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In Vitro Covalent Binding of 3-[^{14}C]Methylindole Metabolites in Goat Tissues[1] (41841)

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Abstract. Covalent binding of 3-[^{14}C]methylindole (3[^{14}C]MI) in crude microsomal preparations of goat lung, liver, and kidney was measured to determine if a reactive intermediate was formed during the *in vitro* metabolism of 3-methylindole (3MI). The bound radioactivity was highest in lung compared to liver and kidney. The amount of bound radioactivity per nanomole of cytochrome *P*-450 was approximately 10 times higher in the lung compared to liver. No detectable bound radioactivity was found when 3-[^{3}H]methyloxindole was used as the substrate. Cofactor requirements and the effects of inhibitors indicate that a mixed function oxidase (MFO) system is involved in formation of a reactive intermediate. Inhibitors and conjugating agents that are known to reduce the severity of 3MI-induced lung injury such as piperonyl butoxide (MFO inhibitor) and glutathione (conjugating agent) significantly decreased the *in vitro* binding of 3[^{14}C]MI. The results indicate that a reactive intermediate is produced during the metabolism of 3MI by the MFO system. The organ specificity in binding suggests that covalent binding by lung microsomes may be related to the mechanism of 3MI-induced lung injury.

3-Methylindole (3MI) is the main ruminal fermentation product of tryptophan which causes acute pulmonary edema and interstitial emphysema in cattle, sheep, and goats (1–3). 3MI is also a bacterial degradation product of dietary tryptophan in the large intestine of rats, pigs, and man (4). Cigarette smoke contains approximately 4 to 50 μg 3MI per cigarette formed by pyrolysis of tryptophan in tobacco leaves (5). The toxic effects of 3MI in ruminants are limited to the lung. The effects of 3MI administration are rapid and cell selective with the most severe effects on nonciliated bronchiolar epithelial (Clara) cells and alveolar Type I cells (6).

3MI is rapidly metabolized by the mixed function oxidases (MFO) and at least 10 metabolites are excreted in the urine, the majority of which are 3-methyloxindole (3MOI) and its derivatives (7). The parent compound (3MI) is not directly responsible for toxicity since MFO inducers and inhibitors can alter 3MI metabolism and the severity of lung injury (8). Also, infusion of 3MOI or indole-3-carbinol does not induce lung damage (9). These results suggest that a metabolic step prior to the formation of 3MOI is responsible for the toxicity.

There is ample evidence that MFO metabolism can result in activation of nontoxic parent compounds to reactive intermediates with potent mutagenic, carcinogenic, or cytotoxic effects. These effects may be mediated by the formation of reactive electrophilic compounds which covalently bind to cellular macromolecules and interfere with normal cellular function. Covalent binding of radioactive metabolites to macromolecules in *in vitro* microsomal preparations or *in vivo* systems has been widely used as an indication of the formation of reactive intermediates (10, 11). The objectives of this experiment were (a) to determine the extent of covalent binding of 3MI and 3MOI in an *in vitro* microsomal system, (b) to study the organ(s) specificity of covalent binding, and (c) to investigate the effect of MFO inhibitors and conjugating agents on the covalent binding.

Materials and Methods. *Materials.* Methyl-3-[^{14}C]methylindole was obtained by commercial synthesis, from New England Nuclear, Boston, Massachusetts. 3-[G-^{3}H]methyloxindole was obtained by synthesis of 3MOI (12), followed by custom tritium labeling using the tritium gas exposure method (New England Nuclear). Glucose-6-phosphate, glucose-6-

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phosphate dehydrogenase, NADP, nicotinamide, reduced glutathione (GSH), and UDP-glucuronic acid were obtained from Sigma Chemical Company, St. Louis, Missouri. Piperonyl butoxide was obtained from ICN Pharmaceuticals, Plainview, New York, and SKF-525A was a gift from Smith, Kline and French, Philadelphia, Pennsylvania. Liquid scintillation counting fluid (Bray's solution) and tissue solubilizer (Protosol) were obtained from New England Nuclear, Boston, Massachusetts.

Animals. Five crossbred yearling wether goats, weighing 20 to 30 kg were maintained on pelleted alfalfa and given access to trace mineralized salt and water. The goats were killed by an intravenous dose of sodium pentobarbital. The blood was drained immediately, and the lung, liver, and kidneys were removed and placed in ice for the determination of covalent binding and enzyme assays.

Determination of covalent binding. Freshly removed tissues (liver, lung, or kidney) were homogenized in 3 vol of 1.15% KCl–0.1 *M* phosphate buffer, pH 7.4, at 4°C using a Polytron (Brinkman Instruments, Inc., Westbury, N.Y.), and centrifuged at 10,000*g* for 25 min. To approximate equal amounts of protein in the incubation mixtures, 1 ml of liver supernatant or 2.0 ml of lung and kidney supernatant were used. The substrates (2 ml), 3[^{14}C]MI or 3[^{3}H]MOI, were added in an amount of 2 μmole which contained 0.26 μCi of radioactivity. A NADPH generating system consisting of 1.30 μmole NADP, 50 μmole nicotinamide, 20 μmole glucose-6-phosphate, and 25 μmole $MgCl_2$ in 2 ml of 0.1 *M*, pH 7.4, phosphate buffer was used. When crude microsomal preparation (10,000*g* supernatant) was used, no exogenous glucose-6-phosphate dehydrogenase was added. When microsomes (resuspension of 100,000*g* pellet) were used, 2 units of glucose-6-phosphate dehydrogenase was added in the system. In addition, one of the following MFO inhibitors or conjugating agents (0.5 ml) was added to the incubation mixtures, piperonyl butoxide, 1.5 μmole; SKF-525A, 5.7 μmole; GSH, 7.5 μmole, or UDP-glucuronic acid, 4.3 μmole. The incubation mixtures with a total volume of 6.5 ml was incubated at 37°C for 30 min. The reaction was stopped by the addition of 0.5 ml of 14% perchloric acid ($HClO_4$) followed by centrifugation at 10,000*g* for 10 min. The precipitate was extracted with acetone (7 × 20 ml) and methanol (3 × 20 ml). The precipitated protein was solubilized with 3 ml of Protosol and an aliquot of the covalently bound radioactivity was counted in 10 ml of Bray's solution. Preliminary experiments determined that covalent binding of 3[^{14}C]MI to $HClO_4$ precipitates increased linearly when using mixtures containing up to 3 μmole 3MI and with incubation times up to 1 hr. In order to measure the degree of nonspecific binding, a control tube for each animal was treated in an identical manner except that the 10,000*g* supernatant was boiled for 5 min to inactivate the MFO system. Other control tubes in which the NADPH generating system was omitted produced similar results. Counting efficiency was determined, using the channels-ratio method of quench correction for liquid scintillation counting.

Localization of covalent binding. The 10,000*g* supernatant (crude microsomes) was usually used as the source of MFO in this study. To determine if the covalent binding is catalyzed by the enzyme in microsomal fraction only, microsomal fraction (resuspension of 100,000*g* pellet) or soluble fraction (100,000*g* supernatant) was incubated individually with 3[^{14}C]MI and cofactors as described in previous section. The covalent binding of 3[^{14}C]MI metabolite in each fraction was determined. To investigate if the reactive 3[^{14}C]MI metabolite covalently binds microsomal protein preferentially, after 30 min of incubation with 3[^{14}C]MI, the crude microsomal preparations were further fractionated into 100,000*g* pellets and 100,000*g* supernatant by ultracentrifugation for 1 hr. The proteins in the microsomal and soluble fractions were precipitated with 1 ml of 14% $HClO_4$. The distributions of covalently bound radioactivity in the protein of subfractions from goat liver, lung, and kidney were determined as described in the aforementioned section.

MFO enzyme assays. Microsomes used for enzyme assays were from liver, lung, and kidney of each goat isolated by a differential centrifugation method (13). The protein content of the microsomal fraction was determined by the method of Lowry *et al.* (14). The cytochrome *P*-450 contents of the lung and liver

TABLE I. COVALENT BINDING OF RADIOACTIVE SUBSTRATES IN CRUDE MICROSOMAL PREPARATIONS FROM GOAT LIVER, LUNG, AND KIDNEY

Substrates	Covalently bound radioactivity[a] (nmole/10 mg protein/30 min)		
	Liver	Lung	Kidney
3-[^{14}C]Methylindole	9.0 ± 2.5[b]	32.1 ± 6.0[c]	2.0 ± 1.0[d]
3-[^{3}H]Methyloxindole	2.0 ± 1.0	ND[e]	ND

[a] Means ± SEM of five animals per group.
[b-d] Values in the same row with different superscripts are significantly different ($P < 0.05$; Duncan's multiple-range test).
[e] ND, not detectable.

were determined by difference spectrophotometry using the extinction coefficient of 91 mM^{-1} cm^{-1} (15). The activity of antipyrine *N*-demethylase was determined by quantifying the production of formaldehyde (16). Aniline hydroxylase activity was determined by the method of Imai *et al.* (17) in which *p*-aminophenol formation was measured.

Statistical analysis. Standard errors of the mean were determined for each data set, and either Student's *t* test or Duncan's multiple-range test was used to test for statistical differences (18).

Results and Discussion. Radioactivity expressed as nanomoles of 3[^{14}C]MI or 3[H^{3}]MOI covalently bound to crude microsomal protein of goat liver, lung or kidney is presented in Table I. When 3[^{14}C]MI was used as the substrate, significant covalent binding occurred in the lung, liver, and kidney, and the lung contained the highest binding. When 3[^{3}H]MOI was used as the substrate, covalent binding was not observed in lung and kidney tissues, and a trace amount was present in the liver. These results indicate that a reactive intermediate, capable of binding macromolecules, is formed during the metabolism of 3MI. The higher covalent binding of ^{14}C radioactivity in lung microsomal preparations compared to liver or kidney may be related to the tissue specificity of 3MI toxicity, suggesting that the MFO metabolism of 3MI in the lung may be responsible for organ specificity. The lack of binding by 3[^{3}H]MOI also may reflect the relative *in vivo* lung toxicity of these substrates. Preliminary experiments determined that binding of ^{14}C radioactivity was not observed when the NADPH generating system was deleted from the incubation media. Carbon monoxide treatment or boiling of the en-

TABLE II. THE EFFECT OF MFO INHIBITORS AND CONJUGATING AGENTS ON THE COVALENT BINDING OF 3-[^{14}C]MI METABOLITES IN CRUDE MICROSOMAL PREPARATIONS OF GOAT LIVER, LUNG, AND KIDNEY

Treatment	Covalently bound radioactivity[a] (nmole/10 mg protein/30 min)		
	Liver	Lung	Kidney
Control	9.0 ± 2.5	32.1 ± 6.0	2.0 ± 1.0
MFO inhibitors			
Piperonyl butoxide	3.0 ± 1.1[b]	3.5 ± 0.4[b]	ND[c]
SKF-525A	11.0 ± 3.4	40.5 ± 7.4	ND
Conjugating agents			
Glutathione	2.0 ± 1.0[b]	5.2 ± 1.5[b]	ND
UDP-glucuronic acid	10.5 ± 3.5	31.5 ± 2.3	ND

[a] Means ± SEM of five animals per treatment.
[b] Values are significantly different ($P < 0.05$) from the control in the same tissue by Student's *t* test.
[c] ND, not detectable.

TABLE III. COVALENTLY BOUND RADIOACTIVITY OF 3[^{14}C]MI METABOLITE IN MICROSOMES AND SOLUBLE FRACTIONS OF GOAT LIVER, LUNG, AND KIDNEY

	Covalently bound radioactivity[a] (nmoles/10 mg protein/30 min)		
Fraction	Liver	Lung	Kidney
Microsomes	48.0 ± 1.0[b]	131.0 ± 10.0[c]	10.0 ± 1.0[d]
Soluble	0.01 ± 0.00	0.05 ± 0.03	0.02 ± 0.01

[a] Means ± SEM of four incubations per group.
[b-d] Values in the same row with different superscripts are significantly different ($P < 0.01$; Duncan's multiple-range test).

zyme also inhibited covalent binding. Thus, an NADPH-dependent MFO is most likely the enzyme system in the 10,000*g* supernatant responsible for the activation of 3MI. This is also supported by *in vivo* experiments in which induction or inhibition of the MFO system with phenobarbital or piperonyl butoxide altered the metabolism of 3MI and the susceptibility to 3MI-induced lung disease in goats (8). Hanafy and Bogan (19) have also shown that 3[^{14}C]MI is covalently bound to microsomal protein when incubated with bovine lung microsomes and that the binding is cytochrome *P*-450 dependent.

Table II shows the effect of MFO inhibitors or conjugating agents on the covalent binding of 3[^{14}C]MI in the crude microsomal preparation from goat liver, lung, and kidney. The addition of piperonyl butoxide or GSH significantly inhibited covalent binding in the lung and liver, but SKF-525A or UDP-glucuronic acid had no effect at the concentration used. No binding of 3[^{14}C]MI occurred when kidney crude microsomal preparations were used. Piperonyl butoxide is often used as a synergist with insecticides because of its ability to covalently bind to the terminal oxidase of MFO system (20). SKF-525A is also a known MFO inhibitor, but it had no effect on the 3[^{14}C]MI binding in both liver and lung of goats. When bovine lung microsomes were used, SKF-525A inhibited 64% of the covalent binding of 3MI metabolites (19). The lack of inhibition of covalent binding by SKF-525A in goat tissues may reflect the species difference in the substrate specificity of the MFO system involved.

GSH is a nucleophilic tripeptide of ubiquitous tissue distribution (21). Tissue GSH concentration can influence the toxicity and organ specificity of xenobiotics (22). The reduction in covalent binding of 3[^{14}C]MI metabolite by GSH addition suggests that the 3MI metabolite may be electrophilic. The activated 3MI metabolite could be conjugated directly with GSH molecule *in vitro*. UDP-glucuronic acid is quantitatively an important conjugation agent using glucose as the precursor (23). However, addition of UDP-glucuronic acid in the microsomal incubation mixture did not affect the covalent binding of 3[^{14}C]MI suggesting that glucuronide conjugation to 3MI does not occur *in vitro*.

Two experiments were carried out to determine, first, if the activation of 3[^{14}C]MI to reactive intermediate is specific by the enzyme in the microsomal fraction, second, if the reactive intermediate will attack the macromolecules in microsomal fraction preferentially when the crude microsomes were used. Table III shows the covalently bound radio-

TABLE IV. COVALENTLY BOUND RADIOACTIVITY OF 3-[^{14}C]METHYLINDOLE IN SUBFRACTIONS OF CRUDE MICROSOMAL PREPARATIONS OF GOAT LIVER, LUNG, AND KIDNEY

	Covalently bound radioactivity[a] (nmole/10 mg protein/30 min)		
Subfractions	Liver	Lung	Kidney
100,000*g* Pellet	11.2 ± 1.4[b]	33.0 ± 2.3[c]	2.4 ± 1.1[d]
100,000*g* Supernatant	ND[e]	0.57 ± 0.06	0.17 ± 0.02

[a] Means ± SEM of three incubation samples per group.
[b-d] Values in the same row with different superscript letters are significantly different ($P < 0.05$) by Duncan's multiple-range test.
[e] ND, not detectable.

TABLE V. CYTOCHROME *P*-450 CONTENT AND MFO ACTIVITIES IN GOAT LIVER, LUNG, AND KIDNEY

	Liver	Lung	Kidney
Cytochrome *P*-450 (nmole/mg protein)	1.21 ± 0.32[a]	0.44 ± 0.10[b]	ND[c]
Aniline hydroxylase (nmole product formed/ 10 mg protein/min)	4.5 ± 1.2[a]	1.9 ± 0.6[b]	ND
Aminopyrine demethylase (nmole product formed/10 mg protein/min)	4.3 ± 0.8[a]	0.8 ± 0.2[b]	ND

[a,b] Means ± SEM of five animals per group. Values in same row with different superscripts are significantly different ($P < 0.05$; Student's t test).
[c] ND, not detectable.

activity of 3[^{14}C]MI in microsomal and soluble fractions when the incubation medium consists of either 100,000*g* pellet or 100,000*g* supernatant. The data clearly indicated that the microsomal fraction is mainly responsible for the activation of 3MI. The soluble fraction does not contain enzyme that could activate 3MI as indicated by the trace amount of radioactivity in soluble fractions of all three types of tissues. Again, the lung has the highest covalently bound radioactivity in the microsomal proteins compared to liver and kidney. Table IV depicts the distribution of covalently bound radioactivity in protein of microsomal and soluble fractions when 3[^{14}C]MI was incubated with crude microsomal mixtures. The result indicated that the microsomal fraction has high affinity for 3[^{14}C]MI metabolite or the binding is specific for microsomal protein. The higher covalent binding in the microsomal fractions may also suggest an electrophilic 3MI metabolite is produced which reacts with macromolecules adjacent to its site of formation. The lung microsomes have higher radioactivity than that of liver or kidney which is consistent with the results obtained when 3[^{14}C]MI was incubated with crude microsomal preparation (Table I) or 100,000*g* pellet (Table III).

The cytochrome *P*-450 content and the NADPH-dependent microsomal enzyme activities in liver, lung, and kidney are summarized in Table V. The pulmonary cytochrome *P*-450 content is one-third that of liver. The activity of aniline hydroxylase and aminopyrine demethylase in the lung is one-half or one-fifth that of the liver levels, respectively. Even though there was significantly more binding in lung microsomes compared to liver, the total cytochrome *P*-450 content and enzyme activities were significantly lower in lung than in liver. The ratio of nanomoles of covalently bound 3[^{14}C]MI of cytochrome *P*-450 was 7.4 in the liver and 72.7 in the lung. This suggests that a form of cytochrome *P*-450 with high affinity to 3MI as substrate may be present in lung. This form of cytochrome *P*-450 may be responsible for the production of the reactive intermediate. Alternatively, differences in the tissue concentrations of conjugating agents or enzymes (i.e., tissue GSH level and GSH-S-transferase activity) could also influence the detoxification of 3MI and the degree of covalent binding in these tissues.

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