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STUDIES ON THE ACTIVATION OF A MODEL FURAN COMPOUND—TOXICITY AND COVALENT BINDING OF 2-(*N*-ETHYL-CARBAMOYLHYDROXYMETHYL)FURAN*

F. PETER GUENGERICH

Department of Biochemistry and the Center in Environmental Toxicology, Vanderbilt University School of Medicine, Nashville, TN 37232, U.S.A.

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Abstract—2-(*N*-ethylcarbamoylhydroxymethyl)furan was studied as a simple model for the entire class of toxic furans. This compound is toxic to both the lung and liver of the rat—covalent binding occurs predominantly in these target organs. The model was covalently bound to protein and nucleic acids throughout the cell; the enzymes responsible for activating the compound to a form(s) capable of covalently binding to these tissue macromolecules are localized in the microsomal and, to a much lesser extent, nuclear fractions of the cells of the target organs. Binding and toxicity appear to involve the furan moiety of the compound; this binding can be inhibited by added nucleophiles, but not by epoxide hydrase inhibitors. Studies utilizing both microsomes and highly purified reconstituted cytochrome P-450 systems for activation indicate that the reactive metabolite(s) possesses a certain amount of stability, in agreement with the observed distribution of the compound *in vivo*.

The toxicity of a number of naturally occurring furan derivatives is well documented; among such compounds are the aflatoxins [1–8], 4-ipomeanol and related compounds [9–11], sterigmatocystin [12], and ngione [13]. The aflatoxins are also potent mutagens and carcinogens [1, 5]. Recently, a number of simple furans have been shown to be hepatotoxic [14–16]. Because of the widespread occurrence of this family of chemicals in the environment [1, 10, 12–14, 16, 17], a simple furan was sought that could serve as a model for the entire class of compounds. Since Seawright and Mattocks [15] had demonstrated the toxicity of certain *N*-substituted carbamate esters of 3-hydroxymethylfuran. studies were initiated with 2-(*N*-ethylcarbamoylhydroxymethyl)furan (CMF), which can be readily prepared with ^{3}H or ^{14}C labels to aid in studying metabolism.

This report provides evidence that CMF is an appropriate model for the toxic furans. Studies on the toxicity *in vivo* to liver and lung, mutagenicity, the relationship of covalent binding to these phenomena, and the enzymic mechanism of activation are presented.

MATERIALS AND METHODS

Materials. *d*-Benzphetamine and pregnenolone-16α-carbonitrile were donated by Dr. P. W. O'Connell of the Upjohn Co.

Synthesis of CMF. A solution of 50 g (0.51 mole) of 2-hydroxymethylfuran in 200 ml benzene was stirred and swept with dry nitrogen; to this was added a few drops of triethylamine and 50 g (0.72 mole) ethyl isocyanate over a period of 1 hr. The reaction was heated under gentle reflux for 1 hr, cooled, and concentrated *in vacuo*. The residue was dissolved in ether and washed four times with water. The ether layer was dried with Na_2SO_4 and the solvent was removed *in vacuo* to give the product in 89 per cent yield. Upon cooling to −20°, the product solidified—a portion was recrystallized from benzene–petroleum ether to give off-white crystals: m.p. 29–30° (uncorrected); calcd. for $C_8H_{11}NO_3$: C, 56.77, H, 6.56, N, 8.28—found: C, 56.87, H, 6.70, N, 8.11 (Galbraith Laboratories, Knoxville, TN); mass spectrum (electron ionization, 70 eV)—m/e (relative intensity): 169 (2, M^+), 98 (38), 97 (10), 81 (100), and 56 (21). The tetrahydro derivative of CMF (m.p. 77–80°) was prepared in the same manner from tetrahydrofurfuryl alcohol.

In the synthesis of [^{3}H] CMF, 0.5 g (5.4 m-mole) of freshly distilled 2-furaldehyde was stirred with 0.2 mg (0.05 m-mole) $NaB^{3}H_4$ (25 mCi) in 20 ml of dry ethanol for 2 hr at 25°. Carrier $NaBH_4$ (0.26 g, 6.7 m-mole) was added and stirring continued for an additional 2 hr. The reaction mixture was concentrated to dryness *in vacuo*, washed with benzene, and reconcentrated to dryness; the benzene treatment was repeated. The residue was dissolved in water and the aqueous solution was extracted five times with ether. The combined organic layers were dried with Na_2SO_4 and the solvent was removed *in vacuo*; the residue was esterified as described above to give 0.42 g [^{3}H]CMF (50 per cent yield); the specific activity was 8.2 mCi/m-mole.

For the synthesis of [^{14}C]CMF, $LiAlH_4$ (75 mg; 2.0 m-moles) was suspended in 20 ml of dry ether; to the solution was added (with stirring) 225 mg (2.0 m-moles) [*carboxyl*-^{14}C]-2-furoic acid (0.1 mCi, Research Products International) dissolved in 5 ml of dry ether. The reaction was stirred for 90 min at 25°,

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stopped by the addition of ethyl acetate, diluted with CH_3OH, and filtered through Celite. The residue was washed with CH_3OH and ether; the combined filtrates were concentrated *in vacuo* and esterified as described above. The material was purified by preparative thin-layer chromatography (t.l.c.) (Silica gel G; benzene–CH_3OH, 9:1 (v/v); elution with acetone) to give [^{14}C]CMF in 41 per cent yield; the specific activity was 0.047 mCi/m-mole.

The ^{3}H-, ^{14}C-, and non-radioactive materials each gave only a single peak upon analysis by gas–liquid chromatography (g.l.c.) (3% OV-17, program from 80 to 200°) and only a single I_2-reactive spot with t.l.c. (Silica gel G; benzene–CH_3OH, 8:1 (v/v); R_f 0.55); all of the radioactivity of the labelled materials migrated in a single peak (coincident with the I_2-positive spot) in the t.l.c. system.

Animals. Male Sprague–Dawley rats (80–100 g) were fed a commercial diet *ad lib.* Injections of CMF were made in 0.5 ml of corn oil intraperitoneally. Rats were induced with phenobarbital by allowing animals to drink a 0.1% solution for 5 days. 3-Methylcholanthrene (20 mg/kg) was injected intraperitoneally (in 0.5 ml corn oil) once each day for 3 days. Pregnenolone-16α-carbonitrile (100 mg/kg) was injected intraperitoneally (in 1 ml water with the addition of Tween 80) twice daily for 2 days prior to sacrifice.

Subcellular fractions. All steps were carried out at 0–4°. Rats were stunned by a blow to the head and decapitated; organs of interest were excised and washed in 1.15% KCl. The tissues were minced in 0.1 M Tris–acetate buffer (pH 7.4) containing 0.1 M KCl, 1 mM EDTA, and 20 μM butylated hydroxytoluene [18] and homogenized in the same buffer with eight strokes of a motor-driven Teflon–glass homogenizer. Nuclei and cell debris were pelleted by centrifugation for 10 min at 1000 *g*—the resulting supernatant was centrifuged at 10,000 *g* for 30 min to obtain the mitochondrial fraction as a pellet. This supernatant was centrifuged at 105,000 *g* for 60 min: the resulting supernatant was used as the cytosol, and the microsomal pellet was homogenized in 0.1 M potassium pyrophosphate (pH 7.4) buffer containing 1 mM EDTA and 20 μM butylated hydroxytoluene and recentrifuged at 105,000 *g* for 60 min to obtain the microsomal fraction. Microsomes were stored in 10 mM Tris–acetate buffer (pH 7.4) containing 1 mM EDTA and 20% glycerol at −20° for up to 3 weeks without loss of activity.

Purified liver nuclei were prepared essentially by the hypertonic sucrose method of Spelsberg *et al.* [19], except that the Triton X-100 steps were omitted.

Enzymes. Cytochromes P-450 from phenobarbital-treated rabbits [20] and rats were purified as described*; the preparations used here contained 13.3 and 15.4 nmoles cytochrome P-450/mg of protein respectively. NADPH–cytochrome P-450 reductase was purified as before*; the preparation catalyzed the reduction of 12.8 μmoles cytochrome *c*/min/mg of protein.

Assays. Protein [21] and cytochrome P-450 [22] concentrations were measured as previously described. Polyacrylamide gel electrophoresis was carried out essentially according to Laemmli [23]. All incubations (37°) *in vitro* utilized 50 mM potassium *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonate (Hepes) buffer (pH 7.7) containing 15 mM $MgCl_2$; the NADPH-generating system consisted of 10 mM glucose 6-phosphate, 0.5 mM $NADP^+$, and 1 unit/ml of yeast glucose 6-phosphate dehydrogenase. Binding of [^{3}H]CMF *in vitro* was measured after thorough washing with 95% ethanol (homogenization with a Sorvall Omni-mixer at top speed for 1 min followed by centrifugation at 20,000 *g* for 10 min) until supernatant radioactivity was at the background level (usually 3–4 cycles); the precipated microsomes were solubilized and assayed for protein and bound radioactivity as described [24]. Binding *in vivo* utilized similar washing procedures for the cell fractions. All assays *in vitro* were done in triplicate and results are expressed as means ± S.D.

* F. Guengerich, manuscript submitted for publication.

RESULTS

Pulmonary and hepatic damage due to CMF. The LD_{50} (after 16 hr) for CMF was found to be about 80 mg/kg for male rats; death appeared to be due chiefly to severe lung damage. Out of eleven animals that survived doses of CMF greater than 75 mg/kg, ten showed extensive lung damage, while only one showed liver damage. The lung damage (observed after 24 hr) was characterized chiefly by edema, hemorrhaging and consolation. The liver damage (observed after 96 hr) was characterized as centrilobular necrosis. (All observations were confirmed by microscopy.) When animals were pretreated with phenobarbital prior to injection of CMF, it was found that all five of the animals surviving a dose of 80 mg/kg of CMF exhibited gross and microscopic hepatic necrosis. In both the untreated and the phenobarbital-treated rats, doses of 40–50 mg/kg produced no observable effects. No damage to the kidney or to other major organs was observed in any of the above work.

Covalent binding of CMF in vivo. Preliminary experiments showed that when rats were injected with [^{3}H]CMF and sacrificed after 24 hr, most of the compound was tightly bound to the target organs, namely the liver and lung, and, to a lesser extent, the kidney (Table 1). In these and subsequent experiments (*vide infra*), it was found that the bound radioactivity could

Table 1. Covalent binding of [^{3}H]CMF to various tissues*

Tissue	[^{3}H]CMF (nmoles/ mg protein)	[^{3}H]CMF (nmoles/g wet wt)
Liver	1.15	91
Lung	0.90	59
Kidney	0.20	13
Spleen	0.04	4
Intestine	0.05	3
Stomach	0.04	3
Heart	0.07	5
Brain	0.02	1

* Measured 24 hr after injection of 70 mg/kg. Results are the mean values for three animals; in all cases, the S.D. was less than ±30 per cent.

Table 2. Covalent binding *in vivo* of CMF to macromolecules of subcellular fractions*

Tissue	Fraction	CMF (nmoles/mg protein)	Per cent of total bound to: Lipid	RNA	DNA	Protein
Liver	Nuclei	1.15	1	29	30	40
	Mitochondria	0.40	1	43	18	38
	Microsomes	0.19	1	42	21	37
	Cytosol	0.24	0	79	14	7
Lung	Nuclei	0.41	0	9	13	78
	Mitochondria	0.30	1	74	17	8
	Microsomes	0.23	2	68	19	11
	Cytosol	0.26	0	62	14	24
Kidney	Nuclei	0.06				
	Mitochondria	0.08				
	Microsomes	0.09				
	Cytosol	0.09				

* Rats were injected with 40 mg/kg [^{3}H]CMF and sacrificed after 24 hr. Subcellular fractions were prepared as described in Materials and Methods, and lipid, RNA, DNA and protein were fractioned as described [25]. Results are the means of three experiments and the S.D. was $\leq \pm 25$ per cent in all cases except for the lipid values ($\leq \pm 100$ per cent).

not be removed by extensive homogenization or stirring under reflux with various organic solvents, dialysis, gel filtration, or electrophoresis; thus, the label is assumed to be covalently bound.

The results presented in Table 2 demonstrate that CMF is covalently bound to all subcellular fractions of the liver and lung and, to a lesser extent, the kidney. Very little of the material was found in the lipid fraction, but instead was tightly bound to RNA, DNA and protein, with variation among the subcellular fractions.

Distribution of the enzymatic capacity to catalyze covalent binding of CMF to protein. In preliminary experiments, it was found that liver microsomal fractions catalyzed the NADPH-dependent binding of CMF to protein. Under identical conditions, [carboxyl-^{14}C]-2-furoic acid was bound at less than 5 per cent of the rate of [^{3}H]CMF. 2-Hydroxy[^{3}H]methylfuran was bound to protein, but the binding did not require NADPH and is presumably due to the instability of this compound in water. The results presented in Table 3 show that the enzymatic ability to activate CMF is concentrated in the microsomal fraction, with some activity being present in the crude nuclear fraction. When the microsomes prepared from several organs were examined for activity (Table 4), it was found that only those from the target organs (liver and lung) were able to catalyze the covalent binding.

Table 3. Covalent binding of CMF to protein by different subcellular liver fractions*

Fraction†	CMF (nmoles bound/min/mg protein)
Nuclei	0.40 ± 0.01
Mitochondria	0.05 ± 0.06
Microsomes	2.88 ± 0.81
Cytosol	0.01 ± 0.05

* Each fraction was assayed for its ability to catalyze covalent binding of [^{3}H]CMF (as described in Materials and Methods); assays were for 15 min.

† Added at a protein concentration of 1.5 mg/ml.

Table 4. NADPH-dependent covalent binding of [^{3}H]-CMF to microsomes isolated from various rat tissues*

Source	[^{3}H]CMF (nmoles bound/min/mg protein)
Liver	1.55 ± 0.22
Lung	0.19 ± 0.04
Kidney	0.01 ± 0.06
Spleen	0.04 ± 0.10
Brain	0.02 ± 0.04

* See Materials and Methods for experimental details.

Microsomal metabolism and binding of CMF to protein. In preliminary experiments, it was found that after rat liver microsomes were incubated with CMF for 2 hr, about 15 per cent of the substrate was covalently bound to protein; subsequent analysis of the resulting supernatant by t.l.c. showed that at least two polar metabolites had been produced, accounting for 8 and 43 per cent of the initial amount of substrate. The production of these presently uncharacterized metabolites, as well as the covalent binding to protein, was dependent upon NADPH.

In order to ascertain whether the furan or the *N*-ethylcarbamate moiety was involved in covalent binding, microsomes were incubated with [^{14}C]CMF and an NADPH-generating system; unbound radioactivity was removed by repeated homogenization in 95% ethanol and centrifugation, followed by stirring in methanol under reflux. Although hydrolysis of CMF in aqueous base destroys the furan ring under conditions sufficient to hydrolyze the carbamate ester, it was found that 2-hydroxymethylfuran could be quantitatively recovered (as judged by g.l.c.) after heating CMF with 0.5 N KOH in dry methanol under reflux overnight. When the [^{14}C]CMF–protein complex was subjected to such hydrolytic conditions, no decrease in the amount of bound radioactivity (980 dis./min/mg of protein) was observed. Thus, it

Table 5. Covalent binding of CMF to microsomes and induction of activity by pretreatment of rats*

Pretreatment	P-450 (nmoles/mg protein)	CMF (nmoles bound/min/mg protein)
Control	1.08	0.85 ± 0.07
Phenobarbital	2.14	1.48 ± 0.12
3-Methylcholanthrene	1.55	0.92 ± 0.03
Pregnenolone-16α-carbonitrile	1.34	0.75 ± 0.10

* Rats (four per group) were pretreated, and cytochrome P-450, protein and binding were measured as described in Materials and Methods.

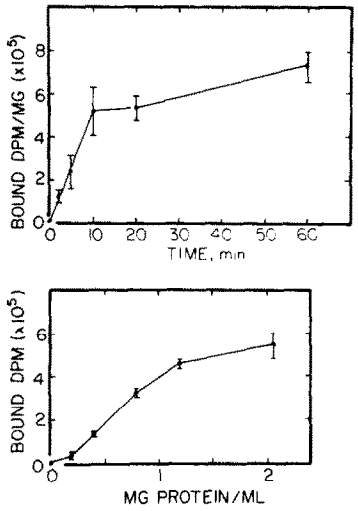

Fig. 1. Covalent binding of CMF to rat liver microsomes: kinetics of binding and dependence on protein concentration. In the upper panel, liver microsomes (1.5 mg/ml) from phenobarbital-induced rats were incubated with [^{3}H]CMF in the presence of an NADPH-generating system as described in Materials and Methods. In the lower panel, the concentration of microsomal protein in the assay was varied; all incubations were for 10 min.

would appear that CMF is bound to protein via the furan ring or the α-carbon, but not via the *N*-ethylcarbamate moiety.

After studies to establish the linearity of the protein binding assay with respect to time and concentration of microsomal protein (Fig. 1), all subsequent experiments were carried out with approximately 1 mg protein/ml for 10 min. The K_m for the microsomal binding of CMF was found to be 0.16 mM; no multiphasicity was apparent in the K_m plots.

The microsomal binding activity was found to be induced by treating rats with phenobarbital, but not with 3-methylcholanthrene or pregnenolone-16α-carbonitrile (Table 5). These findings are in agreement with results obtained using highly purified cytochrome P-450 systems: two P-450 fractions (A and B) from the microsomes of phenobarbital-treated rats had much higher turnover numbers for catalyzing the covalent binding of CMF to albumin than did any of the other fractions isolated from untreated or phenobarbital- or 3-methylcholanthrene-treated rats.* Among the different rabbit cytochromes P-450 tested, the major liver fraction induced by phenobarbital ($P\text{-}450_{LM\text{-}2}$) and the lung fraction were clearly most active. All of the cytochrome P-450 fractions catalyzed significant levels of CMF binding when NADPH and NADPH–cytochrome P-450 reductase were replaced with cumene hydroperoxide [26].

When rats were injected with a single dose of CMF (40 mg/kg) 48 hr prior to sacrifice, no significant differences were observed in the specific content (nmoles/mg of protein) of cytochrome P-450 in either the lung or the liver microsomes; moreover, no differences were detected when the metabolism of benzphetamine [18] and CMF were compared using the microsomes of these organs. Only an apparent "type II" difference spectrum was observed upon the binding of CMF to either microsomal suspensions or to purified rat liver cytochrome P-450, with a peak at 420 nm and a trough at 390 nm. Since the apparent K_s in both cases was 10 mM, it is felt that these spectral changes are unrelated to the metabolism of CMF.

The covalent binding of CMF to microsomes could be abolished completely by boiling and could be inhibited by diethylaminoethyl-2,2-diphenylvalerate (SKF-525A) (a presumably competitive inhibitor) or by cysteine or reduced glutathione (which presumably react with an activated metabolite before reaction with tissue nucleophiles) (Table 6).

Since the hypothesis has been advanced that an epoxide is the active metabolite of the furan derivative aflatoxin B_1 [1, 7, 8, 12], experiments were set up to examine the possible role of the enzyme epoxide hydrase [27] in the activation of CMF. If an epoxide is produced which can be hydrated by epoxide hydrase, then inhibition of epoxide hydrase (with a substrate or competitive inhibitor) should enhance the

Table 6. Inhibition of covalent binding of [^{3}H]CMF to rat liver microsomes*

System	[^{3}H]CMF (nmoles bound/min/mg protein)
Complete	1.48 ± 0.27
Complete, microsomes boiled	0.01 ± 0.01
Complete, + SKF-525A	0.56 ± 0.38
Complete, + cysteine	0.35 ± 0.10
Complete, + glutathione	0.02 ± 0.01

* Microsomes were incubated as described in Materials and Methods (complete system). Boiling of microsomes proceeded for 10 min. SKF-525A, cysteine and reduced glutathione were all added to the incubation mixtures at 1.0 mM.

* F. Guengerich, manuscript submitted for publication.

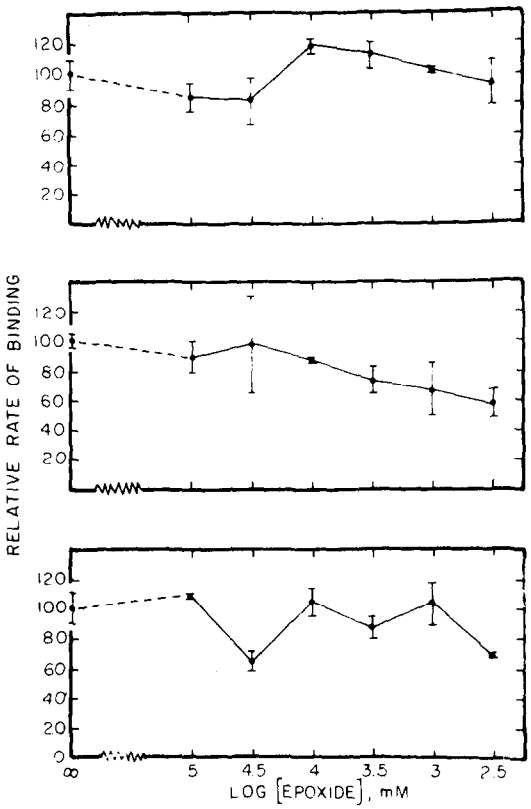

Fig. 2. Attempted enhancement of covalent binding of [^{3}H]CMF to rat liver microsomes with epoxide hydrase substrates. Incubations and assays were carried out as described in Materials and Methods. The incubations contained varying concentrations of cyclohexene oxide (upper panel), 1,2-epoxy-3,3,3-trichloropropane (middle panel), and styrene oxide (lower panel).

level of furan epoxide that can react with and bind to tissue nucleophiles. The results presented in Fig. 2 show that the addition of varying levels of cyclohexene oxide, 1,2-epoxy-3,3,3-trichloropropane, or styrene oxide did not significantly enhance microsomal binding to protein. Several explanations can be proposed for the finding: (a) an epoxide is formed which is not a substrate for any of the epoxide hydrases which bind the epoxides used in this work, (b) an epoxide is not formed, or (c) an epoxide is formed, but is so reactive that it cannot leave the activating enzyme to interact with an epoxide hydrase.

The latter possibility was considered, although the experiment of Table 3 demonstrates that the activating enzymes are concentrated in the microsomes while a previous experiment (Table 2) indicates that CMF is distributed throughout the cell, consistent with the hypothesis that the activated metabolite(s) is somewhat stable. Microsomes were incubated with [^{3}H]CMF, either in the presence of NADPH or cumene hydroperoxide, and highly purified rabbit liver cytochrome P-450$_{LM-2}$ was incubated with [^{3}H]CMF and excess albumin, either in the presence of cumene hydroperoxide or NADPH plus NADPH–cytochrome P-450 reductase; in all cases, excess substrate was removed by repeated dialysis, followed by gel filtration, and the samples were electrophoresed on polyacrylamide gels in the presence of sodium dodecyl sulfate (Fig. 3). With microsomes, CMF was bound to a variety of proteins, while the purified systems, most of the CMF was bound to albumin. [The large amount of radioactivity migrating with the tracking dye in panel B is unexplained; the finding is repeatable, but was not observed with the purified system (panel D).]

The half-life of the activated form of CMF was estimated by incubating liver microsomes (1 mg/ml) with [^{3}H]CMF and NADPH for 10 min; protein was rapidly removed (elapsed time = 1 min) by filtration of the mixture through packed Celite and ultrafiltration (Amicon XM-50A). Aliquots of the filtrate were added to albumin at various intervals and incubated for 60 min; covalent binding was determined. The half-life (of the material that could be bound) was estimated to be between 30 and 50 min.

Covalent binding in vitro *of CMF and DNA and nuclear proteins.* Because of the covalent binding of CMF to nuclear macromolecules observed *in vivo*, experiments were carried out to assess the role of the nuclei in activating CMF. The results presented in Table 7 indicate that rat liver microsomes are capable of catalyzing the covalent binding of large amounts of CMF (as high as one residue per several hundred DNA base residues under these conditions) to both nuclear and exogenous DNA; microsomes also catalyzed the covalent binding of similar levels of CMF to nuclear proteins. Nuclei catalyzed the binding of

Table 7. Covalent binding of CMF to calf thymus DNA, rat liver nuclear DNA, and rat liver microsomal and nuclear proteins catalyzed by rat liver microsomes and nuclei*

System	CMF (nmoles bound/ μmole DNA)†	CMF (nmoles bound/mg microsomal protein)	CMF (nmoles bound/mg nuclear protein)
Microsomes plus thymus DNA	9.77 ± 0.95	6.50 ± 1.30	
Nuclei	0.02 ± 0.10		1.32 ± 0.10
Microsomes plus nuclei	1.77 ± 0.40	6.60 ± 0.90	4.97 ± 0.53

* Incubations contained 6 mg of rat liver microsomes, purified rat liver nuclei [20], and/or calf thymus DNA in 3 ml plus an NADPH-generating system, Hepes–$MgCl_2$ buffer, and [^{3}H]CMF as described in Materials and Methods. After 40 min, DNA and proteins were isolated as described [25, 28]; blanks consisted of boiled proteins with all other components present.

† Results are expressed as nmoles [^{3}H]CMF covalently bound/μmole of DNA-phosphate, using $\epsilon_{260} = 6.6\ mM^{-1}\ cm^{-1}$.

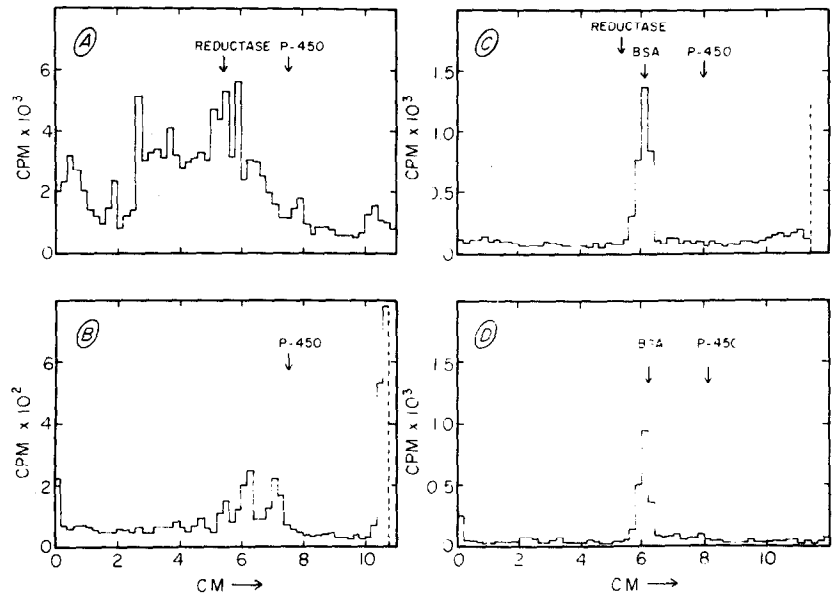

Fig. 3. Distribution of [^{3}H]CMF after binding and polyacrylamide gel electrophoresis. The migration front (bromphenol blue) is at either the dashed line or the right ordinate in each case. The locations of standard proteins run on separate gels in the same electrophoretic run are marked (rabbit liver cytochrome P-450$_{LM_12}$, mol. wt 50,000; NADPH–cytochrome P-450 reductase, mol. wt 79,000; bovine serum albumin (BSA), mol. wt 67,000). The incubates applied to the gels contained components as follows. A: microsomes, [^{3}H]CMF, and NADPH; B: microsomes, cumene hydroperoxide [25], and [^{3}H]CMF; C: rabbit liver cytochrome P-450, NADPH–cytochrome P-450 reductase, dilauroylglyceryl-3-phosphorylcholine (GPC), BSA, deoxycholate (DOC), [^{3}H]CMF, and NADPH [18]; and D: rabbit cytochrome P-450, GPC, BSA, DOC, and cumene hydroperoxide [26].

CMF to nuclear proteins, but apparently not to nuclear DNA—these results appear to be inconsistent with the binding patterns obtained in the presence of microsomes, suggesting that the nuclear CMF metabolite(s) may differ from the microsomal CMF metabolite(s). (Similar results were obtained using liver microsomes and nuclei from phenobarbital-treated rats.)

DISCUSSION

The results presented here demonstrate that a rather simple furan, CMF, possesses many of the toxic and mutagenic properties of a variety of naturally occurring and synthetic furan derivatives found in the environment. Toxicity was not observed with low doses of CMF, in line with reported observation of a "threshold" for liver toxicity with the drug furosemide [16]. However, CMF has been found to be mutagenic to *Salmonella typhimurium* TA 100 at low concentrations in preliminary experiments; at doses of CMF not apparently toxic to rats, significant binding to nucleic acids and nuclear proteins occurs (see Table 2) and this compound should be regarded as a potential carcinogen. These findings support the hypothesis [16] that all furan compounds are, because of the inherent nature of the ring system, potentially toxic and possibly carcinogenic.

Toxicity *in vivo*, covalent binding *in vivo* (see Table 1), and NADPH-dependent covalent binding of CMF *in vitro* (see Table 4) seem to be correlated, as judged by the observed tissue specificity. Liver necrosis, the level of liver microsomal covalent binding to protein *in vitro* (see Table 5), and the liver microsomal cytochrome P-450 species most active in activating CMF to a covalently bound form(s) are all induced by phenobarbital.* These findings argue that the toxicity of CMF (and, by extension, all furans) is a result of covalent binding of an activated form(s) produced enzymatically by cytochrome P-450-dependent systems.

Chemical evidence has been presented here that the carbamate moiety is not directly involved in covalent binding; moreover, treatment of either untreated or phenobarbital-treated rats with the tetrahydro derivative of CMF (100 mg/kg) produced no apparent effects in seven animals tested (observed up to 5 days after injection).

The observed half-life of the activated metabolite(s) (at least 30 min) was unexpected; however, the experiment was done three times with similar results, using microsomes from either untreated or phenobarbital-treated rats. Recently, Wood *et al.* [29] has estimated the half-life of an isomer of 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene to be 6–13 min; this compound was the most active of a number of benzo(a)-pyrene epoxides tested in transforming Chinese hamster V79 cells (these measurements may underestimate stability, as a finite time required for transport to the target for transformation is not included in the calculations). Thus, the stability of the activated CMF metabolite(s) in the absence of nucleophiles is not inconsistent with a role as a biologically important electrophile. Hopefully, the stability of this metabolite(s) can be utilized in carrying out studies (with bacteria and mammalian cells) with CMF as

* F. Guengerich, manuscript submitted for publication.

a model for chemical mutation and carcinogenesis.

Experiments are presently in progress to elucidate the chemical nature of the activated form(s) of CMF. The active species presumably arises from insertion of an oxygen atom or atoms enzymatically, and although epoxides have been suggested as intermediates in the covalent binding of furans [1, 7, 8, 12], these postulated compounds may be too unstable [28] to explain several observations—the active metabolite(s) is apparently stable enough to leave the main site of activation (the microsomal cytochrome P-450) to interact with a wide variety of nucleophiles in the cell, as judged by the experiments *in vitro* and *in vivo* described. Even if the active metabolite is an epoxide, the action of the form(s) of epoxide hydrase concerned with the hydration of styrene oxide and cyclohexene oxide (and inhibited by 1,2-epoxy-3,3,3-trichloropropane) seems to be negligible with regard to the metabolism of CMF (see Fig. 3); similar findings have been reported with the metabolism of aflatoxin B_1 [1].

The work of Vaught and Bresnick [30] and Jernström *et al.* [31] suggests that nuclei play a major role in the activation of benzo(a)pyrene to forms covalently bound to nuclear DNA, while the results of Alexandrov *et al.* [32] argue against such a hypothesis. A major role for nuclear activation in the inhibition of rat liver RNA polymerase by aflatoxin B_1 has also been suggested [33]. The work presented in this paper argues that nuclear enzymes play only a minor role in activating CMF to forms covalently bound to nuclear DNA; such findings are consistent with the view that the metabolite(s) of CMF produced by liver microsomal cytochromes P-450 is rather stable and can cross the membrane to bind to nuclear components. The high level of covalent binding of CMF to nuclear components is postulated to be related to the mutagenicity of CMF; the levels of both DNA binding and mutagenicity approach those of the dangerous carcinogen aflatoxin B_1 under assay conditions utilized in this work.

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