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Microsomal Metabolism of Furosemide
Evidence for the Nature of the Reactive Intermediate Involved in Covalent Binding

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SUMMARY

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Pathways for the cytochrome P-450 monooxygenase-mediated covalent binding of furosemide to microsomal protein were examined. Two pathways were considered: one leading to a highly electrophilic imine by *C*- or *N*-hydroxylation followed by dehydration, the other an epoxidation pathway leading to formation of an arene oxide. Microsomal covalent binding of [α-^{3}H]furosemide, [^{35}S]furosemide, [α-^{2}H]furosemide, and [α,α'-^{2}H]furosemide was the same, indicating that formation of an imine intermediate is unlikely and that the α-carbon is not a site of metabolic activation. Covalent binding to microsomal protein was enhanced in the presence of an epoxide hydrase inhibitor, 1,2-epoxy-3,3,3-trichloropropane, and did not occur when tetrahydro[^{35}S]furosemide was used as substrate. The results indicate that the covalent binding is mediated by an arene oxide intermediate. The potential significance of a subsequent rearrangement of the arene oxide to other electrophilic species cannot presently be evaluated. Furosemide covalent binding assay results using microsomes from the inbred C57BL/6N (B6) and DBA/2N (D2) strains of mice which had been treated with 3-methylcholanthrene and phenobarbital were compared. Phenobarbital treatment increased covalent binding ($p < 0.05$) and 3-methylcholanthrene treatment had no effect, suggesting that a cytochrome P-450, rather than P-448, pathway is predominantly involved in mediating the covalent binding of furosemide.

INTRODUCTION

Furosemide (Lasix), an important diuretic used in the treatment of cardiovascular diseases, has been shown to potentiate renal injury when used with cephaloridine (1, 2) and, because of its teratogenic potential, is contraindicated during pregnancy (3). Recently Mitchell *et al.* (4) demonstrated that large doses of furosemide caused hepatic necrosis in mice. They also showed that furosemide is metabolized by the cytochrome P-450-dependent monooxygenases in liver microsomes (mouse and human) to a reactive intermediate which is covalently bound to liver protein, and that the degree of covalent binding correlates well with the extent of

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hepatic necrosis in mice.

Earlier work (5), both *in vitro* and *in vivo*, demonstrated that the entire furosemide molecule is covalently bound to protein and that covalent binding to these proteins most likely occurs through the furanylmethyl[2] group. However, only incomplete data are available concerning the nature of the metabolically activated intermediate responsible for this covalent binding. To determine the structure of this reactive metabolite, we incubated furosemide labeled in several different positions with liver microsomes, and determined the amount of furosemide covalently bound to microsomal protein.

MATERIALS AND METHODS

Treatment of animals. Adult male N:GP(SW) mice (20–24 g) were obtained from the University of Washington Vivarium. Male mice from the random-bred N:GP(SW) strain and from inbred strains C57BL/6N (B6) and DBA/2N (D2) were obtained from the National Institutes of Health Animal Supply. Some animals received sodium phenobarbital (75 mg/kg, administered intraperitoneally daily for 3 days), 3-methylcholanthrene (80 mg/kg in corn oil intraperitoneally, 40 hr before use), or cobalt chloride ($CoCl_2 \cdot 6H_2O$, 60 mg/kg intraperitoneally, daily for 2 days). Control mice were treated with 0.9% sodium chloride or corn oil alone for the same periods.

Preparation of liver microsomes. Animals were killed by cervical dislocation, and all subsequent steps were performed at 0–4° using ice-cold solutions and glassware. Livers were removed, minced, washed as free from hemoglobin as possible with 1.15% KCl, and homogenized with a motor-driven glass-Teflon homogenizer in 4 volumes of 1.15% KCl. The homogenate was centrifuged for 20 min at 9000 × *g*, and the supernatant fraction was decanted and centrifuged for 60 min at 105,000 × *g*. The microsomal pellet was washed and resuspended in 1.15% KCl and recentrifuged at 105,000 × *g* for 60 min. The washed microsomal pellet was suspended in 0.1 M potassium phosphate buffer, pH 7.4, prior to incubations. Protein concentrations were determined according to Lowry *et al.* (6), using crystalline bovine serum albumin as standard.

Covalent binding assay. The complete incubation mixtures contained the following in a final volume of 3.0 ml: 1.0 ml of liver microsomal suspension (containing 3.0 mg of protein), furosemide labeled with ^3H or ^{35}S at various concentrations, and 0.5 ml of an NADPH-generating system containing NADP (1 μmole), $MgCl_2$ (15 μmoles), glucose 6-phosphate (25 μmoles), and glucose 6-phosphate dehydrogenase (2 enzyme units) in 0.1 M potassium phosphate buffer, pH 7.4. Prior to incubation the reaction mixture (without NADPH-generating system) was kept at 0°. After incubation for 2 min in a Dubnoff shaking incubator at 37°, the reaction was initiated by adding the NADPH-generating system. Control mixtures, used to assay for noncofactor-dependent covalent binding, included 0.5 ml of 0.1 M potassium phosphate buffer, pH 7.4, in place of the NADPH-generating system. In studies using an epoxide hydrase inhibitor, the inhibitor was added to the reaction mixtures in 10 μl of spectrophotometric grade acetonitrile (Aldrich Chemical Company) with vigorous mixing immediately prior to addition of the NADPH-generating system. Control mixtures received 10 μl of acetonitrile alone.

The incubations were terminated after 15 min by the addition of 0.8 ml of 3 M TCA.[3] The incubation mixture was then centrifuged at 1000 × *g* for 10 min at room temperature. The supernatant fraction was discarded, and the precipitated protein was washed twice with 3.0 ml of 0.6 M TCA and then with methanol (3-ml portions) until no further radioactivity could be removed (usually four methanol extractions).

The washed protein was dissolved in 0.5 ml of 1 N NaOH, and a 250-μl aliquot was added to 10 ml of scintillation fluid [14.0 g

[2] Furanylmethyl is accepted *Chemical Abstracts* nomenclature for furfuryl, i.e., the furan ring with its adjacent methylene group.

[3] The abbreviations used are: TCA, trichloracetic acid; CSA, 4-chloro-5-sulfamoylanthranilic acid; 3-MC, 3-methylcholanthrene.

of 2,5-bis[2'-(5-*tert*-butylbenzoxazolyl)]-thiophene (Amersham/Searle), 280 g of naphthalene, 1400 ml of methyl Cellosolve, and 2000 ml of toluene] and counted. Radioactivity was corrected for background and quenching (external standardization). Protein concentration was determined on an aliquot of the NaOH-solubilized protein. Covalent binding was expressed as nanomoles of furosemide bound per milligram of protein.

Covalently bound furosemide was subjected to hydrolysis experiments in which aliquots of reacted protein dissolved in 1 N NaOH were added to 0.1 N HCl in aqueous methanol (opaque solution) and heated at 75° for 2 hr. After neutralization of the reaction mixture with sodium carbonate, the volume of solvent was reduced by rotary evaporation and protein was precipitated again using 0.6 M TCA. The precipitate was washed (TCA and methanol), and covalently bound radiolabel was determined as described above. In control experiments furosemide was converted to CSA under these conditions in 30–45 min, as shown by thin-layer chromatography on silica gel GF (R_F value for furosemide, 0.53; and for CSA, 0.19 in chloroform–methanol–acetic acid (89:6:5).

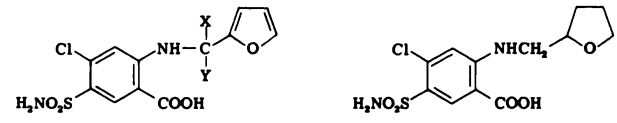
Labeled compounds and other reagents. Furosemide (2-furylmethylamino-4-chloro-5-sulfamoylbenzoic acid) was provided by Hoechst-Roussel Pharmaceuticals, Inc. [^{35}S]Furosemide (initial specific activity, 330 μCi/mmole) was provided by Drs. Ralph E. Cutler and Andrew D. Blair, Department of Medicine, Harborview Medical Center, University of Washington. [^{3}H]Furosemide (furanylmethyl generally labeled) was provided by Dr. Jerry R. Mitchell, Laboratory of Chemical Pharmacology, National Heart and Lung Institute. [α-^{2}H]Furosemide and [α-^{3}H]furosemide (Fig. 1) (specific activity, 9.32 mCi/mmole) were prepared by the method of Nelson *et al.* (7). 2-Tetrahydrofuranylmethylamino-4-chloro-5-sulfamoylbenzoic acid (tetrahydrofurosemide, Fig. 1) was prepared by the method of Seidel *et al.* (8). Tetrahydro-[^{35}S]furosemide (specific activity, 120 μCi/mmole) was similarly prepared from [^{35}S]furosemide. [α,α'-^{2}H]Furosemide (Fig. 1) was prepared by the procedure of Sturm *et al.* (9), using [α,α'-^{2}H]-furanylmethylamine prepared by $LiAl^{2}H_4$ reduction of 2-cyanofuran. [α,α'-2H]Furosemide had the same R_F value as furosemide on thin-layer chromatography, and was further characterized by chemical ionization (methane) mass spectrometry (MH^+ = 333). A nuclear magnetic resonance spectrum of [α,α'-^{2}H]furosemide showed no α-methylene proton resonances.

The purity of the radiolabeled compounds was greater than 99.9% as shown by thin-layer chromatography on silica gel in two solvent systems (ethyl acetate–methanol–ammonium hydroxide, 65:25:10, and chloroform–methanol–acetic acid, 89:6:5), using a Berthold 6000-1 radiochromatogram scanner to locate the radioactive compounds.

1,2-Epoxy-3,3,3-trichloropropane was obtained from Aldrich Chemical Company and was distilled prior to use. All other reagents either were of analytical reagent grade or were distilled prior to use.

RESULTS

Assay for covalent binding of furosemide. In plots of S/v vs. S (10, 11), rates of covalent binding followed classical Michaelis-Menten kinetics (Fig. 2). Covalent binding was a linear function of time and



Furosemide X = Y = H
[α-^{3}H] Furosemide X = ^{3}H, Y = H
[α-^{3}H] Furosemide X = ^{3}H, Y = H
[α,α'-^{3}H] Furosemide X = Y = ^{3}H

Tetrahydrofurosemide

FIG. 1. *Structures of furosemide substrates*

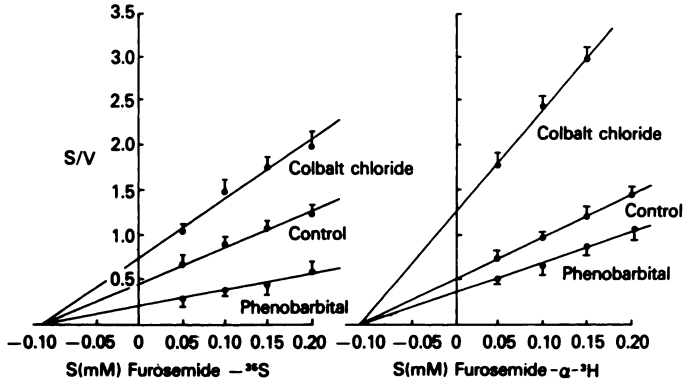


FIG. 2. *Microsomal covalent binding of furosemide in vitro, using [35S]furosemide and [α-3H]furosemide.* Covalent binding was determined as described in MATERIALS AND METHODS. Each point represents the mean and standard error of at least three incubations (non-cofactor-dependent covalent binding subtracted). The differences in effects of phenobarbital and $CoCl_2$ result from incubations done with microsomes prepared on different occasions. Because of limitations on the number of incubations carried out at any one time, experiments with control microsomes were performed simultaneously, using differently labeled substrates. All microsomes were prepared from N:GP(SW)-UW mice. Incubations using microsomes prepared from animals treated with phenobarbital and cobalt chloride were performed with one of the labeled substrates simultaneously with incubations using control microsomes and both labeled substrates. Velocity (v) is expressed as nanomoles of substrate bound per milligram of protein per minute.

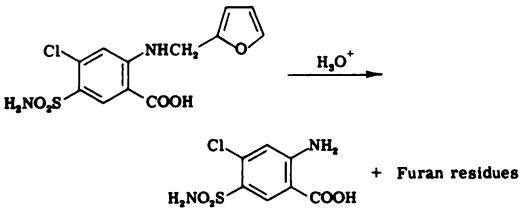
microsomal protein concentration, as previously described (5). The extent of covalent binding, as determined by radiolabel, was quantitatively the same whether furosemide labeled with ^{35}S in the sulfamoyl group attached to the benzene ring or labeled with tritium in the α-position of the furanylmethyl group was used. The apparent Michaelis constant (K_m) was 0.11 mM, and the maximal velocity (V_{max}) was 0.21–0.24 nmole bound per milligram of protein per minute. Prior treatment with phenobarbital, an inducer of microsomal cytochrome P-450 monooxygenase(s), doubled the extent of covalent binding (V_{max} = 0.4–0.5 nmole bound per milligram of protein per minute). Cobalt chloride, an inhibitor of microsomal cytochrome P-450 monooxygenase synthesis (12), decreased the extent of covalent binding to 0.09–0.12 nmole/mg of protein per minute. Neither treatment affected the apparent K_m. Because the degree of covalent binding was quantitatively the same for both labeled substrates, the entire molecule, not a fragment containing only one of the aromatic rings, must be bound to protein. When mixtures of [^{35}S]furosemide and [α-^{3}H]-furosemide were used together as substrates, the total binding was the same as when either was used alone. The alternative possibility of independent reactions, each involving one fragment of the molecule and proceeding at the same rate, is thus excluded. To show that the covalent binding was not a result of any acid-catalyzed process occurring during termination of the incubations with TCA, we terminated some incubations with acetone. No difference in the amount of bound radiolabel was noted.

Nature of reactive intermediate. To determine what part of the furosemide molecule is covalently bound to protein, we subjected samples of reacted protein to aqueous acid hydrolysis to cleave the furan ring and break the furanylmethyl carbon–amine nitrogen bond. Following reprecipitation of the protein, the amount of covalently bound radiolabel was again determined. Nearly all the tritium, but less than one-half (41%) the ^{35}S label, remained covalently bound (Table 1). At least a portion of the persistent ^{35}S label is thought to be due to incomplete hydrolysis of protein-bound furosemide, occurring be-

TABLE 1

Labilities of covalently bound [^{35}S]furosemide and [α-^{3}H]furosemide to hydrolytic conditions in vitro

Microsomes were prepared from N:GP(SW)-NIH mice. Precipitated protein obtained from at least three separate 3.0-ml incubations was combined and suspended as an opaque solution in 0.1 N HCl in methanol-water at 75° for 2 hr, after which the conversion of furosemide (solution) to CSA was measured as described in MATERIALS AND METHODS. Microsomal covalent binding of [^{35}S]furosemide and [α-^{3}H]furosemide was quantitatively the same before hydrolysis (see Table 2). Values are the means and standard errors of the three determinations.



Protein binding reaction	Covalent binding
	nmoles/mg protein/15 min (% control)
[^{35}S]Furosemide	2.97 ± 0.11
After hydrolysis	1.22 ± 0.10 (41%)
[α-^{3}H]Furosemide	2.75 ± 0.10
After hydrolysis	2.39 ± 0.10 (87%)

cause of poor solubility of the protein under the experimental conditions of hydrolysis. This finding suggests that the primary site of covalent binding must be in the furanylmethyl moiety. Similar results were obtained by Mitchell *et al.* (5), who compared the amount of furosemide covalently bound to protein *in vivo* using [*carboxyl*-^{14}C]furosemide and [*furanylmethyl G*-^{3}H]furosemide.

Based on these results, a number of alternative electrophilic intermediates, each arising from the metabolism of the furanylmethyl group, must be considered (Fig. 3). The two most likely possibilities are (a) a pathway leading to a highly electrophilic imine of (b) arene oxide formation. The formation of the imine [*N*-(2-furylmethylene)-4-chloro-5-sulfonamoylanthranilic acid] requires prior hydroxylation at either the amine nitrogen or the α-carbon, followed by dehydration. The resulting electrophilic imine would be most susceptible to nucleophilic attack at the α-carbon or at positions 3′ and 5′ of the furan ring. With regard to arene oxide formation, epoxidation at either the 2′,3′- or 4′,5′-positions is the most probable pathway.

To determine which of these pathways occurs, we used suitably labeled furosemide derivatives as substrates in the covalent binding assay (Table 2). No change in amount of bound radiolabel was observed when comparing [α-^{3}H]furosemide with [^{35}S]furosemide and [*furanylmethyl G*-^{3}H]furosemide as substrates, indicating that the pathway leading to the imine is not very likely. If this pathway were operative, at least some decrease in the radioactivity of covalently bound furosemide would be expected (compared with [^{35}S]-furosemide), since, following either *C*-hydroxylation or *N*-hydroxylation and subsequent dehydration, some amount of tritium would be lost from the α-carbon. The possibility remains, however, that the presence of a large kinetic isotope effect could result in preferential removal of hydrogen rather than tritium. This possibility was evaluated using the deuterated analogues [α-^{2}H]furosemide and [α,α'-^{2}H]-furosemide. The results, also in Table 2, show no difference in the amount of bound radiolabel, as determined using trace amounts of [^{35}S]furosemide as markers in the presence of these deuterated compounds.

To evaluate the importance of the aromatic furan ring in this process, we studied an additional radiolabeled analogue, tetrahydro[^{35}S]furosemide. This analogue has solubility and oil-water distribution characteristics similar to furosemide, but lacks the aromatic furan ring. Practically no covalent binding occurred following incubation with this compound (Table 2), demonstrating that the aromatic furan ring is necessary for covalent binding of furosemide.

To confirm that an arene oxide of the furan ring is the reactive species in covalent binding, the effect of 1,2-epoxy-3,3,3-trichloropropane, a potent inhibitor of epoxide hydrase (13), on covalent binding was examined (Table 3). As has been shown for the covalent binding of polycyclic aromatic hydrocarbons to DNA (14), we expected that, by preventing the enzymatic hydration of a furan epoxide, the

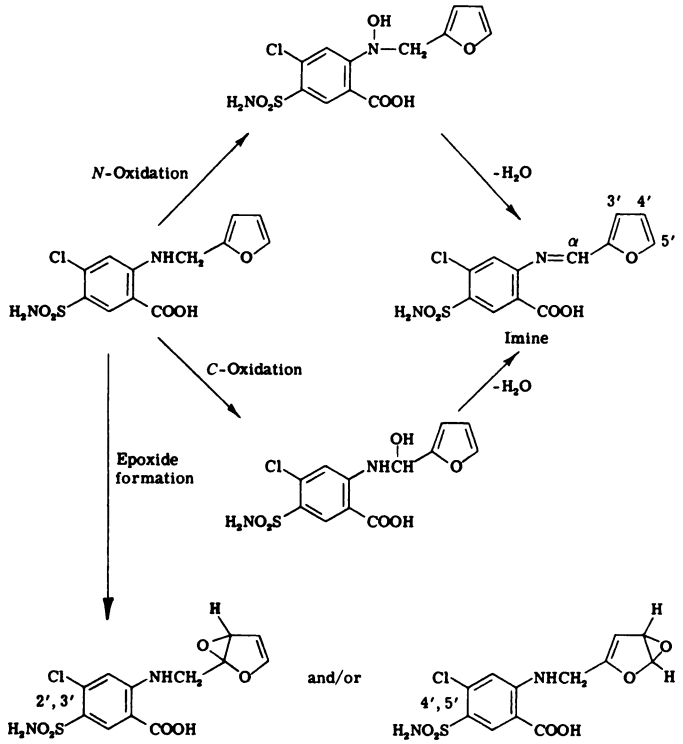


FIG. 3. *Possible routes of microsomal oxidation of furosemide to electrophilic intermediates*

steady-state level of this intermediate would be increased and would result in a greater degree of covalent binding. A concentration of 1.0 mM 1,2-epoxy-3,3,3-trichloropropane produced a nearly 2-fold increase in covalent binding (Table 3). The relatively smaller increases at higher concentrations indicate that the epoxide hydrase inhibitor may also inhibit arene oxide formation (15).

Nature of monooxygenase system involved in furosemide activation. There are at least two forms of cytochrome P-450 which support monooxygenase activities. Treatment of animals with phenobarbital increases cytochrome content without changing the spectral characteristics of control microsomes, whereas treatment with polycyclic aromatic hydrocarbons such as 3-MC gives rise to a new, predominant form with unique spectral characteristics, known as cytochrome P_1-450 (16) or P-448 (17). In certain inbred strains of mice, the ability to form P-448 is genetically controlled and is specifically associated with increases in at least 10 monooxygenase activities, including arene oxide formation, *N*-hydroxylation, and *N*- and *O*-dealkylation reactions (18). The comparison of two inbred strains of mice, C57BL/6N (B6), which is responsive to 3-MC treatment, and DBA/2N (D2), which is nonresponsive, provides a useful model system for determining the cytochrome involved in a given reaction (18).

Microsomes from B6 and D2 mice which had been treated with 3-MC or phenobarbital were compared for their ability to catalyze covalent binding of furosemide (Table 4). Treatment of mice with 3-MC caused no detectable increase in covalent binding, whereas phenobarbital treatment resulted in significant increases ($p < 0.05$) in all strains tested. These results suggest that a cytochrome P-450 rather than a P-448 pathway is predominantly involved in mediating the covalent binding of furosemide.

DISCUSSION

These results confirm the earlier finding (5) that the covalent binding of furosemide to protein is mediated by a cytochrome P-450 monooxygenase activity, and provide additional insight into the nature of the molecular species which is covalently bound. The metabolite which is covalently bound must retain the essential elements of the furosemide molecule, since labels from either aromatic ring system [^{35}S or ^{14}C on the benzene ring (5) and ^{3}H in the furanylmethyl group] are bound to the same extent. Additionally, the site of covalent binding is primarily in the furanylmethyl group, as indicated by the results of the hydrolysis experiments (Table 2).

To account for the covalent binding of furosemide through a metabolic activation process, two potential pathways were considered most likely (Fig. 3): (a) imine formation and (b) arene oxide formation. Each pathway is an oxygen- and NADPH-requiring process, and each fulfills the requirement of retaining the entire molecule upon covalent binding. The imine path-

TABLE 2

Microsomal covalent binding of furosemide analogues

Preparation of liver microsomes from N:GP(SW)-NIH mice, the incubation procedure, and determination of covalent binding are described in MATERIALS AND METHODS. All substrate concentrations were 0.1 mM, and the apparent K_m for all substrates was 0.11 mM. Values are the means and standard errors of at least three determinations (less non-cofactor-dependent covalent binding).

Analogue	Covalent binding
	nmoles/mg protein/15 min
[*furanylmethyl G*-^{3}H]Furosemide	1.26 ± 0.10
[^{35}S]Furosemide	1.36 ± 0.09
[α-^{3}H]Furosemide	1.36 ± 0.15
[α-^{2}H]Furosemide[a]	1.30 ± 0.10
[α,α'-^{2}H]Furosemide[a]	1.48 ± 0.20
Tetrahydro[^{35}S]furosemide	0.04 ± 0.01

[a] Mixed with less than 1.0% (w/w) [^{35}S]-furosemide.

TABLE 3

Effect of 1,2-epoxy-3,3,3-trichloropropane on microsomal covalent binding of [α-^{3}H]furosemide

Preparation of liver microsomes from N:GP(SW)-NIH mice, the incubation procedure, and determination of covalent binding are described in MATERIALS AND METHODS. The furosemide concentration was 0.4 mM, and 10 μl of acetonitrile, when used, were added to each 3.0-ml incubation. 1,2-Epoxy-3,3,3-trichloropropane was added in 10 μl of acetonitrile. Values are the means and standard errors of at least three determinations (less non-cofactor-dependent covalent binding).

Conditions	Covalent binding
	nmoles/mg protein/15 min (% control)
Control	
[α-^{3}H]Furosemide, buffer	2.56 ± 0.21
[α-^{3}H]Furosemide, acetonitrile	2.81 ± 0.19 (100%)
1,2-Epoxy-3,3,3-trichloropropane	
1.0 mM	4.80 ± 0.29[a] (170%)
2.5 mM	4.32 ± 0.20[a] (154%)
5.0 mM	3.91 ± 0.15 (139%)
10.0 mM	3.26 ± 0.30 (116%)

[a] Significantly different from control ($p < 0.05$).

TABLE 4

Comparison of microsomal covalent binding of [^{35}S]furosemide from various strains of mice

Treatments of mice, preparation of liver microsomes, incubation procedure, and determination of covalent binding are described in MATERIALS AND METHODS. The concentration of furosemide was 0.5 mM. Values are the means and standard errors of at least three determinations (less non-cofactor-dependent covalent binding).

Mouse Strain	Treatment of mice		
	None	3-MC	Phenobarbital
	nmoles bound/mg protein/15 min		
DBA/2N	2.18 ± 0.09	1.98 ± 0.10	3.31 ± 0.10
C57BL/6N	2.17 ± 0.15	2.26 ± 0.13	3.84 ± 0.08
N:GP(SW)-NIH	1.75 ± 0.07	1.80 ± 0.03	2.70 ± 0.10

way, however, is not compatible with the results obtained using α-labeled derivatives of furosemide. Similarly, the *N*-hydroxyl and *C*-hydroxyl intermediates, which are potential electrophiles in their own right, and might be further activated by esterification as has been proposed for *N*-hydroxyl-2-acetylaminofluorene (19, 20), are not possible. Neither the *N*-hydroxyl nor *C*-hydroxyl species could lead to the imine without loss of tritium label from the α-carbon (Table 2). Although the *C*-hydroxyl species could theoretically retain tritium label if a large kinetic isotope effect were operative, no such process was identified when binding of [α-^{2}H]- and [α,α'-^{2}H]furosemide was compared (Table 2). These experiments should be sufficiently sensitive to detect a kinetic isotope effect, since similar oxidations of other compounds show kinetic isotope effects of 1.4–2.0 (21, 22), although values as large as 7–10 have been reported (23, 24). No change in the apparent K_m was observed when the deuterated compounds were used as substrates (Table 2), which is evidence against an isotope effect and against the imine pathway.

The failure of tetrahydro[^{35}S]furosemide to bind significantly and the enhancement of binding of furosemide in the presence of the epoxide hydrase inhibitor (Table 3) further support our conclusion that formation of an arene oxide in the furan ring is the initial step in activating furosemide. This epoxide, be it the 2′,3′-oxide or the 4′,5′-oxide, rather than reacting with protein directly, might itself be an intermediate leading to the formation of other electrophiles by prototropic shifts (25). A number of potential subsequent intermediates are illustrated in Fig. 4. For instance, the α,β-unsaturated ketones and α,β-unsaturated lactones are isomeric with the arene oxide structures. These proposed arene oxide intermediates could be converted into highly electrophilic ketones or lactones by the known epoxide-carbonyl rearrangement (25), requiring only prototropic shifts. The conversions of arene oxides to phenols (NIH shift) are very closely related rearrangements (26). A number of α,β-unsaturated carbonyl derivatives of tetrahydrofuran sytems are known to be alkylating agents which react readily with biological nucleophiles such as cysteine, and are carcinogens (27) or potential antileukemic agents (28). Some related α-methylene γ-lactones, which also are α,β-unsaturated carbonyl compounds, produce liver toxicity, which is decreased following conjugation of the toxin with cysteine (29). Related electrophilic derivatives of a dihydrofuran moiety have been proposed as intermediates in the binding of aflatoxin B_1 2,3-dichloride (a carcinogen related to the

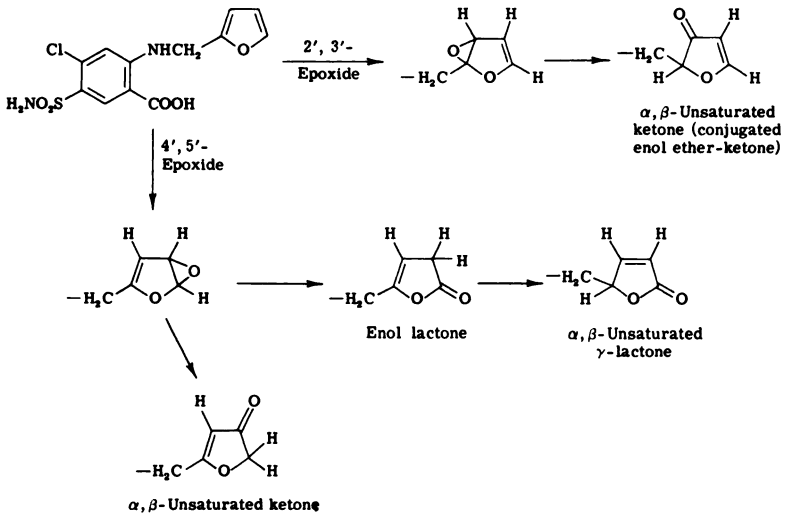


FIG. 4. *Possible electrophilic species formed from epoxide intermediates*

2,3-oxide of aflatoxin B_1) with nucleic acids and proteins (30).

The activation pathway mediating covalent binding is most likely associated with a form of cytochrome P-450 other than P-448, since covalent binding did not increase when microsomes from 3-MC-treated mice were used. Because 3-MC does not induce epoxide hydrase in the three strains of mice used (31), the observed lack of increase in covalent binding is not likely to be the result of increased destruction of an epoxide intermediate. However, phenobarbital does induce epoxide hydrase in B6 mice, but not in the D2 or N:GP(SW) strains (31). The observed increase in covalent binding in all three strains after phenobarbital induction may result because the intermediate epoxide is a poor substrate for epoxide hydrase, since more than one epoxide hydrase may be present, as has been found in partially purified rat microsomal fractions (32). On balance, the data presently support a cytochrome P-450-mediated pathway.

In summary, the results of this study clearly indicate that a furan epoxide is the most likely intermediate responsible for the cytochrome P-450-mediated covalent binding of furosemide to microsomal protein. Since it was previously shown that the extent of covalent binding parallels furosemide-induced liver necrosis in mice (5), it is likely that such an intermediate is the species responsible for the observed toxicity.

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