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ENZYMATIC FORMATION OF CHEMICALLY REACTIVE METABOLITES OF *N*-NITROSO-DESMETHYL TRIPELENNAMINE BY A MECHANISM OTHER THAN *N*-DEALKYLATION*

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Abstract—Benzyl-^{14}C and *N*-methyl-^{3}H labeled *N*-nitroso derivatives (NDT) of tripelennamine were synthesized by reacting the corresponding labeled parent drug with sodium nitrite at pH 1–2, and their covalent binding to rat liver microsomes was compared with that of radiolabeled tripelennamine. The covalent binding of these substances is mediated by liver microsomal cytochrome P-450 enzymes; it required an NADPH-generating system and oxygen, and was inhibited by $CO:O_2$ (8:2). Reduced glutathione (1 mM) also inhibited the covalent binding. The covalent binding of NDT to liver microsomal proteins from phenobarbital-pretreated rats was ten times greater than that of tripelennamine. A K_m of 60 μM and a V_{max} of 1 nmole/mg of protein/min were obtained for the covalent binding of NDT. Phenobarbital and acetone pretreatments of rats increased covalent binding of both the drug and its nitroso derivative, while pretreatment with 3-methyl-cholanthrene decreased their binding. These results suggest that chemically reactive intermediates of tripelennamine and NDT are involved in the covalent binding. Under the conditions *in vitro* employed, the rate of *N*-demethylation of NDT was lower than that of the parent drug. Moreover, the covalent binding of the *N*-methyl-^{3}H labeled nitroso derivative was equivalent to that of the benzyl-^{14}C labeled derivative. Thus, *N*-demethylation is not a requisite for the covalent binding of tripelennamine and its *N*-nitroso derivative. The mechanism of the covalent binding of the *N*-nitroso derivative of tripelennamine, therefore, differs from that of dimethylnitrosamine.

When administered orally with sodium nitrite, drugs containing secondary and tertiary amino groups can be converted to their *N*-nitroso derivatives in the stomach where suitable acidic conditions may prevail [1–4]. Since many *N*-nitroso compounds are known to produce liver necrosis and other tissue lesions including tumors [5–9], it seemed possible that the drug toxicities might be initiated through the formation of these derivatives [10].

Tripelennamine (Pyribenzamine) (I) is an antihistaminic agent which forms *N*-nitrosodesmethyltripelennamine (NDT) (II) when treated with sodium nitrite at pH 1–2. NDT is also formed in the rat stomach when the drug is orally administered with sodium nitrite [11]. The present paper describes the effect of *N*-nitrosation on the covalent binding *in vitro* of tripelennamine to rat liver microsomal proteins.

MATERIALS AND METHODS

Radiochemicals. Benzyl-^{14}C labeled tripelennamine HCl (sp. act. 4·5 mCi/m-mole) and *N,N*-dimethyl-^{3}H labeled tripelennamine (sp. act. 8·67 mCi/m-mole) were purchased from New England Nuclear Corp. The labeled *N*-nitroso derivatives of tripelennamine were synthesized as described below.

Synthesis of benzyl-^{14}C labeled NDT. A solution of benzyl-^{14}C labeled tripelennamine HCl (50 mg; 5 μCi/mg) in 2 ml of 1 M HCl was mixed with 2 ml of 2 M $NaNO_2$, and the reaction mixture was heated for 1 hr at 60° in a water bath. The reaction mixture was then extracted with chloroform (3 × 5 ml), and the chloroform extract was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness. The yellow residue from the chloroform extract was streaked on a preparative thin-layer chromatographic (TLC) plate (E. Merck Silica gel F-254, layer thickness 0·5 mm, 20 × 20 cm) and the chromatograph developed in an ethyl acetate–methanol (1:1) solvent mixture. The band corresponding to R_f = 0·65 was scraped off the plate and extracted with chloroform (3 × 5 ml). The extract was filtered, and evaporated to dryness to yield benzyl-^{14}C-NDT as a yellow viscous liquid (0·13 mCi; 27·8 mg). NDT was identified by mass spectrometry as described previously [11].

The radiochemical purity of ^{14}C-NDT was found to be greater than 99 per cent by radiochromatography in three Silica gel TLC systems: ethyl acetate–methanol (1:1), ethyl ether–benzene (5:2), and ethyl

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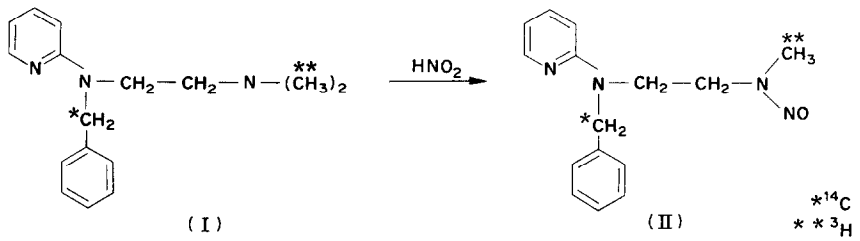

Fig. 1. Synthesis of radioactive *N*-nitrosodesmethyltripelennamine.

acetate–benzene (7:3) and one paper chromatographic system (Whatman 3 MM paper): *n*-butanol–water–glacial acetic acid (4:2:1).

Synthesis of N-methyl-^{3}H labeled NDT (Fig. 1). *N,N*-dimethyl-^{3}H labeled tripelennamine (50 mg; 34 μCi/mg) in 2 ml of 1 M HCl was allowed to react with 2 ml of 2 M $NaNO_2$ to form *N*-methyl-^{3}H-NDT as a yellow viscous liquid (0·76 mCi; 29·1 mg) as described above. The radiochemical purity of *N*-methyl-^{3}H labeled NDT was observed to be greater than 99 per cent by radiochromatography in four solvent systems, as in the case of benzyl-^{14}C labeled NDT.

Animals. Male Sprague–Dawley rats, weighing 120–200 g, were employed in all experiments and were maintained on Purina rat chow and tap water *ad lib.*

Drug pretreatments. Rats were pretreated daily with phenobarbital (80 mg/kg, in water, i.p.) or 3-methylcholanthrene (40 mg/kg, in sesame oil, i.p.) for 3 consecutive days and were killed 24 hr after the last dose. Pretreatment with acetone was carried out by a single oral dose (2·5 ml/kg, as 25% solution in water) 16 hr prior to the experiments [12].

Liver microsomal preparations. Rat liver microsomes were prepared from 10,000 *g* supernatant of rat liver homogenates by the calcium precipitation method of Kamath and Rubin [13]. Washed microsomal pellets were suspended in 1·15% KCl–20 mM Tris-HCl buffer, pH 7·4, by gentle homogenization to yield approximately 5 mg protein/ml.

Protein estimation. Protein content was determined by the method of Lowry *et al.* [14]. Bovine serum albumin was used as the protein standard.

Covalent binding studies. Covalent binding studies *in vitro* were carried out with rat liver microsomal preparation according to the procedure of Krishna *et al.* [15]. In a typical experiment, the labeled compounds (1 μCi/ml, 0·1 mM) were incubated at 37° with microsomes (1 mg protein/ml) in the presence of NADH, and an NADPH-generating system (0·124 mM NADH, 0·2 mM NADP, 2 mM nicotinamide, 2 mM glucose 6-phosphate and 1 unit of glucose 6-phosphate dehydrogenase) in a total volume of 1 ml for 10 min. The reaction was stopped by addition of 1 ml of 10% trichloroacetic acid (TCA) and the precipitate was subjected to ten consecutive washings with a hot (60°) ether–methanol (1:3) mixture to remove noncovalently bound test compounds. The TCA precipitate was then dissolved in 1 N NaOH (1 ml) and the covalent binding was determined by measuring the radio-activity and protein in the NaOH solution. A blank incubation consisting of denatured microsomes was also performed with these incubations.

Extraction with phenol (8%) according to the procedure of Reid *et al.* [16] reveased that virtually all of the radioactivity in the TCA precipitate was bound to proteins rather than to nucleic acids.

N-demethylation assay. *N*-demethylation activity was determined by measuring the rate of formaldehyde (HCHO) formation. The incubations were performed as described above, using varying concentrations of unlabeled tripelennamine and NDT up to 1 mM. Formaldehyde was determined according to the method of Nash [17].

Table 1. Effect of pretreatment of rats with various chemicals on the covalent binding of tripelennamine and NDT to liver microsomal proteins*

	Covalent binding (pmoles/mg protein/10 min)			
	Tripelennamine		NDT	
Pretreatment	^{14}C	^{3}H†	^{14}C	^{3}H
Control	40 ± 5‡	43 ± 7§	205 ± 18‡	200 ± 21§
Phenobarbital	215 ± 17‡	221 ± 20§	2170 ± 110‡	2159 ± 131§
Acetone	83 ± 8‖		413 ± 20‖	
3-Methylcholanthrene	34 ± 2‖		167 ± 15‖	

* Pretreatment of rats with various chemicals and covalent binding studies with labeled tripelennamine and NDT (0·1 mM) were performed as described under Materials and Methods.

† Calculations were based on the assumption that both methyl groups of tripelennamine were retained in the covalently bound metabolite.

‡ Results represent the mean ± S. E. M. of six determinations.

§ Mean of triplicate determinations ± S. E. M.

‖ Mean of duplicate determinations ± S. E. M.

Table 2. Effect of various cytochrome P-450 inhibitors and GSH on the covalent binding of tripelennamine and NDT to liver microsomal proteins from phenobarbital-pretreated rats*

Experimental condition	Covalent binding (pmoles/mg protein/10 min)			
	Tripelennamine		NDT	
	^{14}C	^{3}H†	^{14}C	^{3}H
Under air	215 ± 17‡	221 ± 20	2170 ± 110‡	2159 ± 131
Under nitrogen§	8 ± 3	11 ± 4	91 ± 3	25 ± 8
Under $CO:O_2$ (8:2)§	2 ± 2		32 ± 4	
Absence of NADPH-generating system	0	0	0	0
Presence of GSH (1 mM)	10 ± 4	14 ± 5	68 ± 2	59 ± 6

* Covalent binding studies with labeled tripelennamine and NDT (0·1 mM) were performed as outlined under Materials and Methods.

† Calculations were based on the assumption that both methyl groups of tripelennamine were retained by the covalently bound metabolite.

‡ Mean of six determinations ± S. E. M. All the remaining results represent the mean ± S. E. M. of triplicate determinations.

§ Air was replaced by nitrogen or a $CO:O_2$ (8:2) mixture.

RESULTS

Covalent binding of tripelennamine and its nitroso derivative, NDT. As shown in Table 1, both tripelennamine and NDT are covalently bound by rat liver microsomes in the presence of an NADPH-generating system. But the covalent binding of NDT was much greater than that of tripelennamine. Pretreatment of rats with phenobarbital increased the covalent binding of tripelennamine 5-fold, while it increased the binding of NDT 10-fold. Pretreatment of the animals with acetone increased the covalent binding of both compounds about 2-fold. By contrast, pretreatment with 3-methylcholanthrene decreased the covalent binding by about 15–19 per cent.

As shown in Table 2, the covalent binding of tripelennamine and NDT by liver microsomes from phenobarbitol-pretreated rats was almost completely inhibited when the substrates were incubated under anerobic conditions, in the absence of an NADPH-generating system, or in the presence of a carbon monoxide–oxygen (8:2) atmosphere. Moreover, glutathione markedly inhibited the covalent binding.

A Lineweaver–Burk plot for the covalent binding of NDT to liver microsomes from phenobarbital-pretreated rats is shown in Fig. 2. A K_m value of 60 μM and a V_{nby} value of 1 nmole/mg of protein/min for the covalent binding of NDT were calculated. However, the rate of covalent binding did not increase at NDT concentrations higher than 0·1 mM.

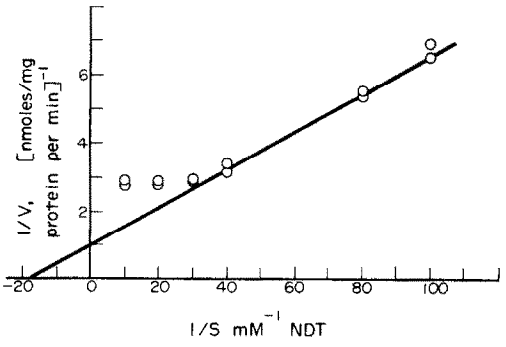

Fig. 2. Lineweaver–Burk plot for the covalent binding of *N*-nitrosodesmethyltripelennamine (NDT) to liver microsomal proteins from phenobarbital-pretreated rats.

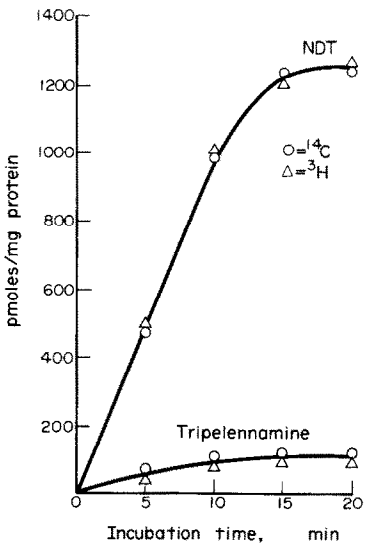

Fig. 3. Covalent binding of an equimolar (0·1 mM) mixture consisting of 0·5 μCi ^{14}C-labeled and 0·5 μCi ^{3}H-labeled tripelennamine and a similar equimolar (0·1 mM) mixture of the double-labeled *N*-nitroso derivative (NDT) to liver microsomal proteins from phenobarbital-pretreated rats.

Studies with double-labeled compounds. Based on the specific activities of the benzyl-^{14}C and *N,N*-dimethyl-^{3}H labeled tripelennamine preparations, the covalently bound tripelennamine retains both of its methyl groups as well as its benzyl group. As shown in Table 1, equivalent amounts of the benzyl-^{14}C and *N,N*-dimethyl-^{3}H label were bound when liver microsomes from untreated and phenobarbital-pretreated animals were used. Even when the two preparations were present in the same incubation mixtures, the rates of incorporation of the two labels were identical (Fig. 3).

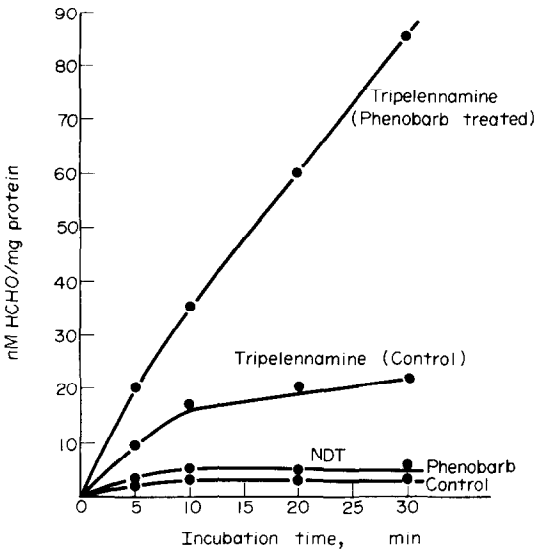

Fig. 4. *N*-demethylation of tripelennamine (0·1 mM) and its *N*-nitroso derivative (NDT) (0·1 mM) by control and phenobarbital-pretreated rat liver microsomes.

Similarly, the covalently bound NDT retains its methyl group as well as its benzyl group. Equivalent amounts of benzyl-^{14}C and *N*-methyl-^{3}H labeled NDT were bound when they were incubated in air with liver microsomes from untreated and phenobarbital-pretreated rats. Even when the two labeled preparations of NDT were present in the same incubation mixture, the rate of covalent binding of the benzyl-^{14}C label was identical to that of the *N*-methyl-^{3}H (Fig. 3).

N-demethylation studies. As shown in Fig. 4, the rate of *N*-demethylation of NDT by liver microsomes from untreated rats was slow (0·5 nmole/mg of protein/min) and was linear for only 10 min. Moreover, the rate of *N*-demethylation of NDT was not significantly increased by pretreatment of the rats with phenobarbital. By contrast, the rate of *N*-demethylation of tripelennamine was high (3·5 nmoles/mg of protein/min) and was increased about 3-fold by pretreatment of the animals with phenobarbital.

DISCUSSION

The covalent binding of both tripelennamine and NDT to liver microsomal protein is apparently mediated by chemically reactive intermediates formed by cytochrome P-450 enzyme systems [18]. The covalent binding of both compounds require an NADPH-generating system and oxygen and is inhibited by a carbon monoxide–oxygen atmosphere (Table 2). Moreover, pretreatment of the animals with phenobarbital, which induces cytochrome P-450 enzyme systems, increases the rate of covalent binding by 5- to 10-fold (Table 1). In addition, glutathione, which inhibits the covalent binding of many other drugs [19], also inhibited the covalent binding of tripelennamine and NDT.

There is considerable evidence that dialkylnitrosamines, such as dimethylnitrosamine, are *N*-dealkylated to form chemically reactive intermediates which have properties similar to diazoalkanes [20]. If the reactive metabolite of NDT were formed by this mechanism, however, the covalently bound NDT might retain either the benzyl-^{14}C or the methyl-^{3}H radiolabel depending on which mechanism of dealkylation predominated. But the finding that the covalently bound metabolite of NDT contains both radiolabels in exactly the same amounts (Table 1, Fig. 3) indicates that *N*-dealkylation is not a requisite for the formation of the reactive metabolite and that the activation must occur with some other moiety of NDT. Thus it cannot be automatically assumed that all nitrosamines are converted to chemically reactive metabolites by the same mechanism as dimethylnitrosamine, even when the nitrosamine derivative is covalently bound more rapidly than its parent compound.

The nature of the chemically reactive intermediates of tripelennamine and NDT remains to be elucidated. Since the covalently bound metabolite of tripelennamine retains not only the benzyl moiety but also both methyl groups (Table 1, Fig. 3), the formation of the tripelennamine-reactive metabolite cannot be mediated by an *N*-demethylation reaction, even though the drug is rapidly metabolized by this reaction. It seems possible, however, that the chemically reactive metabolites of both tripelennamine and NDT may be arene oxides. Indeed the finding that *m,p*-dihydroxybenzyl (catechol) derivatives are urinary metabolites of both tripelennamine and NDT [21] lends support for the view that these substances undergo epoxidation, because arene oxides may be converted to catechols by being hydrated to dihydrodiols and then dehydrogenated to catechols. Moreover, arene oxide intermediates are thought to be the chemically reactive metabolites formed from various hepatotoxic halobenzenes [22–24]. It is still possible, however, that the catechols, themselves, may also be converted to chemically reactive *ortho*-quinone derivatives in the body, but this reaction probably does not occur in our experiments becasue the dehydrogenation of dihydro-diols is catalyzed by soluble enzymes in liver and not in liver microsomes [25].

It seems plausible that under certain circumstances the *N*-nitrosation of therapeutic agents resulting from their interaction with dietary nitrite in the stomach may modify the effectiveness of the drug as well as enhance their capacity to bind covalently with tissue macromolecules. Indeed, preliminary pharmacological studies with NDT revealed it to be devoid of both central stimulant and anti-histaminic activities of the parent drug, tripelennamine [11].

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