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Rabbit Liver and Lung Microsomal Metabolism of β-Nicotyrine: Isozyme Specificities toward the Oxidation of β-Nicotyrine.

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Studies on the biodisposition of β-nicotyrine by lung and liver microsomes was examined in order to provide a better understanding of its fate in this tissue. β-nicotyrine (100 μM) was incubated with microsomes (1 mg/ml) prepared from New Zealand White rabbits. The rate of oxidation observed in lung microsomal incubations was 1.7 nmoles β-nicotyrine oxidized mg^{-1} min^{-1} compared with 2.7 nmoles β-nicotyrine oxidized mg^{-1} min^{-1} by the liver microsomal preparation. However, when these rates were expressed as a function of cytochrome P-450 content, the specific activity of the metabolic oxidation catalyzed by lung (8.3 nmoles β-nicotyrine oxidized nmole cytochrome P-450^{-1} min^{-1}) was approxiamtely 4 times greater than liver microsomes (2.3 nmoles β-nicotyrine oxidized nmole cytochrome P-450^{-1} min^{-1}). Isozyme studies on the oxidation of β-nicotyrine employed several methods of altering activities of specific isozymes present in pulmonary microsomes, including the use of the isozyme 2 and 6 specific inhibitor α-methyl ABT, metabolic inhibitor (MI) complex formation. The results of this inhibition study would appear to indicate the β-nicotyrine is metabolized predominantly by pulmonary isozyme 5.

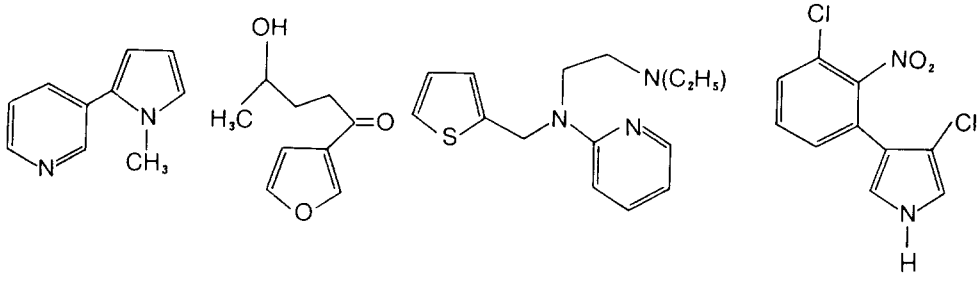
INTRODUCTION

β-nicotyrine (1) is a member of the cyclopentadienoid heterocyclic class of compounds which include furan, thiopene and pyrrole. Many of the substituted derivatives of this compounds have been studied in detail with regard to their overall biodisposition, the role oxidation metabolism plays in the

ABBREVIATIONS: α-methyl ABT, N-α-methylbenzylaminobenzotriazole; HEPES, N-2 hydroxyethylpiperazine-N'-2-ethanesulfonic acid.

toxicities they induce and enzyme systems required for the expression of these toxicities. 4-ipomeanol (2), a substituted furan, represents the most well studied compound of this class (Durham *et al.*, 1985). The compound undergoes extensive cytochrome P-450 dependent metabolism to reactive intermediates which covalently bind to cellular macromolecules. The expression of the lung specific toxicities observed after administration of this compound is related to oxidative metabolism of the furan ring and involves covalent binding. Chronic toxicity studies on the thiophene containing antihistaminic agent methapyrilene (3) has shown this compound to be a potent hepatocarcinogen (Lijinsky *et al.*, 1980). Structural analogs of methapyrilen, where the thiophen ring is substituted with either paramethoxybenzen or benzen leads to a pharmacologically active antihistaminic without the carcinogenic potency of methapyrilene. It has therefore been suggested that the oxidation of the thiophen moiety to reactive and presumably toxic intermediates is responsible for the carcinogenic effects of methapyrilene. Pyrrole derivatives such as pyrrolnitrin (4) also are metabolically activated to reactive intermediates which covalently bind to thiolating species such as β-mercaptoethanol and presemably sulfhydryl groups on macromelecules (Murphy *et al.*, 1972). By structural analogy to the compounds mentioned above our interest in pursuing the metabolic fate of β-nicotyrine is stimulated by the observation that oxidative metabolism of these substituted cyclopentadienoid heterocycles in many cases leads to the expression of organ specific toxicites.

In our efforts to characterize the metabolic disposition of this minor, but potentially toxic tobacco alkaloid, studies were undertaken to define the interactions between this compound and cytochrome P-450, the effects of inducing agents on the *in vitro* oxidative metabolism by liver and lung microsomes and through the utilization of biochemical probes (isozyme specific inhibitors) the identification of the specific pulmonary cytochrome P-450 isozymes involved in the oxidation of this substituted pyrrole. Rabbit lung homogenates were included in these studies in part because the rabbit lung cytochrome P-450 systems have been characterized in this species (Franklin *et al.*, 1980) and in part because this is the principal organ which is exposed to β-nicotyrine containing tobacco smoke.



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MATERIALS AND METHODS

Synthetic reactions were carried out under a nitrogen atmosphere. Diisopropylamine, n-butyllithium, phenylselenyl chloride, D20, and(s)-nicotine, used in the synthesis of (s)-cotinine (Bowman *et al.*, 1963) and β-nicotyrine tartrate (Frank *et al.*, 1942; Brown *et al.*, 1972) were purchased from the Aldrich Chemical Co. EGTA, glucose-6-phosphate and HEPES were purchased from the Sigma Chemical Co. All other chemicals were reagent grade or HPLC grade. α-methyl ABT was a generous gift of Dr. James M. Mathews (National Institute of Environmental Health Science, NIH).

Sources of Tissue

The liver and lungs of New Zealand White male rabbits (2.5-3 kg) were used for preparation of microsomes.

Preparation of Rabbit Liver Microsomal Fractions

After carbon dioxide asphyxiation, the livers were perfused *in situ* via the portal vein with 250 ml of ice cold 0.25 M sucrose buffered at PH 7.4 and with 0.05 M Tris/0.05 M NaOH. Livers were minced with scissors and the pieces were homogenized in a Potter-Elvehjem apparatus in 3 volumes (w/v) of the same solution. The homogenate was centrifuged at 10,000g for 20 min and the resulting supernatant fraction was centrifuged at 100,000g for 75 min. The pellet was resuspended in 5 ml of 0.15 M KCl buffered at pH 7.4 with 0.02 M KH_2PO_4 and this mixture was centrifuged a second time at 100,000g. The resulting pellet was homogenized in this buffer at a concentration of approximately 50 mg of microsomal protein per ml and stored under nitrogen at $-70^{\circ}C$ for up to 1 month. Protein concentration was determinded by the method of Lowry *et al.*, (1951).

Preparation of Rabbit Lung Microsomes.

Immediately after the animal was sacrificed by carbon dioxide asphyxiation, the lungs were perfused via the pulmonary artery with 10-15 ml of ice cold 0.15M KCl/0.02M KH_2PO_4 buffer containing 100U/ml heparin (Elkin-Sinn, Cherry Hill. NJ). The isolated perfused lungs were coarsely minced in a solution consisting of 0.02 M Tris, 0.15 M KCl, 0.2mM EDTA, and 0.5mM dithiotreitol and the resulting mince was homogenized in a Waring Blendor with two 10-sec bursts. The contents were transferred to Potter Elvehjem homogenizer and homogenized with six passes of a Teflon pestle. The resulting homogenate was centrifuged at 18,000g for 20 min. The postmitochondrial supernatant fraction was centrifuged for an additional 60 min at 100,000g. The microsomal pellet was resuspended in 5 ml of 0.02 M Tris and 0.15 M KCl, the pH was adjusted to 7.4 with 1 N NaOH, and the resulting suspension was centrifuged at 100,000g for an additional 60 min. The pellet was resuspended in this buffer, homogenized, and stored under nitrogen at a concentration of approximately 15-25mg/ml at $-70^{\circ}C$ for up to 1 month.

Determination of Cytochrome P-450 Concentrations

The concentrations of cytochrome P-450 were determined using an Aminco DW-2 UV/visible spectrophotometer by measuring UV absorbance differences between the dithionite-reduced carbon monoxide treated sample and an unreduced carbon monoxide treated reference sample (Estabrook *et al.*, 1972).

Metabolism Studies

Incubation mixtures (final volume 1.0ml) consisted of 1.5 to 2.0 mg of microsomal protein, β-nicotyrine tartrate (154 μg, 0.5 mol), EGTA (1 mM) in 0.1 M HEPES buffer,

pH 7.6, and an NADPH regenerating system (0.5 mM NADP+, 8 mM glucose-6-phosphate, 1 unit/mL glucose-6-phosphate dehydrogenase, and 4mM MgCl) were incubated at 37°C for 1 hour or, in the case of kinetic studies, 10, 20, 30, 40, 50, and 60 minutes in a metabolic shaker. (S)-Nicotine (22μg) was added as an internal standard and the resulting mixtures were extracted with CH_2Cl_2 (1mL), the extracts vortexed for 1 minute, and the phases separated by centrifugation for 3-4 minutes at 1000g. The CH_2Cl_2 layer was transferred to a second vial and followed the procedure described below for the construction of standard curves.

HPLC Analysis

Plots of peak height ratios of analyte to internal standard against analyte concentrations gave straight lines, which were used in estimating analyte concentrations in sample incubation mixtures. Recoveries were estimated to be greater than 95%. The HPLC assay used a Beckman 110A solvent delivery system and a Hitachi 100-10 spectrophotometer/flow cell combination. The precolumn (4.6mm x 5cm) was packed with Lichrosorb Si 60, 30 μm particle size (Merck, Darmstadt, FRG) and the analytical column (4.6mm x 25cm) with 10 m Lichrosorb (or 5 μm Alltech silica). The mobile phase consisted of acetonitrile plus 1% n-propylamine (v/v) and qualitative and quantitative HPLC analyses of metabolic incubation mixtures using (S) nicotine as internal standard and employing UV detection (260 nm) were performed with the same mobile phase (flow rate: 1 mL/min; retention times: (S)-nicotine, 1.9 min; β-nicotyrine, 2.8 min; metabolite, 4.1 min). Samples were run in triplicate.

α-methyl ABT Studies

Incubations with α-metyl ABC utilized concentrations of 1 μM, 2.5 μM and 1mM. α-methyl ABT was dissolved in methanol to a stock concentration of 10mM. Dilutions of the stock solution was made and appropriate volumes of the methanolic solutions were transferred to sample tubes. Prior to adding the other components of the incubation mixture, methanol was evaporated with a gentle stream of nitrogen. Components of this mixture included EGTA, NADP+ and a glucose-6-phosphate based regenerating system, lung microsomes from untreated male NZW rabbits (1mg/ml) and glutathione (1mM) in a final incubation volume of 0.4 ml. Incubation mixtures were preincubated in the presence of the inhibitor for 30 min at 37°C prior to the addition of the substrate β-nicotyrine (100 μM). Samples were worked up as described previously. Each condition was performed in triplicates.

Metabolite Inhibitor (MI) Studies

The effect of MI complex formation on the oxidation of β-nicotyrine was tested by preincubating untreated NZW rabbits (4mg/ml) with the following combinations of inhibitor: 1) 100 μM norbenzphetamine only 2) 667 μM N-hydroxyamphetamine only 3) 100 μM norbenzphetamine plus 667 μM N-hydroxyamphetamine.

RESULTS AND DISCUSSION

β-nicotyrine Metabolism by Liver and Lung Microsomes

Studies on the biodisposition of β-nicotyrine by liver and lung microsomes was investigated in order to provided a better understanding of its fate in these tissues. β-nicotyrine (100 μM) was incubated with microsomes (1 mg/ml)

prepared from NZW rabbits. The time course of oxidation was observed over a 60 minutes period. Plots of β-nicotyrine metabolized by liver and lung microsomal incubation mixtures vs time were linear over the first 20 minutes (Fig. 1). The rate of oxidation observed in lung microsomal incubations was 1.7 nmoles β-nicotyrine oxidized mg^{-1} min^{-1} compared with 2.7 nmoles β-nicotyrine oxidized mg^{-1} min^{-1} by the liver microsomal preparation. However, when these rates were expressed as a function of cytochrome P-450 content, the specific activity of the metabolic oxidation catalyzed by lung (8.3 nmoles β-nicotyrine oxidized nmole cytochrome $p\text{-}450^{-1}$ min^{-1}) was approximately 4 times greater than liver microsomes (2.3 nmoles β-nicotyrine oxidized nmole cytochrome $P\text{-}450^{-1}$ min^{-1}). The differences in the specific activities of the microsomal preparations isolated from these two tissues is consistent with the paticipation of lung isozymes 2 and/or 5 in the oxidation of this compound, since these two forms represent most of the cytochrome P-450 (estimated to account for up to 85%) activity present in lung.

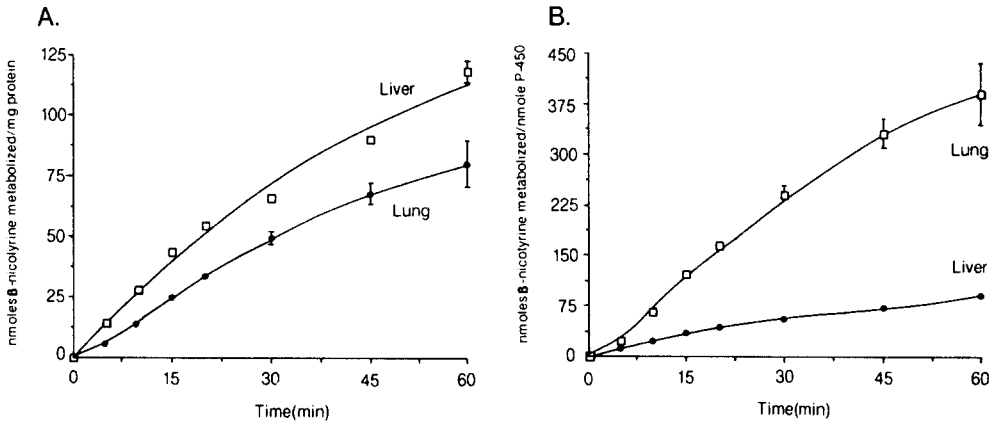

Fig. 1. Rates of metabolism of β-nicotyrine in rabbit lung and liver microsomal preparations ploted per mg microsomal protein (left panel) and per nmole cytochrome cytochrome P-450 (right panel).

Isozyme Studies: Isozyme Specificities Toward the Oxidation of β-nicotyrine

The results of the lung microsomal metabolism of β-nicotyrine encouraged us to pursue studies which could elucidate the isozyme selectivities toward the oxidation of tobacco alkaloid β-nicotyrine. Although the highest microsomal monooxigenase activities are normally found in the liver, the lung also contiains an active P-450 system. The rabbit pulmonary monooxygenase system is composed of two major and one minor P-450 isozyme. The major isozymes are forms 2 and 5, which are present in approximately equal amounts, are indistinguishable from those P-450 isozymes induced in rabbit liver by treatment with phenobarbital and account for up to 80% of total pulmonary

P-450 (Philpot *et al.*, 1982; Domin *et al.*, 1984). The minor form, isozyme 6, is induced in liver and lung of rabbit by treatment with 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin and it occurs in amount of 1 to 3% of total P-450 in pulmonary microsomes prepared from untreated rabbits (Serabjit-Singh *et al.*, 1983; Domin *et al.*, 1984).

Table 1. Effect of α-methy ABT on the metabolism of β-Nicotyrine by UINZW rabbit* lung microsomes.

Condition Time	Sample#	β-Ni/I.S**	Avg±S.E.	S.E. fraction (%)	n moles β-Ni Remains	n moles met	Inh. rate (%)
	1	4.150					
0'	2	4.161	4.195±0.057	±1.3	100	—	
	3	4.275					
	4	3.393					
20'	5	3.205	3.352±0.107	±3.2	99.9	20.1±0.64	
	6	3.457					
	7	3.323					
20'+1μm	8	3.741	3.585±0.186	±5.2	85.5	14.5±0.75	27.9
	9	3.690					
	10	3.423					
20'+2.5μm	11	4.088	3.707±0.291	±8.6	86.8	13.2±1.14	34.3
	12	3.418					
	13	3.924					
20'+1mM	14	3.900	4.022±0.254	±7.8	88.4	11.6±0.90	42.3
	15	3.296					

*uninduced New Zealand White rabbit **ratio of β-Nicotyrine against internal standard

Incubation with α-methyl ABT Utilized Concentration of 1μm, 2.5μm and 1mM.
α-methyl ABT was dissolved in methanol to a stock concentration of 10mM. Prior to adding the other components of the incubation mixture, methanol was evaporated with a gentle stream of nitrogen. Incubation mixtures were preincubated in the presence of the inhibitior for 30 min at 37°C prior to the addition of the Substrate β-nicotyrine.

These studies employed several methods of altering activities of specific isozymes present in pulmonary microsomes, including the use of the isozyme 2 and 6 specific inhibitor α-methyl ABT, the suicide inactivator described previously (Matthews *et al.*, 1986). Lung microsomes isolated from untreated New Zealand White rabbits were preincubated with 1 μM, 2.5 μM and 1 mM α-methyl ABT, concentrations which inhibited 84, 93 and 98% of the isozyme 2 specific n-demethylation of benzphetamine and 41, 70 and 100% of the isozyme 6 catalyzed O-deethylation of ethoxyresolrufin. When similar incubation were attempt with β-nicotyrine as substrate, β-nicotyrine metabolism was, at the concentrations of α-methyl ABT indicated above, was inhibited by only 27.4, 34.3 and 42.3% respectively (Table 1, Fig. 2). The tentative conclusions drawn from this study is that β-nicotyrine is metabolized predominantly by isozyme 5 or an as yet unidentified pulmonary form of cytochrome P-450. To further evaluate the effect of various pulmonary isozyme on the metabolism of this compound, the metabolite intermediate (MI) complex forming agents norbenzphetamine and N-hydroxyamphetamine were utilized. These agents, characterized by their ability to inhibit cytochrome P-450 monooxigenase activity following metabolic oxidation, display a 455 nm chromophore 2 and 5 in lung microsomes (Franklin *et al.*, 1976). These inhibitors, upon oxidation by cytochrome P-450, form stable noncovalent interactions with the heme of P-450 a process which requires the presence of NADPH and 0_2. There is evidence that the complex is a result of a ligand interaction between an unstable nitroso intermediate and the reduced ferrous cytochrome P-450 (Mansuy *et al.*, 1977). As a test of stability of these interactions, *in vivo* administration of these agents result in the inactivation of susceptible P-450 isozymes and which are stable to isolation and purification

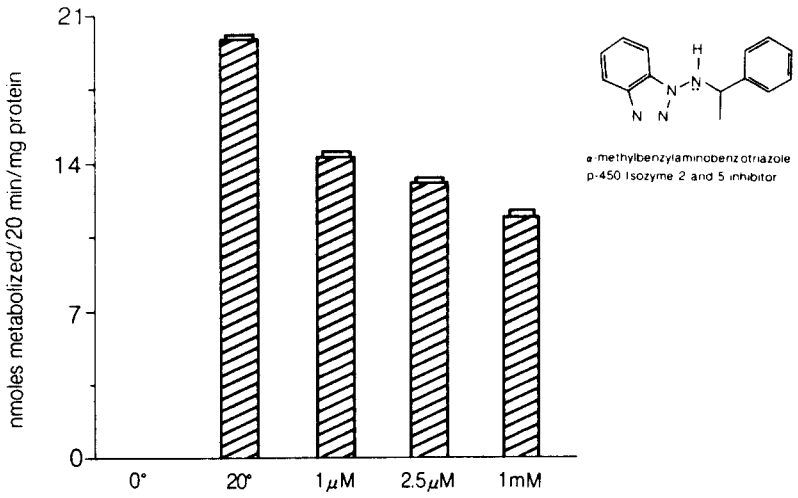

Fig. 2. Effect of α-methyl ABT on the metabolism of β-nicotyrine by UINZW rabbit lung microsomes.

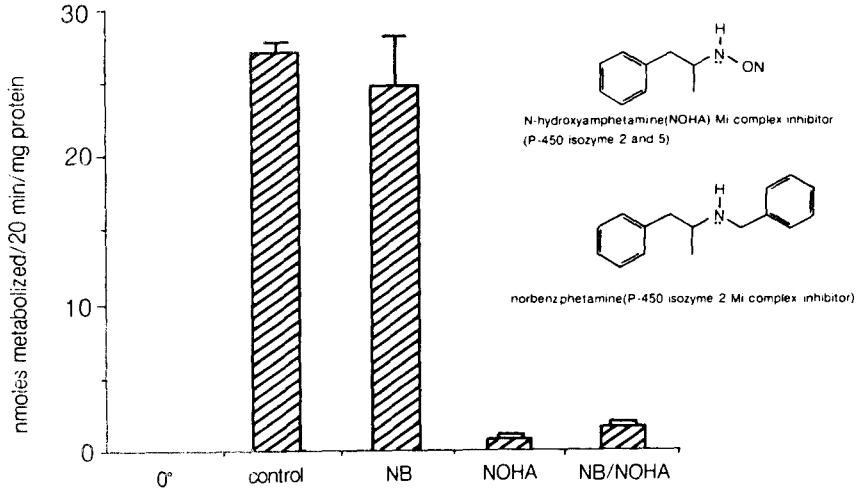

Fig. 3. Effect of M1 inhibitors on the oxidation of β-nicotyrine by UINZW lung microsomes.

of the isozymes. In the case of norbenzphetamine, oxidative metabolism leads to the formation of an intermediate which binds tightly only to pulmonary isozyme 2. In this respect, the inhibitory properties of norbenzphetamine and α-methyl ABT are similar. N-hydroxyamphetamine, on the other hand, is a less specific inhibitor of pulmonary P-450. Metabolism of this compound results in the inhibition of both isozymes 2 and 5. The differential inhibition exhibited by these 2 MI complex substrates can provide information on the role of isozyme 5 in the oxidation of β-nicotyrine.

The effect of MI complex formation on the oxidation of β-nicotyrine was tested by preincubating untreated NZW rabbits (4mg/ml) with the following combinations of inhibitor: 1) 100 μM norbenzphetamine only 2), 667 μM N-hydroxyamphetamine only, 3) 100 μM norbenzphetamine plus 667 μM N-hydroxyamphetamine. The concentration used above were derived from studies which established maximal rates of MI complex formation (Franklin *et al.*, 1980). For maximal inhibition, these compounds were preincubated with NADPH (0.8 M) and lung microsomes for 30 min at room temperature. Following the preincubation, microsomes treated with the respective combination of inhibitor(s) was transferred to two sets of sample tubes, which included the components/cofactors necessary for cytochrome P-450 monooxygenase activity. These incubations, containing 1mg/ml of the MI complexed lung microsomes, were incubated with β-nicotyrine (100 μM) for an additional 20 min at 37°C. This incubation resulted in 8.8, 95.6 and 93.8% inhibition of its overall metabolism at the concentrations of 2 MI complexed substrates described above (Fig. 3). The results of this inhibition study would appear to indicate that β-nicotyrine is metabolized predominantly by pulmonary isozyme 5.

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β-Nicotyrine의 대사연구 : β-Nicotyrine의 산화에 대한 효소특이성

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뉴질랜드 토끼로 부터 얻은 간 및 폐 마이크로좀과 β-nicotyrine(100M)을 배양시킨 결과 폐 마이크로좀에서 배양시킨 β-nicotyrine의 산화율은 1.7nmoles 인데 비해 간에서는 2.7nmoles 였다. 그러나 이를 조직에서의 cytochrome P-450 농도별로 나타나면 폐에 의해 대사된 산화율이 간에서 보다 약 4배 높았다. 또한 폐 마이크로좀에 있는 isozyme 2.6 specific inhibitor 인 α-methyl ABT 와 기타 metabolite inhibitor 를 사용하여 β-nicotyrine의 산화를 실험한 결과 β-nicotyrine은 폐 isozyme 5에 의해 우선적으로 대사되었다.