calf thymus DNA (an equal volume of Buffer 1 was substituted for the DNA in the incubation medium) was used. To this incubation mixture was added 40 µl (1 µCi; corresponding to 0.1 µmol) of N,N-di[^{14}C]methylnitrosamine (Amersham/Searle; 7.5 Ci/mol). The total volume of the incubation mixture was 1 ml. Each different incubation condition (i.e. "complete," minus DNA, etc.) was run in triplicate.

After incubation at 37°C for 60 min, DNA was isolated according to a modification of the procedures of Marmur[27] and Howell.[28] Briefly, the incubation mixture was made 0.15% with respect to NaCl. Protease (Type VI; Sigma), 100 µg/ml incubation volume, and ribonuclease A (Type 1-A; Sigma), 50 µg/ml incubation solution, were then added, and this mixture was incubated for 1 h at 37°C with shaking. An equal volume of chloroform:isoamyl alcohol (24:1) was added, and the mixture was shaken for 20 min at room temperature and centrifuged at 2500 x *g* for 5 min. The top aqueous layer was carefully isolated. The bottom organic phase was re-extracted with an equal volume of Buffer 1 made 0.15% with respect to NaCl by shaking for 20 min at room temperature. This suspension was then centrifuged at 2500 x *g* for 5 min. The top aqueous phase was isolated and combined with the previously isolated aqueous layer. To the combined aqueous phases two volumes of ice-cold ethanol (95%) was added. This mixture was allowed to stand overnight at 4°C. Precipitated DNA was isolated by centrifugation and washed twice with ice-cold ethanol (75%), once with ice-cold ethanol (99%), and once with absolute ethyl ether. The DNA pellet was then dried in a desiccator. The pellet was then dissolved in 0.7-1.0 ml glass-distilled water. Chemical analysis for DNA was according to the procedure of Hinegardner.[29] Radioactivity in the various DNA or protein fractions was determined by plating 0.2 ml of the solutions on glass fiber filters and counting by liquid scintillation.[26] Protein determinations were made by the procedure of Lowry et al.[30]

The enzymatic nature of this *in vitro* binding of ^{14}C-label to exogenous calf thymus DNA and endogenous protein is illustrated in Table 1. Boiling of the microsomes results in a complete inhibition of this binding of ^{14}C-label to DNA and/or protein in this system, which would suggest that some type of enzymatic reaction is occurring. It should be noted that the cpm/mg protein and cpm/mg DNA cannot be compared because a different data base is used in each instance. No information is available on the relative number of sites available in the "protein" or "DNA" fraction. Table 1 illustrates the susceptibility of both exogenous calf thymus DNA and endogenous protein to "alkylation" by DMN in this *in vitro* microsomal system. It is important to observe, however, that DNA, the cellular genome macromolecule, is highly methylated in the complete system by the [^{14}C]DMN.

Table 1. Methylation of protein and exogenously added DNA by N,N-di[^{14}C]methylnitrosamine utilizing a rat liver microsomal fraction

	^{14}C from [^{14}C]DMN onto protein (cpm/mg protein)	^{14}C from [^{14}C]DMN onto DNA (cpm/mg DNA)
Complete system	5700	4000
	8600	2900
	--	2600
Boiled microsomes	230	210
	340	70

The system for the in vitro microsome-mediated "alkylation" of exogenous calf thymus DNA or protein macromolecules in the microsomal fraction by DMN is as described in the text. Each value in the table represents a single observation of combined triplicate samples. The "complete" microsome system for the in vitro metabolism of DMN to an alkylation intermediate and the resulting alkylation of calf thymus DNA or protein was as follows: for ^{14}C-labeling of DNA, 49.8 µmol sodium phosphate buffer (pH 7.4), 33.3 µmol EDTA, 0.808 mg DNA, 0.805 µmol NADPH, 3.3 µmol GSH, 0.666 µmol $MgCl_2$, 0.4 ml microsomal suspension (suspending microsomes from 1 g of liver in 1.4 ml Buffer 1) and 0.04 ml pH 5 enzymes (8 mg protein/ml); for binding of ^{14}C-label to proteins, an equal volume of Buffer 1 was substituted for the calf thymus DNA in this previously described incubation mixture. Three such mixtures were incubated for 60 min at 37°C. At the end of the incubation these triplicate samples were combined and DNA extracted, as described. This resulting pellet was dissolved and analyzed for radioactivity and DNA and protein content. In the "boiled" microsome incubation, microsomes suspended in Buffer 1 were heated in a boiling water bath (100°C) for 10 min and substituted for untreated microsomes as enzyme source. The dash (--) indicates experiment was not performed. It should be noted that the reported cpm/mg DNA represents radioactivity bound only to the exogenously added DNA while cpm/mg protein represents radioactivity bound only to protein in the added microsomal fraction.

The dependence of this binding of ^{14}C-label from N,N-di[^{14}C]methylnitrosamine by DNA on NADPH is dramatically shown in Table 2. This dependency on NADPH is consistent with the mixed function oxidases as mediators of the demethylation of DMN. It is important to note that these data show that the "activation" of N,N-dimethylnitrosamine to a methylating intermediate is absolutely dependent on NADPH and that the methyl group can be transferred to exogenously added DNA.

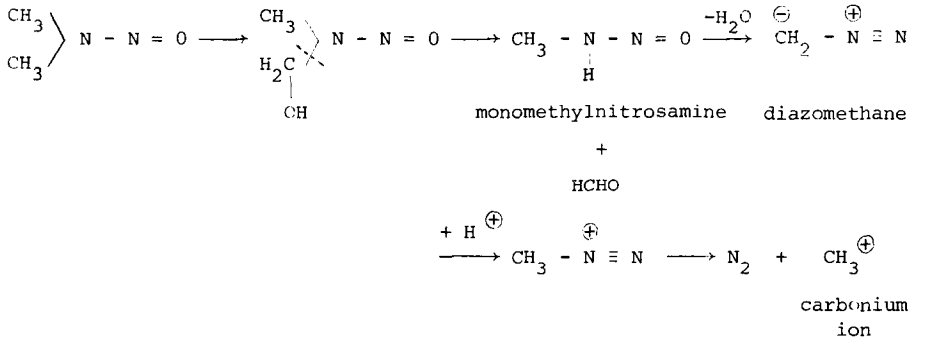
Table 2. Effect of NADPH on methylation of exogenously added DNA by N,N-di[^{14}C]methylnitrosamine utilizing a rat liver microsomal fraction

	^{14}C from [^{14}C]DMN (cpm/mg DNA)	% Decrease in bound radioactivity
Complete system	2600	0
Minus NADPH	20	99
	30	99

Experiments were performed as given in the text and in the legend to Table 1 except that for the "minus NADPH" incubation an equal volume of Buffer 1 was substituted for the NADPH in the incubation medium; cpm/mg DNA represents radioactivity bound only to the exogenously added DNA.

Although not known for certain, it is thought that alkylation of biological macromolecules such as DNA or proteins has an important role in the process of chemical carcinogenesis. In this study we have shown that incubation of ^{14}C-labeled N,N-dimethylnitrosamine with hepatic microsomes *in vitro* results in a binding of ^{14}C to exogenous calf thymus DNA and endogenous protein. This binding appears to be enzymatically mediated and dependent upon NADPH. If this *in vitro* metabolism of DMN proceeds in a fashion similar to what occurs *in vivo*, then it would be safe to assume that this binding of ^{14}C-label to biological macromolecules *in vitro* is due to a methylation process. This, however, remains to be shown by isolation and identification of specific methylated nucleotides from what we believe is methylated DNA.

As an alternative to a methylation process, binding of formaldehyde generated from DMN may also be occurring. However, there are several reasons why we do not believe this to be the case. The postulated mechanism by which DMN is metabolized is as follows (see ref. 31):



If one assumes this to be correct, two moieties are formed in the metabolism of DMN which could conceivably bind to biological macromolecules (i.e. the carbonium ion or formaldehyde).

Methylated bases (i.e. N^7-methylguanine, O^6-methylguanine, etc.) have been isolated from the nucleic acids from animals treated *in vivo* with DMN[32,33] as well as tissue slices *in vitro*.[34] Since we do not know of any mechanism by which an aldehyde (i.e. formaldehyde) can be incorporated into nucleic acid as a methyl group covalently bound to the bases of nucleic acids, this would suggest to us that formaldehyde derived from [^{14}C]DMN would not be responsible for the ^{14}C-label associated with these methylated bases. Again, we stress that isolation of methylated bases from our ^{14}C-labeled DNA would verify this assumption. In any event, we have demonstrated conclusively an NADPH-dependent enzyme-mediated transfer of ^{14}C-label from [^{14}C]DMH to calf thymus DNA *in vitro* which could represent a methylation process. This alkylation of DNA may be the basis for the carcinogenic effect of N,N-dimethylnitrosamine and, by implication, nitrites and other precursors of nitrosamines.

Acknowledgments. This study was supported in part by grants GM-15190 and GM-01781. H.B.B. is a scholar of the Leukemia Society of America. We thank Mr. K. R. Case, Dr. A. C. Spataro, and Dr. I. Evans for helpful criticism and discussion.

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