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FACTORS INFLUENCING THE MICROSOME- AND MITOCHONDRIA-CATALYZED *IN VITRO* BINDING OF DIETHYLNITROSAMINE AND *N*-NITROSOPIPERIDINE TO DEOXYRIBONUCLEIC ACID

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Abstract—[^{14}C]Diethylnitrosamine ([^{14}C]DEN) and [^{14}C]*N*-nitrosopiperidine ([^{14}C]NPiP) bind covalently to calf thymus DNA in an *in vitro* incubation system containing rat liver microsomes. The reaction is NADPH-dependent. Pretreatment of the animals with phenobarbital (PB) enchances the binding of both DEN and NPiP to DNA, whereas the binding of DEN to DNA decreases after 3-methylcholanthrene pretreatment. The PB effect, as observed from the binding of DEN to DNA, is more pronounced in young rats than in the older animals. Addition of cytosol to the incubation system enhances the binding of DEN 3- to 4-fold and the binding of NPiP 2- to 3-fold. Addition of mitochondria to the incubation system increases the binding of [^{14}C]DEN only slighty, but increases the binding of NPiP more than 5-fold. Addition of mitochondria has no effect on the binding of [^{14}C]dimethylnitrosamine ([^{14}C]DMN). Mitochondria alone markedly catalyze the binding of NPiP to DNA. Addition of benzylamine, which is a substrate of mitochondrial monoamine oxidase as well as an inhibitor of DMN-demethylase, inhibits the binding of NPiP catalyzed by microsomes and microsomes plus mitochondria.

In addition to DMN,† some higher nitrosamines and their precursors are present in many processed foods [1], tobacco and its smoke [2], agricultural chemicals and cosmetics [3], as well as in urban air [4] and drinking water [5]. The salient biological effects of nitrosamines (carcinogenicity, mutagenicity and toxicity) have been well established [6,7].

It is generally accepted that both the dialkyl and cyclic nitrosamines require metabolic activation by microsomal mixed-function oxidases [8, 9] to display their carcinogenic or mutagenic activities. The critical initial step in the activation has been hypothesized to be an enzymatic hydroxylation at the α-carbon atom [6], although hydroxylation at carbon atoms other than the α-position is possible [10–13]. The overall process results in dealkylation (or opening of the ring) and formation of an alkylating intermediate which is presumably responsible for their biological activities [14, 15]. Evidence for the formation of an alkylating intermediate was first provided by Magee and Farber [16] who demonstrated the methylation of nucleic acid and protein in the carcinogenesis target tissues of DMN. Alkylation of DNA and RNA in rat tissues by other dialkyl and cyclic nitrosamines has also been reported [10, 11, 17, 18]. However, Lijinsky *et al.* [19, 20] have recently failed to detect alkylation in DNA of rats treated with several cyclic nitrosamines and reported that the positive results in their earlier studies on alkylation of RNA were produced by contaminants. Moreover, the degree of alkylation by some nitrosamines appears not to correlate with their carcinogenic activity [18–22]. The chemical nature of the alkylating intermediates is unclear and remains to be elucidated.

Binding of polycyclic hydrocarbons and other carcinogenic compounds to exogenous DNA has largely been studied using *in vitro* incubation systems [23–25]. However, *in vitro* studies on a nitrosamine have been reported only recently by Chin and Bosman [26] and by Grilli *et al.* [27], showing microsome-mediated methylation of exogenously added DNA by DMN. In the present report, *in vitro* systems were used to study the binding of [^{14}C]DEN and [^{14}C]NPiP, a higher dialkyl and cyclic nitrosamine, respectively, to exogenous DNA.

MATERIALS AND METHODS

Chemicals. [^{14}C]DMN (sp. act. 26 mCi/mmole), [*ethyl*-1-^{14}C]DEN (sp. act. 13.5 mCi/mmole) and [2,6-^{14}C]NPiP (sp. act. 18.8 mCi/mmole) were purchased from New England Nuclear (Boston, MA); their radiochemical purity was greater than 97 per cent. All labeled nitrosamines were stored at −20° in the dark. Unlabeled DMN was obtained from the Aldrich Chemical Co. (Milwaukee, WI); unlabeled DEN was from Eastman (Rochester, NY), calf thymus DNA, ribonuclease A and benzylamine from Sigma (St. Louis, MO) and pronase from Cal-Biochem (San Diego, CA). All chemicals were reagent grade.

Treatment of animals. Male Sprague–Dawley rats (Holtzman, Madison, WI) weighing 120–180 g or 235–280 g were used. They were fed Purina laboratory chow and water *ad lib.* PB was dissolved in 0.9% saline and injected i.p. at 80 mg/kg body wt

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† Abbreviations used are: DMN, dimethylnitrosamine; DEN, diethylnitrosamine; NPiP, *N*-nitrosopiperidine; PB, phenobarbital; and 3-MC, 3-methylcholanthrene.

daily for four consecutive days, the last injection preceding sacrifice by 24 hr. Controls received only saline. The injection volume was 5 ml/kg body wt. 3-MC at 40 mg/kg body wt was administered in a single i.p. injection in corn oil 24 hr prior to sacrifice. Controls were given only corn oil. The injection volume was 10 ml/kg body wt.

Preparation of microsomes. The rats were decapitated and the livers were quickly removed and weighed. Liver homogenates (50%, w/v) were prepared in ice-cold 0.25 M sucrose–0.1 M sodium phosphate buffer (pH 7.4) with a glass–teflon homogenizer. Nuclei and mitochondria were removed by two consecutive centrifugations at 18,000 *g* for 10 min. The post-mitochondrial supernatant fluid was then centrifuged in a Spinco ultracentrifuge at 105,000 *g* for 60 min to sediment the microsomes. The resultant supernatant fluid was designated as cytosol. The microsomal pellet was suspended in ice-cold 50 mM sodium phosphate buffer (pH 7.4). Aliquots were taken for the protein determinations following the method of Lowry *et al.* [28]. All steps in the isolation of microsomes were carried out at 1–4°.

Preparation of mitochondria. Mitochrondria were isolated according to the method of Wu *et al.* [29] with some modifications. Liver homogenates (30%, w/v) were prepared in ice-cold 0.25 M sucrose–0.1 M sodium phosphate buffer (pH 7.4) with a glass–teflon homogenizer. The homogenate was centrifuged twice at 800 *g* for 10 min to remove the nuclei and cell debris. The supernatant fluid was then centrifuged twice at 12,000 *g* to sediment the mitochondria. The mitochondrial pellets were washed twice by homogenizing in 0.25 M sucrose–0.1 M sodium phosphate buffer (pH 7.4) and then centrifuged. The final pellets were suspended in 50 mM sodium phosphate buffer (pH 7.4) and aliquots were taken for the protein determinations following the method of Lowry *et al.* [28].

In vitro *binding to DNA.* This was studied following an adaptation of the method of Gelboin [23] and Woo *et al.* [30]. The complete system contained, in a total volume of 3.0 ml, the following components: 50 mM sodium phosphate buffer (pH 7.4), 100 mM sodium EDTA, 2 mg calf thymus DNA, microsomes equivalent to 4.5–5.0 mg of microsomal protein, an NADPH-generating system (3.33 mM glucose-6-phosphate, 0.33 mM NADP and 1 unit glucose-6-phosphate dehydrogenase) and 0.15 mM [^{14}C]DMN (sp. act. 6.5 mCi/mmole) or 0.15 mM [^{14}C]DEN (sp. act. 9.0 mCi/mmole) or 0.15 mM [^{14}C]NPiP (sp. act. 18.8 mCi/mmole). To examine the dependence on NADPH of the ^{14}C-binding to DNA, the incubation mixture minus the NADPH-generating system was used. In some experiments, cytosol (approximately 20 mg protein) or mitochondria (approximately 3.0 mg protein) was included in the incubation mixture together with microsomes. In one series of experiments only mitochondria were used, in lieu of microsomes. When benzylamine was used, the concentration was 1.0 mM. Incubation was carried out at 37° for 60 min with shaking under subdued light.

DNA was re-isolated from the incubation medium by an adaptation of the procedure of Gelboin [23] as modified by Wu *et al.* [29]. In order to remove tightly bound RNA and protein, the latter modification includes RNase and pronase treatment, which was shown [31] to yield DNA of high purity. Thus the isolated DNA was redissolved in 0.1 × SSC (0.15 M sodium chloride–0.015 M sodium citrate, pH 7.0) and treated with preheated ribonuclease A (50 μg/ml) for 30 min and pronase (100 μg/ml) for 60 min at 37°, and then re-isolated again. The final purified DNA was redissolved in 0.1 × SSC and aliquots of the solution were taken for quantitation of DNA by the diphenylamine method of Burton [32] and for measurement of radioactivity. Counting of radioactivity was carried out in a Beckman liquid scintillation counter (LS-233), using external standardization for quench correction.

RESULTS

Some requirements of the *in vitro* binding of ^{14}C-labeled DEN and NPiP to exogenous calf thymus DNA in the presence of rat liver microsomes are shown in Table 1. The data indicate that both DEN and NPiP become bound to calf thymus DNA in the *in vitro* incubation system. It should be noted that the levels of binding of DEN and of NPiP given in

Table 1. Requirements for hepatic microsome-catalyzed *in vitro* binding of [^{14}C]diethylnitrosamine and [^{14}C]*N*-nitrosopiperidine to DNA*

System	[^{14}C]DEN (dis./min/mg DNA)	[^{14}C]NPiP (dis./min/mg DNA)
Complete system	123 ± 9 (4)	157 ± 6 (4)
Complete system, minus NADPH-generating system	16 ± 3 (4)	45 ± 6 (4)
Complete system, plus cytosol	371 ± 33 (4)	405 ± 40 (4)
Complete system, plus mitochondria	171 ± 17 (3)	671 ± 30 (4)

* The complete system contained, in a total volume of 3.0 ml, the following: 50 mM sodium phosphate (pH 7.4), 100 mM sodium EDTA, 2 mg calf thymus DNA, 4.5–5.0 mg microsomal protein, an NADPH-generating system (3.33 mM G-6-P, 0.33 mM NADP, and 1 unit of G-6-P dehydrogenase), 0.15 mM [^{14}C]DEN (sp. act. 9.0 mCi/mmole) or 0.15 mM [^{14}C]NPiP (sp. act. 18.8 mCi/mmole). The amounts of cytosol and mitochondria added were equivalent to approximately 20 mg protein and 3.0 mg protein respectively. Incubation was carried out at 37° for 60 min. The procedures for isolation and purification of DNA are referred to in Materials and Methods. Values represent means ± S.E.; numbers in parentheses refer to the number of determinations.

Table 2. Effects of phenobarbital and 3-methylcholanthrene pretreatment on the hepatic microsome-catalyzed binding of [^{14}C]diethylnitrosamine to DNA*

Pretreatment	Without cytosol		With cytosol		
	pmoles/mg DNA†	Percent	pmoles/mg DNA†	Percent	Cytosol effect‡
Control§	3.5 ± 0.4 (8)	100	15.6 ± 2.5 (4)	100	4.4
PB§	7.1 ± 0.9 (4)	203	31.9 ± 4.5 (4)	205	4.5
3-MC§	2.1 ± 0.4 (4)	61	3.7 ± 0.2 (4)	23	1.7
Control‖	5.4 ± 0.4 (4)	100	17.9 ± 1.6 (4)	100	3.3
PB‖	25.0 ± 2.4 (3)	461	79.5 ± 10.6 (4)	444	3.2

* Rats were pretreated with saline or corn oil (control), PB or 3-MC according to the schedule described in Materials and Methods. The complete system (see legend to Table 1) containing 0.15 mM [^{14}C]DEN (sp. act. 9.0 mCi/mmole), with or without the addition of cytosol, was used. Incubation was carried out at 37° for 60 min. The procedures for isolation and purification of DNA are referred to in Materials and Methods.

† Values represent means ± S.E.; numbers in parentheses refer to the number of determinations.

‡ Ratio of *in vitro* binding in the presence of cytosol and in the absence of cytosol.

§ Rats weighing 235–280 g.

‖ Rats weighing 120–180 g.

this table cannot be compared because the specific activities of the two compounds used are different (their binding levels are given as pmoles/mg DNA in Tables 2 and 3). The binding reaction is dependent on NADPH, since in the absence of the NADPH-generating system the radioactivity is not significantly different from background. The requirement of NADPH is consistent with the involvement of a microsomal mixed-function oxidase system. However, this does not rule out the possibility that other oxidase(s) may be involved.

Addition of cytosol to the complete incubation system markedly enchanced the binding of [^{14}C]DEN, and to a lesser extent the binding of [^{14}C]NPiP, to DNA. Addition of mitochondria to the complete system increased only slightly the binding of [^{14}C]DEN to DNA. However, the binding of [^{14}C]NPiP to DNA was greatly increased, which is consistent with the presence of various oxidizing enzymes in mitochondria, including certain cytochrome P-450-dependent mixed-function oxidase(s) [33, 34]. On the other hand, mitochondria did not increase the binding of [^{14}C]DMN to DNA; expressed as dis./min/mg DNA, the radioactivity was 1333 ± 50 with the complete system in the absence of mitochondria and 1320 ± 67 with mitochondria.

Administration of PB and 3-MC is well known to induce many, and repress some, hepatic microsomal mixed-function oxidases [35, 36]. The effect of pretreatment of rats with PB and 3-MC on the binding of [^{14}C]DEN to DNA is presented in Table 2. The table shows that in older rats PB pretreatment doubled the binding of [^{14}C]DEN; on the other hand, 3-MC pretreatment reduced the binding values. Although cytosol only slightly enhanced the binding in the 3-MC-pretreated system, the binding was increased 4.4-fold in the control system and 4.5-fold in the phenobarbital-pretreated system due to the inclusion of cytosol. The table also shows that the effect of PB on the binding of DEN to DNA was more pronounced when young adult (120–180 g), rather than older rats (235–280 g), were used. The binding levels were 461 and 444 per cent compared to 203 and 205 per cent (Table 2) of the controls as observed in the absence and in the presence of cytosol respectively. Thus, the results obtained in this study parallel the finding that induction by PB of several microsomal enzymes was the highest in 33-day-old rats weighing 100–120 g [37]. The highest binding of [^{14}C]DEN to DNA was observed in these PB-pretreated animals with the inclusion of cytosol, since the binding was increased 3.2-fold in this system.

The *in vitro* binding of [^{14}C]NPiP to DNA in the

Table 3. Effects of phenobarbital pretreatment on the hepatic microsome-catalyzed binding of [^{14}C]*N*-nitrosopiperidine to DNA*

Pretreatment	Without cytosol		With cytosol		
	pmoles/mg DNA†	Percent	pmoles/mg DNA†	Percent	Cytosol effect‡
Control	2.7 ± 0.2 (4)	100	8.7 ± 1.0 (4)	100	3.2
PB	4.9 ± 0.7 (3)	181	10.1 ± 2.5 (3)	115	2.1

* Rats (120–180 g) were injected i.p. with 0.9% saline (control) or PB (80 mg/kg body wt) for four consecutive days prior to sacrifice. Microsomes were isolated from rat livers as described under Materials and Methods. The complete system (see legend to Table 1) containing 0.15 mM [^{14}C]NPiP (sp. act. 18.8 mCi/mmole), with or without the addition of cytosol, was used. Incubation was carried out at 37° for 60 min. The procedures for isolation and purification of DNA are referred to in Materials and Methods.

† Values represent means ± S.E.; numbers in parentheses refer to the number of determinations.

‡ Ratio of *in vitro* binding in the presence of cytosol and in the absence of cytosol.

untreated control system was 2.7 pmoles/mg DNA (Table 3). Cytosol was also effective, although to a lesser extent than with DEN, in enhancing the binding of NPiP to DNA; binding was increased 3.2-fold in the control system and 2.1-fold in the PB-pretreated system. Binding of [^{14}C]NPiP was only slightly enhanced by pretreatment with PB; the values were 115 and 181 per cent of the controls in the presence and in the absence of cytosol, respectively.

Considerable increase in the binding of [^{14}C]NPiP to DNA was noted when mitochondria were included together with microsomes in either the control or PB-pretreated system (Table 4); the increase observed was never less than 5- to 6-fold. Data not included in the table show that mitochondria alone, in the absence of microsomes, catalyze the binding of NPiP to the extent of 3.5 ± 0.1 pmoles/mg DNA. Thus, the combined effect of microsomes and mitochondria appears to be synergistic. Amine oxidase has recently been suggested by Lake *et al.* [38, 39] to be involved in the metabolism of another nitrosamine, DMN, *in vivo*, in addition to the microsomal cytochrome P-450-dependent mixed-function oxidase system. Most of the amine oxidase activity of rat liver, however, is located in the mitochondrial fraction [40]. Thus, in order to investigate if the combined effect of microsomes and mitochondria may be attributed to the presence of mitochondrial monoamine oxidase, the binding of NPiP was also studied with the inclusion of benzylamine, a typical monoamine oxidase substrate. The data in Table 4 indicate that addition of benzylamine to the system containing microsomes and mitochondria produces 39 and 45 per cent inhibition of binding in the control system and the PB-pretreated system, respectively. As the table shows, the binding catalyzed by microsomes alone is also inhibited by benzylamine (19 per cent in the control system and 35 per cent in the PB-pretreated system, respectively). This shows excellent consistency with the observation* that benzylamine is a very potent inhibitor of DMN-demethylase and of microsome-catalyzed *in vitro* binding of DMN to DNA (despite the absence of monoamine oxidase activity in the microsomes), and these effects are concentration dependent.

Since nuclei are involved in the metabolic activation of polycyclic hydrocarbons [41, 42], their potential role in the activation of DEN and NPiP has been studied. This was carried out by measuring the *in vitro* binding of [^{14}C]DEN and [^{14}C]NPiP to various nuclear components (DNA, RNA, histone, non-histone proteins, and soluble proteins). Preliminary results (D. Y. Lai *et al.*, unpublished observations), obtained by using essentially the method by Vaught and Bresnick [25], show no evidence for nucleus-catalyzed covalent binding of DEN and NPiP to any of the nuclear fractions.

Table 4. Effects of mitochondria on the hepatic microsome-catalyzed binding of [^{14}C]*N*-nitrosopiperidine to DNA*

Pretreatment	Microsomes		Microsomes + benzylamine		Microsomes + mitochondria		Microsomes + mitochondria + benzylamine		Mitochondrial effect‡
	pmoles/mg DNA†	Percent	pmoles/mg DNA†	Percent	pmoles/mg DNA†	Percent	pmoles/mg DNA†	Percent	
Control	2.7 ± 0.2 (4)	100	2.2 ± 0.1 (3)	100	15.1 ± 0.7 (4)	100	9.2 ± 0.9 (3)	100	5.6
PB	4.9 ± 0.7 (3)	181	3.2 ± 0.1 (3)	145	26.7 ± 4.8 (3)	176	14.6 ± 2.0 (3)	158	5.5

* Rats (120–180 g) were injected i.p. with 0.9% saline (control) or PB (80 mg/kg body wt) for four consecutive days prior to sacrifice. Microsomes and mitochondria were isolated from rat livers as described in Materials and Methods. The complete system (see legend to Table 1) containing 0.15 mM [^{14}C]NPiP (sp. act. 18.8 mCi/mmole), with or without the addition of mitochondria, was used. Benzylamine (1.0 mM) was present in some systems as indicated. Incubation was carried out at 37° for 60 min. The procedures for isolation and purification of DNA are referred to in Materials and Methods.

† Values represent means ± S.E.; numbers in parentheses refer to the number of determinations.

‡ Ratio of *in vitro* binding in the presence of mitochondria and in the absence of mitochondria.

DISCUSSION

Requirements for binding. The results show that

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the microsomal enzyme systems mediating the binding of ^{14}C-containing segments from DEN and NPiP require NADPH. This is consistent with the requirement of NADPH for the mixed-function oxidase-catalyzed dealkylation of DMN [43] and DEN [44], and for binding of DMN to DNA.* Consistent with the α-hydroxylation of dialkylnitrosamines, the production of reactive intermediate from NPiP probably involves initial oxidation at the carbon α to the nitroso group [45].

Addition of cytosol brings about a 3.3- to 4.4-fold increase in the binding of DEN and a 3.2-fold increase in the binding of NPiP. This parallels the effect of cytosol on the *in vitro* binding of ^{14}C-*methyl* from DMN to DNA*. The effect of cytosol was not enhanced by PB. These observations are also in accord with reports that the cytosol stimulates various microsomal *N*-demethylation reactions [e.g. Refs. 46–48] and that the stimulatory factor in the cytosol is not inducible by PB [49].

Effects of 3-MC and PB on binding. The enhancement by PB pretreatment of the binding of [^{14}C]DEN, and its inhibition by 3-MC pretreatment, are in excellent agreement with the respective increase and decrease of DEN-deethylase activity following these pretreatments [44]. The increased binding of DEN and NPiP following PB pretreatment is also consistent with the increased mutagenicity of the two nitrosamines following PB pretreatment [50, 51]. Moreover, the PB effect on binding correlates with its effect on the carcinogenic activity of DEN. Pitot *et al.* [52] have shown that a single < 30 mg/kg dose of DEN induces hepatic tumors in rats only if the DEN administration is followed by PB administration. Pretreatment with PB enhances drug metabolism by inducing cytochrome P-450 and NADPH-cytochrome *c* reductase [35, 53]. The identity of NADPH-cytochrome *c* reductase with NADPH-cytochrome P-450 reductase has been demonstrated [54, 55].

The greater effect of PB on the binding of DEN, when younger rats (120–180 g) rather than older rats (235–280 g) were used, parallels the observations of others that the PB induction of mixed-function oxidases is the highest in 33-day-old rats weighing 100–120 g [37]. The age-dependence of DMN-demethylase activity has also been documented [56].

Possible participation of monoamine oxidase. The observations that addition of mitochondria stimulates the microsome-catalyzed binding of DEN and NPiP and, furthermore, that mitochondria alone can catalyze the binding of NPiP, support the possibility that these nitrosamines may be metabolized, at least in part, by enzymes unrelated to cytochrome P-450-requiring mixed-function oxidases. The mitochondrial stimulation of binding is nil with DMN, small (1.4-fold from data in Table 1) with DEN, but considerable (4.3- to 5.6-fold from data in Tables 1 and 4) with NPiP; thus, the mitochondrial enhancement of binding appears to be a function of the alkyl chain length of the nitrosamine. The binding of NPiP is reduced by benzylamine, a typical substrate of mitochondrial monoamine oxidase.

Lake *et al.* [38, 39] suggested that an amine-oxidase resembling monoamine oxidase may be involved in the metabolism of DMN. Although DMN does not appear to be a substrate for either microsomal amine-oxidase (from different sources) or mitochondria [57,*], the present results suggest that amine-oxidase pathway(s) possibly play a role in the metabolism of higher nitrosamines. Distinct enzymic forms of monoamine oxidase display striking differences in their relative activities between different animal species and between different organs of the same species [58]. If later investigations would establish that monoamine oxidase participates in the metabolism of NPiP, this could provide an explanation for the organotropic properties of this carcinogen.

The possibility also exists that other mitochondrial enzyme systems are involved in the degradation of NPiP. Cytochrome P-450 present in adrenal mitochondria has been shown to hydroxylate many steroid compounds [33, 34] and a mitochondrial cytochrome P-450 may also be involved in the metabolism of cyclic nitrosamines. The synergistic mitochondrial effect on NPiP binding may also be attributed to the presence of NADH in mitochondria, since it has been reported [41, 59] that NADH increases the activity of cytochrome P-450 in the presence of a saturating concentration of NADPH. The synergism by NADH of NADPH-supported mixed-function oxidation has been reported by others [60, 61]. The reason for the stimulation by NADH is unclear. It has been suggested that NADH protects NADPH from inactivation by NADPH-pyrophosphatase, or NADH-dependent flavoproteins may compete with NADPH-cytochrome *c* reductase for the reduction of endogenous substrates, such as cytochrome b_5 [41].

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