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EFFECT OF THE STRUCTURAL ISOMER *N*-3-FLUORENYLACETAMIDE ON MICROSOMAL BINDING AND HYDROXYLATION OF THE CARCINOGEN *N*-2-FLUORENYLACETAMIDE*,†

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Abstract—*N*-2-Fluorenylacetamide (2-FAA), a carcinogen, or *N*-3-fluorenylacetamide (3-FAA), the noncarcinogenic isomer, when added to hepatic microsomes from 3-methylcholanthrene (3-MC)-treated rats, in phosphate buffer–1,2-propanediol, exhibited equal affinity for cytochrome P_1-450 as indicated by equal binding constants (K_s). Both isomers were bound to the same site on cytochrome P_1-450 and displaced each other from the common binding site, as shown by competitive inhibition. Microsomal hydroxylation of 2-FAA to *N*-OH-2-FAA and to 7-OH-2-FAA was also inhibited by 3-FAA. The inhibitory effect was enhanced by omission of 1,2-propanediol from the incubation system. In contrast to the inhibition of the binding of 2-FAA by 3-FAA, the inhibition of hydroxylation of 2-FAA by 3-FAA was noncompetitive or uncompetitive. The contrasting patterns of inhibition of hydroxylation of 2-FAA by 3-FAA and of binding of 2-FAA by the isomer indicated that the binding site of 2-FAA is separate from the site of hydroxylation. This conclusion was supported by (1) a 10^3-fold difference in the values of K_s and K_m of 2-FAA, and (2) opposite effects of 1,2-propanediol on binding and hydroxylation of 2-FAA. Whereas binding of 2-FAA to cytochrome P_1-450 was increased with increasing concentrations of 1,2-propanediol, the microsomal formation of *N*-OH-2-FAA and of 7-OH-2-FAA was markedly diminished under the same conditions.

Previous work from this laboratory has shown that addition of *N*-2-fluorenylacetamide (2-FAA)§, a carcinogen, to hepatic microsomes of 3-MC-treated rats or guinea pigs results in a characteristic type I binding spectrum [1]. Similarly, *N*-3-fluorenylacetamide (3-FAA) [2], the noncarcinogenic isomer, gave a type I binding spectrum on addition to hepatic microsomes of the two species [1]. The appearances and magnitudes of the binding spectra of 2-FAA and 3-FAA suggested that both isomers bind equally to cytochrome P_1-450. Equal affinity was confirmed in this study by determination of the spectral dissociation constants (K_s). These findings raised the question of whether both arylamides bind to the same site on cytochrome P_1-450 and whether the isomer inhibits binding of the carcinogen. If the binding site of 2-FAA on cytochrome P_1-450 were the site of the hydroxylation of 2-FAA, a structural isomer, such as 3-FAA, might conceivably inhibit the microsomal hydroxylation of 2-FAA by displacement of 2-FAA from the binding site. Complete or partial inhibition of the formation of *N*-OH-2-FAA, the proximate carcinogen of 2-FAA [3], might diminish or abolish the carcinogenicity of 2-FAA. A parallel study of the microsomal binding and metabolism of 2-FAA would also provide evidence whether the site on cytochrome P_1-450 to which 2-FAA binds is also the site where hydroxylation of 2-FAA occurs. Because it has been shown that alcohols inhibit microsomal hydroxylation [4], we examined the effects of 1,2-propanediol, an alcohol used as a solvent in these investigations, on the microsomal binding and hydroxylation of 2-FAA. The results of these experiments and the conclusions derived therefrom form the basis of this report.

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§ Abbreviations: 2-FAA, *N*-2-fluorenylacetamide; 3-FAA, *N*-3-fluorenylacetamide; *N*-OH-2-FAA, *N*-2-fluorenylacetohydroxamic acid; 7-OH-2-FAA, *N*-(7-hydroxy)2-fluorenylacetamide; 5-OH-2-FAA, *N*-(5-hydroxy)2-fluorenylacetamide; 3-OH-2-FAA, *N*-(3-hydroxy)2-fluorenylacetamide; 9-OH-3-FAA, *N*-3-acetamidofluoren-9-ol; 9-oxo-3-FAA, *N*-3-acetamidofluoren-9-one; 3-MC, 3-methylcholanthrene; t.l.c., thin-layer chromatography; and h.p.l.c., high pressure liquid chromatography.

MATERIALS AND METHODS

Reagents. Silica gel GF_{254} for preparative and analytical t.l.c. was obtained from Brinkmann Instruments, Inc., Westbury, NY, U.S.A. Radiochromatograms were scanned with a thin-layer scanner (model LB 271, Berthold Laboratories, Wildbad, West Germany). 3-MC, m.p. 181–182°, was obtained from Distillation Products Industries, Division of Eastman Kodak Co., Rochester, NY, U.S.A.

NADPH was purchased from the Sigma Chemical Co., St. Louis, MO, U.S.A.

Preparation of labeled and unlabeled compounds. 2-FAA-[1'-^{14}C] was prepared by addition of 2-fluorenamine (75 mg) in benzene (5 ml) to a 20% solution of [^{14}C-carbonyl]acetic anhydride (40.8 mg; 5 mCi) (New England Nuclear Corp., Boston, MA, U.S.A.) in benzene (0.2 ml). The precipitate that formed on addition of triethylamine (0.02 ml) was collected and purified by preparative t.l.c. on silica gel GF_{254} with $CHCl_3$–methanol (9:1) as a solvent. 2-FAA-[1'-^{14}C] was eluted from the gel with methanol and recrystallized from ethanol/water; m.p. 196–198°; sp. act. 4.49 mCi/mmole. The compound gave a single radioactive peak on t.l.c. with $CHCl_3$–MeOH (9:1). 2-FAA, m.p. 194–195° (Aldrich Chemical Co., Milwaukee, WI, U.S.A.), gave a single fluorescence-quenching spot on t.l.c. with MeOH–$CHCl_3$ (95:5) as a solvent. *N*-OH-2-FAA, m.p. 150–151° [5], 7-OH-2-FAA, m.p. 230–232° [6], 5-OH-2-FAA, m.p. 217–218° [7], 3-OH-2-FAA, m.p. 247–249° [8], 3-FAA, m.p. 194–196° [9], 9-OH-3-FAA, m.p. 210–211° [10], and 9-oxo-3-FAA, m.p. 219–221° [10], were prepared by the published procedures and were found to be pure by t.l.c. on silica gel GF_{254}.

Preparation of hepatic microsomes. Male rats (180–200 g) (Holtzman Company, Madison, WI, U.S.A.) were maintained on Purina Chow and had free access to food and water. 3-MC (2.0 mg/100 g body weight) in corn oil was injected i.p. 48 and 24 hr prior to the preparation of the microsomes. The rats were decapitated, and the livers were perfused *in situ* with cold 0.9% NaCl, excised, and minced. A 20% homogenate (w/v) in a 0.25 M sucrose–0.05 M phosphate buffer (pH 7.4) was prepared from the minced tissue, and microsomes were obtained by differential centrifugation of the homogenate. The pellet was washed once with 0.05 M phosphate–0.025 M KCl buffer (pH 7.4) and the microsomes were resuspended in the buffer by gentle homogenization with a Dounce homogenizer. The concentration of protein in the microsomal suspension was determined colorimetrically with the use of crystalline bovine serum albumin as the standard [11]. Cytochrome P-450 was measured spectrophotometrically at 448–450 nm with the use of an extinction coefficient of 91 mM^{-1} [12]. Hepatic microsomes of male Holtzman rats injected twice with 3-MC, as described above, usually contained ~2 nmoles cytochrome P-450/mg protein.

Measurement of binding spectra. For determinations of the difference spectra of 2-FAA or 3-FAA, reference and sample cuvettes contained microsomes [8 nmoles cytochrome P-450 (Fig. 3) or 12 nmoles cytochrome P-450 (Figs. 4 and 5)], 0.05 M phosphate–0.025 M KCl buffer and 1,2-propanediol. Appropriate amounts of arylamide dissolved in 1,2-propanediol–buffer (1:1) were added to the sample cuvette to give the concentrations of arylamide shown in the figures. The final concentrations of 1,2-propanediol in both cuvettes were 4.5 M (Figs. 4 and 5), 1.1, 1.8, 3.2 and 4.8 M (Fig. 3). The total volume of the mixture in the cuvettes was 3.0 ml. When the effect of an isomer on the microsomal binding of 2-FAA or 3-FAA was studied, the isomer was added to reference and sample cuvettes to give the final concentrations indicated in Figs. 4 and 5. Difference spectra of 2-FAA or of 3-FAA were recorded at room temperature between 480 and 350 nm.

Conditions of incubation of arylamides with microsomes for metabolic experiments. The incubation mixtures used routinely for metabolic studies contained hepatic microsomes of 3-MC-treated male rats (12 mg protein) and NADPH (7.5 μmoles) in 1,2-propanediol and 0.05 M phosphate–0.025 M KCl buffer (pH 7.4). A standard amount of 2-FAA-[1'-^{14}C] (10^5 dpm/incubation mixture) and appropriate amounts of unlabeled 2-FAA were added to obtain the final substrate concentrations indicated in the figures and tables. Unless indicated otherwise, the final concentration of 1,2-propanediol was 4.5 M. The total volume of each incubation mixture was adjusted to 3.0 ml with buffer. When the effect of 3-FAA on microsomal *N*- and *C*-hydroxylation was investigated, the required amounts of 3-FAA were added to give the final concentrations indicated in the figures.

The mixtures were incubated in a rotary shaker at 37° for 10 min. The formation of *N*-OH-2-FAA and of 7-OH-2-FAA from 2-FAA was nearly linear during the first 10 min of incubation, and the amounts of metabolites produced from all concentrations of substrate during this time were adequate for quantitation. At the end of the incubation, *N*-OH-2-FAA (0.63 μmole) and 7-OH-2-FAA (0.17 μmole) were added as carriers. The reaction was terminated by adding 1 N HCl (0.25 ml). The mixtures were frozen immediately in dry ice/acetone and stored at −20°.

Separation and quantitative determination of microsomal metabolites of 2-*FAA*-[1'-^{14}C]. After the frozen incubation mixtures had been thawed, the metabolites and the remaining substrates were extracted from the mixtures with diethyl ether and

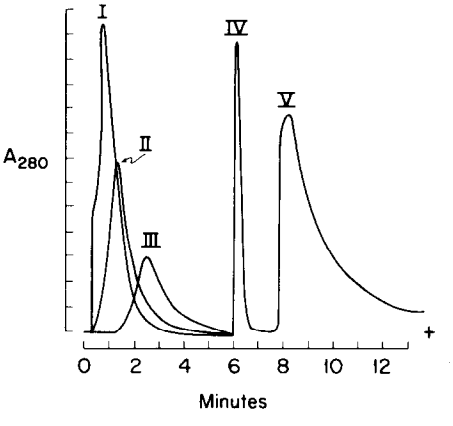

Fig. 1. Separation of *N*-OH-2-FAA (V) from 2-FAA (IV), 3-OH-2-FAA (III), 5-OH-2-FAA (II), and 7-OH-2-FAA (I) by h.p.l.c. on a Permaphase ODS column. The compounds (0.5 to 1.5 μg) were applied to the column in methanol (0.05 ml) and eluted at a pressure of 1000 psi and at a temperature of 60°. The eluent for the amidofluorenols was H_2O. 2-FAA and *N*-OH-2-FAA were eluted successively with a linear gradient in which the low concentration eluent was 40% MeOH and the high concentration eluent was 100% MeOH.

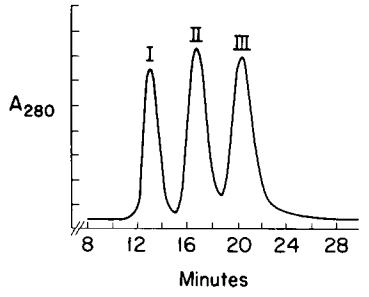

Fig. 2. Separation of a mixture of 3-OH-2-FAA (I), 5-OH-2-FAA (II), and 7-OH-2-FAA (III) by h.p.l.c. on a Corasil II column. The compounds (0.5 to 1.0 μg) in ethyl acetate (0.05 ml) were applied to the column and eluted with ethyl acetate–*n*–hexane (1:1) at a pressure of 100 psi and at room temperature.

partitioned by solvent extractions into neutral and acidic/phenolic fractions [13]. The acidic/phenolic fractions were evaporated under nitrogen at 50°. The residues were dissolved in 0.10 ml of methanol, and aliquots of this solution (up to 0.05 ml) were injected into a Permaphase ODS column (0.5 m × 2.1 mm) attached to a DuPont high pressure liquid chromatograph [gradient mixer (no. 838), pump module (no. 848) and spectrophotometer (no. 887)]. The column was kept at 60° and at a pressure of 1000 psi. *C*-Hydroxy metabolites were eluted with H_2O for 6 min (Fig. 1). A linear gradient in which the low concentration eluent was 40% MeOH and the high concentration eluent was 100% MeOH was then used to separate *N*-OH-2-FAA from any remaining substrate (Fig. 1). The u.v. spectra of the fractions containing *N*-OH-2-FAA were in all cases identical with those of authentic *N*-OH-2-FAA. The amounts of compound in the various fractions were determined from the extinction at 302 nm. The quantities of *N*-OH-2-FAA formed during the incubation were calculated by the method of inverse isotopic dilution

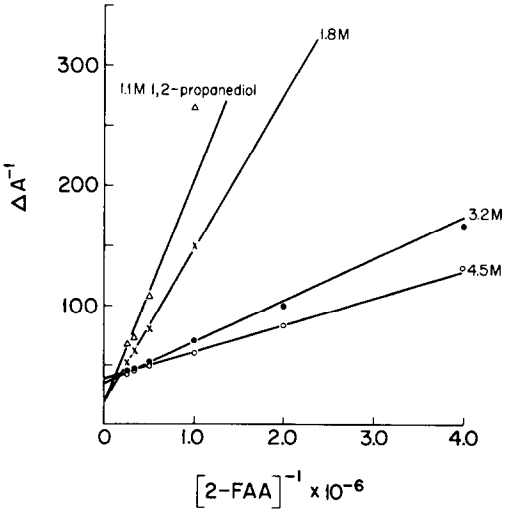

Fig. 3. Double reciprocal plots showing the effect of concentrations of 1,2-propanediol on the binding of 2-FAA to cytochrome P_1-450 of hepatic microsomes. The composition of the mixtures in reference and sample cuvettes were those described under Materials and Methods.

from the radioactivity of the fraction and the amount of carrier recovered. The water in the fraction containing the *C*-hydroxy metabolites was removed. The residue was dissolved in ethyl acetate and the metabolites were separated by h.p.l.c. on a Corasil II column as shown in Fig. 2 [14]. The fraction containing 7-OH-2-FAA was collected, and the radioactivity of this fraction was determined. The quantity of eluted carrier was calculated from a standard curve. The amount of 7-OH-2-FAA formed was calculated by the method of inverse isotopic dilution.

Radioactivity measurements. All fractions containing eluted *N*-OH-2-FAA or 7-OH-2-FAA were taken to dryness. The residues were dissolved in scintillation liquid. The radioactivity was measured in a Packard liquid scintillation spectrometer (model 3375). Counts were corrected for quenching by means of external standards. The counting efficiency was approximately 80 per cent. All samples were counted with an error not exceeding 5 per cent.

Calculations. The values of ΔA_{max}, K_s and $K_s/\Delta A_{max}$ of the binding experiments and of V_{max}, K_m and K_m/V_{max} of the metabolic experiments were calculated on a Hewlett–Packard 9830A calculator with a progam using the weighted least-squares method. The progam was supplied by Wang Laboratories, Inc. (1970). The double reciprocal plots were obtained with a Hewlett–Packard 9862A calculator plotter.

RESULTS AND DISCUSSION

Effect of solvent on the binding of 2-FAA to cytochrome P_1-450 of hepatic microsomes of the rat. 2-FAA and 3-FAA are insoluble in the aqueous media commonly used in studies of the binding of exogenous compounds to cytochrome P-450. The arylamides, however, were found to be soluble in the mixture of phosphate buffer and 1,2-propanediol used in this study and in a previous investigation [1]. It was also found that the binding of 2-FAA to cytochrome P_1-450, represented by the type I binding spectrum ($\Delta A_{385\text{-}417\,nm}$) [1], increased with increasing concentrations of 1,2-propanediol (Fig. 3). Consistent difference spectra were obtained when the concentration of 1,2-propanediol in the mixtures was 4.5 M. Although the absolute values of $\Delta A_{385\text{–}417\,nm}$ varied with each microsomal preparation, the values of the spectral dissociation constant (K_s) of 2-FAA at this concentration of diol were found to fall within a relatively narrow range (3.0×10^{-7} to 6.0×10^{-7} M).

The effect of 1,2-propanediol on the binding of 2-FAA to cytochrome P_1-450 deserves comment. If 1,2-propanediol would function only as a solvent, the magnitude of the difference spectra of 2-FAA as well as the K_s values of 2-FAA should be independent of the concentration of diol. Since the K_s values of 2-FAA decreased with increasing concentrations of 1,2-propanediol, the data (Fig. 3) suggested that 1,2-propanediol facilitated binding of 2-FAA to the hemoprotein by unmasking previously inaccessible binding sites with different spectral characteristics. A possible explanation for the above effect was the interaction of 1,2-propanediol with cytochrome P_1-450. In fact, 1,2-propanediol was

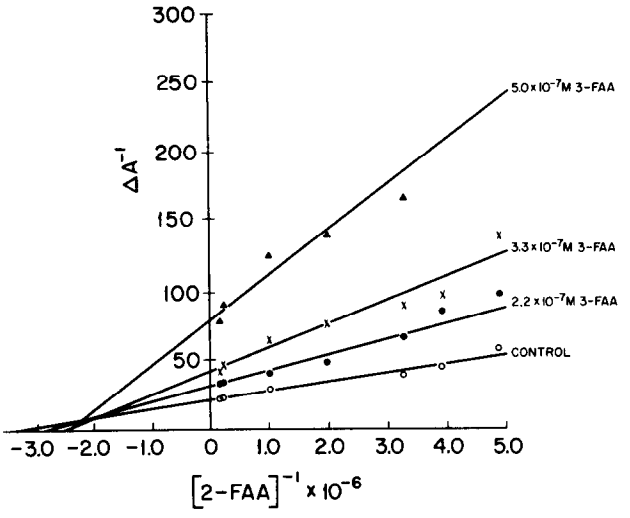

Fig. 4. Double reciprocal plots showing the effects of 3-FAA on the binding of 2-FAA to cytochrome P_1-450. The compositions of the mixtures in reference and sample cuvettes were those described under Materials and Methods.

found to bind to cytochrome P_1-450, as shown by a type II spectrum with a minimum at 380 nm and a maximum at 416 nm ($K_s \sim 0.8$ M). We feel, however, that the binding of 1,2-propanediol to cytochrome P_1-450 did not account for the increased binding of 2-FAA for the following reasons. First, it has been demonstrated that type II compounds inhibit the binding of other type I or type II compounds to cytochrome P-450 [15]. Second, the K_s of 1,2-propanediol was 0.8 M. This value is the concentration of diol at which one-half of the type II binding sites are occupied. If the binding spectrum of 2-FAA was related to the saturation of the type II binding sites by the diol, one would expect that a consistent type I binding spectrum of 2-FAA would be obtained at much lower concentrations of 1,2-propanediol than 4.5 M. It seems reasonable to assume that the effects of 4.5 M 1,2-propanediol on the binding of 2-FAA were due to nonspecific membrane perturbation or protein denaturation. We have also observed that increasing concentrations of dimethyl sulfoxide, another solvent miscible with water, increased binding of 2-FAA to cytochrome P_1-450 in the same way as 1,2-propanediol. These observations on two structurally unrelated organic solvents suggest that the increased binding of 2-FAA was due to a disturbance of the membrane or protein structure of the microsomes.

Mutual inhibition of the binding of 2-FAA and of 3-FAA to cytochrome P_1-450 of hepatic microsomes. We have shown previously that 2-FAA and 3-FAA added to hepatic microsomes of 3-MC-treated rats gave characteristic type I binding spectra [1]. The spectral dissociation constants (K_s) of the arylam-

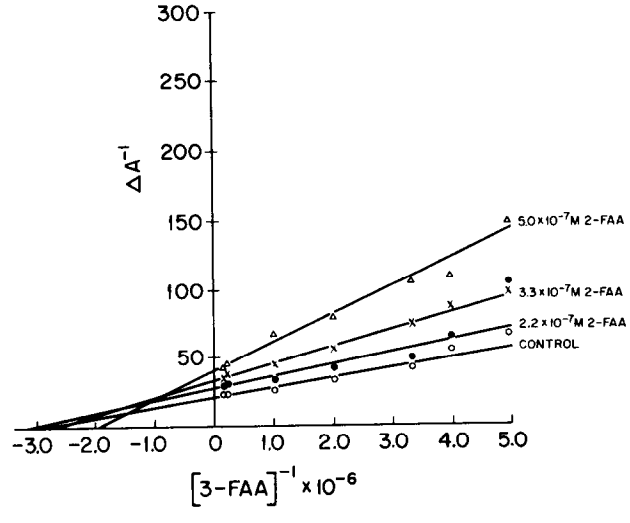

Fig. 5. Double reciprocal plots, showing the effects of 2-FAA on the binding of 3-FAA to cytochrome P_1-450. The composition of the mixtures in reference and sample cuvettes were those described under Materials and Methods.

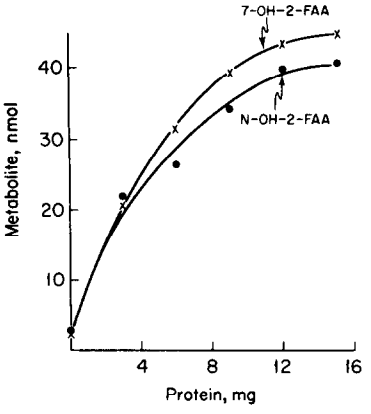

Fig. 6. Formation of 7-OH-2-FAA and *N*-OH-2-FAA from 2-FAA by hepatic microsomes as a function of protein concentration. The incubation system (3.0 ml) consisted of microsomes (12 mg protein), NADPH (7.5 μmoles), and 2-FAA-[1'-^{14}C] (3.3×10^{-4} M, 10^5 dpm) in 1,2-propanediol (4.5 M) and phosphate–KCl buffer. The incubations and the determinations of the metabolites were carried out as described under Materials and Methods.

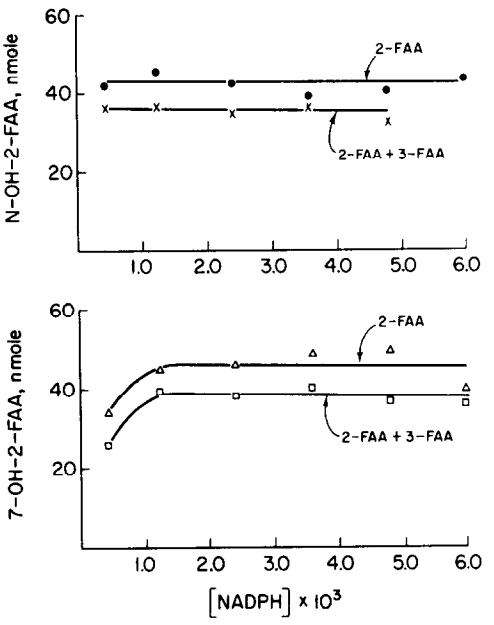

Fig. 7. Formation of *N*-OH-2-FAA and of 7-OH-2-FAA from 2-FAA-[1'-^{14}C] (3.3×10^{-4} M, 10^5 dpm) by hepatic microsomes (12 mg protein) as a function of the concentration of NADPH in the presence and absence of 3-FAA (3.3×10^{14} M). The incubations and the determinations of the metabolites were carried out as described under Materials and Methods.

ides, determined in this study from double reciprocal plots of ΔA versus substrate concentration (Figs. 4 and 5), were 3.0×10^{-7} M and 3.6×10^{-7} M for 2-FAA and 3-FAA respectively. This indicated that both arylamides have equal affinity for cytochrome P_1-450, and it suggested that the two isomers might compete for the same binding site on cytochrome P_1-450. Addition of increasing amounts of 3-FAA did, in fact, decrease the binding of 2-FAA to cytochrome P_1-450 (Fig. 4). Similarly, binding of 3-FAA to cytochrome P_1-450 was decreased by addition of 2-FAA (Fig. 5). In either case, the inhibition did not fit the conventional competitive pattern according to which ΔA_{max} is independent of inhibitor concentration. There was a gradual decrease of ΔA_{max} and a continuous increase of K_s as the concentration of inhibitors increased. It has been shown by van den Berg *et al.* [16] that this change of ΔA_{max} and of K_s is indicative of the competitive inhibition of two compounds with the same type of binding spectra. Therefore, the data support the view that 2-FAA and 3-FAA are bound to the same site on cytochrome P_1-450.

Dependence of N- *and* C-*hydroxylation of 2-FAA on protein concentration and on NADPH.* The *N*- and *C*-hydroxylation of 2-FAA by hepatic microsomes of 3-MC-treated rats was a function of protein concentration (Fig. 6). On the basis of these data, 12 mg of microsomal protein was used in all subsequent incubations. This amount of protein was equiv-

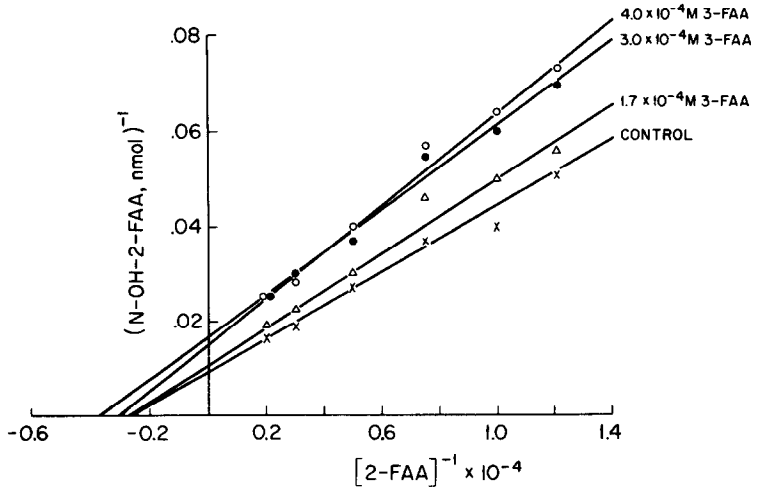

Fig. 8. Double reciprocal plots showing the inhibition of the *N*-hydroxylation of 2-FAA by 3-FAA. The incubation system consisted of hepatic microsomes (12 mg protein), NADPH (7.5 μmoles), 2-FAA-[1'-^{14}C] and 1,2-propanediol (4.5 M) in phosphate–KCl buffer.

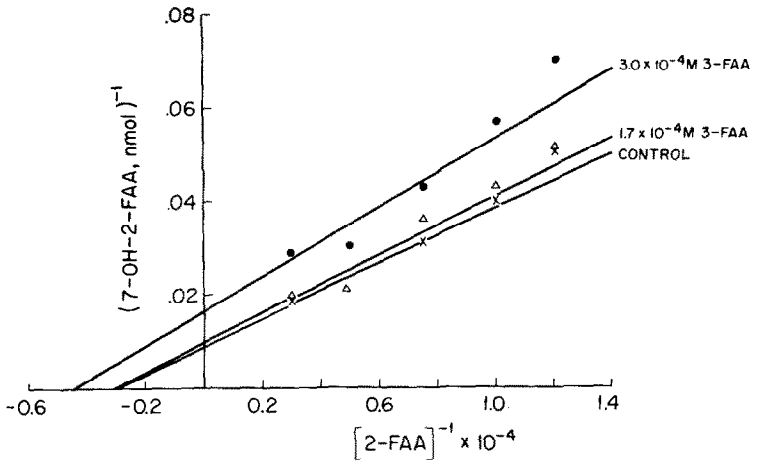

Fig. 9. Double reciprocal plots showing the inhibition of the formation of 7-OH-2-FAA from 2-FAA by 3-FAA. The incubation mixtures were the same as those used for the experiments depicted in Fig. 8.

alent to ~ 24 nmoles cytochrome P-450 and close to the quantities that gave maximum hydroxylation.

The hydroxylation of 2-FAA requires NADPH or an NADPH-generating system [1]. The dependence of *N*- and *C*-hydroxylation on NADPH concentration was, therefore, investigated with the amount of protein used routinely in this study (12 mg) (Fig. 7). The data show that NADPH at a concentration of 1.3×10^{-3} M was sufficient for maximum hydroxylation. In the presence of NADPH, 3-FAA is oxidized to 9-OH-3-FAA and 9-oxo-3-FAA [17], and these reactions proceed simultaneously with the *N*- and *C*-hydroxylation of 2-FAA. The yield of *N*-OH-2-FAA and of 7-OH-2-FAA, however, remained constant in the presence of 3-FAA, provided that the concentration of NADPH in the incubation system was equal to or greater than 1.3×10^{-3} M (Fig. 7). To ensure that any effect of 3-FAA on the hydroxylation of 2-FAA was not due to a depletion of NADPH by the simultaneous oxidation of 3-FAA, a concentration of 2.5×10^{-3} M NADPH was used in all metabolic experiments.

Inhibition of the microsomal hydroxylation of 2-FAA by 3-FAA in the presence of 1-2-propanediol. These experiments were designed to test whether 3-FAA inhibits the hydroxylation of 2-FAA and whether the hydroxylation of 2-FAA proceeds at the same site as the binding of 2-FAA that gives rise to a type I spectrum. The concentration of buffer and of 1,2-propanediol in these metabolic experiments were, therefore, the same as those used in the binding studies. Under these conditions, 3-FAA inhibited the formation of *N*-OH-2-FAA (Fig. 8) was well as that of 7-OH-2-FAA (Fig. 9). The type of inhibition was dependent on the concentration of 3-FAA. As shown by double reciprocal plots, the inhibition of the hydroxylation of 2-FAA at low concentrations of 3-FAA was noncompetitive. At higher concentrations of 3-FAA, the inhibition changed to an uncompetitive pattern and reached a maximum at approximately 3.0×10^{-4} M 3-FAA. Dual modes of inhibition were also observed in the *N*-demethylation of ethylmorphine by mouse liver microsomes when nicotinamide, 2,4-dichloro-6-phenylphenoxyethy-

Table 1. Effects of 9-OH-3-FAA and 9-oxo-3-FAA on the formation of *N*-OH-2-FAA and 7-OH-2-FAA from 2-FAA*

Compounds tested for inhibition	Concn. (10^{-4} M)	*N*-OH-2-FAA formed (μmoles)	7-OH-2-FAA formed (μmoles)
		34.8 ± 3.6	42.6 ± 2.8
9-OH-3-FAA	0.17	37.9 ± 4.1	44.0 ± 3.2
	0.33	36.7 ± 4.5	41.9 ± 0.6
	0.67	36.2 ± 6.3	42.0 ± 5.2
9-oxo-3-FAA	0.17	35.5 ± 3.2	40.0 ± 1.9
	0.33	34.6 ± 4.1	40.2 ± 2.4
	0.67	33.3 ± 2.7	39.6 ± 4.9

* The incubation systems consisted of microsomes (12 mg protein), NADPH (7.5 μmoles), and 2-FAA-[1'-^{14}C] (2.0×10^{-4} M, 10^5 dpm) in phosphate buffer containing 1,2-propanediol (4.5 M) as described under Materials and Methods. 9-OH-3-FAA and 9-oxo-3-FAA were present in the concentrations indicated. The conditions of incubation and the determinations of *N*-OH-2-FAA and 7-OH-2-FAA are described in the text. The values shown are the means ± S.D. of two separate experiments.

Table 2. Effect of 1,2-propanediol on the formation of *N*-OH-2-FAA and 7-OH-2-FAA from 2-FAA*

2-FAA conc (10^{-4} M)	*N*-OH-2-FAA formed (nmoles)				7-OH-2-FAA formed (nmoles)			
	In absence of diol	In presence of 1.8 M diol	In presence of 4.5 M diol	Inhibition†	In absence of diol	In presence of 1.8 M diol	In presence of 4.5 M diol	Inhibition† (%)
0.83	25 ± 1	22 ± 3	17 ± 2	32	64 ± 9	31 ± 3	19 ± 5	70
1.00	29 ± 6	26 ± 1	22 ± 4	24	75 ± 5	39 ± 4	24 ± 4	78
1.33	37 ± 4	27 ± 2	26 ± 3	30	98 ± 12	47 ± 7	28 ± 4	71
2.00	48 ± 5	32 ± 3	31 ± 3	35	205 ± 48	63 ± 10	39 ± 6	81
3.33	66 ± 8	41 ± 3	39 ± 5	41		79 ± 6	55 ± 8	

* The incubation mixture consisted of microsomes (12 mg protein), NADPH (7.5 μmoles) and 2-FAA-[1'-^{14}C] in phosphate buffer in the absence or presence of 1,2-propanediol as indicated. The conditions of incubation and the method of the determinations of *N*-OH-2-FAA were those described in the text. The values shown are the means ± S.D. of two or three experiments.

† These values are calculated from the formation of the metabolite in the absence of diol and in the presence of 4.5 M diol.

lamine, or aminoethyldiphenylpropylacetate were the inhibitors [18].

Since the inhibition of the hydroxylation of 2-FAA by 3-FAA was not competitive, it appeared that 3-FAA did not displace 2-FAA from the active site on the enzyme. Moreover, the difference in the pattern of inhibition exerted by 3-FAA on the binding and on the hydroxylation of 2-FAA indicated that the site at which 2-FAA is bound to cytochrome P_1-450 to give a type I binding spectrum was not the site where *N*-OH-2-FAA and 7-OH-2-FAA were formed. The same conclusion was reached by a comparison of the K_m and K_s values of 2-FAA. The K_m values for the formation of *N*-OH-2-FAA and 7-OH-2-FAA were 3.6×10^{-4} M and 3.2×10^{-4} M, respectively, while the K_s value of 2-FAA was 3.0×10^{-7} M. The 10^3-fold difference in the magnitude of the values of K_m and K_s indicate that the difference spectra from which the K_s value was derived are not representative of the enzyme–2-FAA complex that yields *N*-OH-2-FAA and 7-OH-2-FAA. Striking differences between K_m and K_s values for the hydroxylation of type I as well as of type II compounds have been reported [19].

Microsomal hydroxylation of 2-FAA in the presence of 9-OH-3-FAA and 9-oxo-3-FAA. With the experimental conditions of this study, 3-FAA is metabolized primarily to 9-OH-2-FAA, which undergoes further oxidation to 9-oxo-3-FAA [17]. It was conceivable, therefore, that the inhibitory action of 3-FAA was attributable to 9-OH-3-FAA or 9-oxo-3-FAA. Accordingly, we compared the formation of *N*-OH-2-FAA and of 7-OH-2-FAA from 2-FAA in the presence of 9-OH-3-FAA and 9-oxo-3-FAA. Since neither 9-OH-3-FAA nor 9-oxo-3-FAA inhibited the hydroxylation of 2-FAA (Table 1), the results indicate that the inhibition of the microsomal hydroxylation of 2-FAA by 3-FAA was due to the isomeric arylamide rather than to its metabolites, 9-hydroxy-3-FAA and 9-oxo-3-FAA.

Effect of 1,2-propanediol on the hydroxylation of 2-FAA. There is conclusive evidence that the oxidative metabolism of compounds exhibiting a type I binding spectrum is inhibited by compounds displaying type II binding spectra [18, 20]. Since 1,2-propanediol was found to give a type II binding spectrum, the above observations prompted us to examine the effect of 1,2-propanediol on the microsomal hydroxylation of 2-FAA. As shown in Table 2, 1,2-propanediol inhibited the formation of *N*-OH-2-FAA as well as that of 7-OH-2-FAA. The inhibition of *N*-hydroxylation appeared to reach a maximum at a concentration of 1.8 M diol. The inhibition of the formation of 7-OH-2-FAA increased further when the concentration of diol was raised from 1.8 M to 4.5 M. This effect of the diol on the hydroxylation of 2-FAA was opposite to the effect of the diol on the binding of 2-FAA. As mentioned above, binding of 2-FAA, determined spectrally, increased with increasing concentrations of diol. These findings strengthen the view that the binding site of 2-FAA on cytochrome P_1-450 is separate from the metabolic site where hydroxylation of 2-FAA to *N*-OH-2-FAA and 7-OH-2-FAA occurs.

Table 3. Inhibitory action of 3-FAA on the *N*-hydroxylation of 2-FAA in the presence and absence of 1,2-propanediol*

2-FAA concn (10^{-4} M)	*N*-OH-2-FAA formed (nmoles) in absence of diol		Inhibition (%)	*N*-OH-2-FAA formed (nmoles) in presence of diol		Inhibition (%)
	−I	+I		−I	+I	
0.83	25	18	28	20	18	10
1.00	36	22	39	25	20	20
1.33	41	39	29	27	22	19
3.33	65	45	31	52	47	10
5.00	90	57	37	60	52	13

* The incubation systems consisted of microsomes (12 mg protein), NADPH (7.5 μmoles), and 2-FAA-[1′-^{14}C] in phosphate buffer in the absence or presence of 1,2-propanediol (4.5 M) as described under Materials and Methods. 3-FAA (I) was added to give a concentration of 1.7×10^{-4} M. The conditions of incubation and the method of determination of *N*-OH-2-FAA were those described in the text. The data are the averages of duplicate determinations.

The data of Table 2 indicate that the formation of 7-OH-2-FAA was appreciably greater than that of *N*-OH-2-FAA over the entire range of substrate concentrations when 1,2-propanediol was absent from the incubation system. This result is in agreement with published observations [21, 22] on the relative amounts of these metabolites formed in the microsomal oxidation of 2-FAA. In contrast, at a concentration of 4.5 M 1,2-propanediol, the yield of 7-OH-2-FAA differed only slightly from that of *N*-OH-2-FAA. The observation that 1,2-propanediol had a greater inhibitory effect on the formation of 7-OH-2-FAA than on the *N*-hydroxylation of 2-FAA and that different concentrations of diol were required for maximum inhibition of the two hydroxylation reactions suggested that the site of hydroxylation of 2-FAA to 7-OH-2-FAA is distinct from the site of *N*-hydroxylation. These two sites may be located on the same form of cytochrome P-450. It is also possible, however, that *N*- and *C*-hydroxylation of 2-FAA are carried out by different forms of cytochrome P-450 that are affected differently by 1,2-propanediol.

For the reasons stated above, the molar concentrations of 1,2-propanediol used in the binding studies of 2-FAA were retained in the metabolic experiments. Because of the high concentrations of diol we believe that the observed effect of diol on the hydroxylation of 2-FAA is largely attributable to nonspecific membrane perturbation and/or protein denaturation rather than to a conformational change of cytochrome P_1-450 due to occupancy of the type II binding sites by 1,2-propanediol. It was beyond the scope of this study to determine whether the nonspecific alterations of the microsomal structure affected cytochrome P-450 alone or also other components of the electron transfer system associated with cytochrome P-450.

Effect of 1,2-propanediol on the inhibitory action of 3-FAA. We suggested above that 1,2-propanediol inhibits the microsomal hydroxylation of 2-FAA by alteration of the membrane lipids and/or the protein structure of the microsomes. In that event, the inhibition of the microsomal hydroxylation of 2-FAA, determined in presence of 4.5 M 1,2-propanediol, might not represent the inhibitory action of 3-FAA on the hydroxylation of 2-FAA by unaltered microsomes. Accordingly, we tested the inhibition of the hydroxylation of 2-FAA by 3-FAA in the absence of diol. It will be seen from the data of Tables 3 and 4 that 3-FAA became a more potent inhibitor of the formation of *N*-OH-2-FAA and

Table 4. Inhibitory action of 3-FAA on the formation of 7-OH-2-FAA in the presence and absence of 1,2-propanediol*

2-FAA concn (10^{-4} M)	7-OH-2-FAA formed (nmoles) in absence of diol		Inhibition (%)	7-OH-2-FAA formed (nmoles) in presence of diol		Inhibition (%)
	−I	+I		−I	+I	
0.83	65	54	17	20	19	5
1.00	74	58	22	25	23	8
1.33	109	83	24	32	27	16
2.00	166	109	34	47	46	2

* The incubation system, conditions of incubation, and the determination of metabolites were those described in Table 3. The data are the averages of duplicate determinations.

7-OH-2-FAA when the microsomes were not exposed to diol. The reduced effectiveness of 3-FAA as an inhibitor of the hydroxylation of 2-FAA at a high concentration of diol may be rationalized by assuming that the diol modifies the metabolic site where hydroxylation of 2-FAA occurs. The inhibition resulting from this modification appears to be of such a magnitude that addition of a second inhibitor, 3-FAA, has only a minor effect.

In conclusion, the principal results of this study may be summarized as follows. First, the binding of 2-FAA to microsomal cytochrome P_1-450, resulting in a type I binding spectrum, and the cytochrome P_1-450-catalyzed hydroxylation of 2-FAA take place on separate sites of the enzyme. This conclusion is based on (1) differences between the patterns of inhibition of microsomal binding and of inhibition of hydroxylation of 2-FAA by the structural isomer 3-FAA, (2) the 10^3-fold difference between the value of the spectral dissociation constant, K_s, and that of the Michaelis–Menten constant, K_m, of 2-FAA, and (3) the opposing effects of 1,2-propanediol on microsomal binding and on hydroxylation of 2-FAA. Second, the action of 1,2-propanediol, an alcohol yielding a type II binding spectrum, on the microsomal hydroxylation of 2-FAA directs attention to the possibility that type II compounds, in high concentrations, may mask the inhibitory effects of type I compounds on microsomal hydroxylation. Third, the observation that a noncarcinogenic isomer, 3-FAA, inhibits the first step (hydroxylation) of the activation sequence of the carcinogen, 2-FAA, encourages the view that the action of 2-FAA and other carcinogens requiring microsomal activation may be inhibited or blocked by noncarcinogenic structural analogues. Further experiments *in vitro* and *in vivo* along these lines are in progress.

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