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Effects of Safrole and Isosafrole Pretreatment on *N*- and Ring-Hydroxylation of 2-Acetamidofluorene by the Rat and Hamster

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1. The effects of safrole and isosafrole pretreatment on both *N*- and ring-hydroxylation of 2-acetamidofluorene were studied in male rats and hamsters. 2. Isosafrole (100mg/day per kg body wt.) pretreatment of rats for 3 days did not have any effect on urinary excretion of hydroxy metabolites of 2-acetamidofluorene. However, similar pretreatment with safrole produced increased urinary excretion of *N*-, 3- and 5-hydroxy derivatives. 3. Similar treatment with these two chemicals for 3 days increased ring-hydroxylation activity by rat liver microsomal material. Increases in *N*-hydroxylation were much less than those in ring-hydroxylation. Isosafrole was twice as effective as safrole. 4. Increases in hydroxylating activity due to safrole or isosafrole treatment were inhibited by simultaneous administration of ethionine. Similarly, ethionine inhibition was almost completely reversed by the simultaneous administration of methionine. 5. Safrole or isosafrole (0.1mM and 1mM) inhibited 7-hydroxylation activity by liver microsomal material from control rats. At 1mM these two chemicals inhibited both 5- and 7-hydroxylation activity by liver microsomal material from 3-methylcholanthrene-pretreated rats. 3-Hydroxylation activity was not inhibited by 1mM concentrations of these two chemicals. 6. A single injection of safrole (50100 or 200mg/kg body wt.) 24h before assay had no appreciable effect on either *N*- or ring-hydroxylation activity by hamster liver microsomal material. However, isosafrole (200mg/kg body wt.) treatment inhibited *N*-, 3- and 5-hydroxylation activities by hamster liver microsomal material; it had no effect on 7-hydroxylation activity.

Safrole (4-allyl-1,2-methylenedioxybenzene), a component of sassafras and many other essential oils, was used as a flavouring agent in soft drinks and some pharmaceutical preparations. Several studies have demonstrated that safrole produces liver tumours in rats and mice (Epstein *et al.*, 1970; Homburger *et al.*, 1961; Long *et al.*, 1963). Safrole and its isomer, isosafrole (4-propenyl-1,2-methylenedioxybenzene), were shown to induce several hepatic microsomal enzymes, including biphenyl hydroxylase (Parke & Rahman, 1970, 1971).

N-Hydroxylation of 2-acetamidofluorene and several other aromatic amines and amides is an activation step in the carcinogenesis by these compounds. However, ring-hydroxylation of 2-acetamidofluorene is an inactivation step (Miller, 1970; Miller & Miller, 1969). Both of these reactions occur in the liver endoplasmic reticulum and require NADPH and molecular oxygen for activity (Booth & Boyland, 1957; Cramer *et al.*, 1960; Irving, 1964; Lotlikar *et al.*, 1967; Seal & Gutmann, 1959). Induction of liver tumours in male rats by 2-acetamidofluorene is decreased to a large extent by the simultaneous administration of a small amount of 3-methylcholanthrene (Miyaji *et al.*, 1953; Miller *et al.*, 1958). From studies *in vivo* and *in vitro*, it appears that this inhibitory effect of 3-methylcholanthrene is primarily caused by induction of the inactivation process (Miller *et al.*, 1960; Lotlikar *et al.*, 1967). In contrast, 3-methylcholanthrene does not inhibit liver tumour induction by 2-acetamidofluorene in hamsters (Enomoto *et al.*, 1968). This is because pretreatment of this species with 3-methylcholanthrene does not induce the inactivation process but instead it produces a severalfold increase in *N*-hydroxylation of 2-acetamidofluorene by liver microsomal material (Lotlikar *et al.*, 1967).

In the present paper we describe the effects of safrole and isosafrole pretreatment on the *N*- and ring-hydroxylation of 2-acetamidofluorene by rat and hamster liver microsomal fractions.

Experimental

Animals

Male albino Wistar rats were obtained from Carworth Farms Co., New City, N.Y., U.S.A. Male Syrian golden hamsters were purchased from Huntingdon Farms Inc., West Conshohocken, Pa.,

U.S.A. All animals were maintained on commerical diets before use in the experiments.

Chemicals

N-Hydroxy-2-acetamidofluorene was prepared as described by Poirier *et al.* (1963). 2-Acetamidofluorene was purchased from Mann Research Laboratories, New York, N.Y., U.S.A. The following compounds were kindly supplied by Dr. James A. Miller of the McArdle Laboratory, University of Wisconsin, Madison, Wisc., U.S.A.: 1-, 3-, 5- and 7-hydroxy-2-acetamidofluorene, 3-methylthio-2-acetamidofluorene and 2-amino-3-methylthiofluorene. Safrole and isosafrole were purchased from K & K Laboratories, Plainview, New York, U.S.A. NADH, NADPH, ATP, DL-ethionine, DL-methionine and β-glucuronidase were products of Sigma Chemical Co., St. Louis, Mo., U.S.A. Takadiastase was purchased from Parke, Davis and Co., Detroit, Mich., U.S.A. 3-Methylcholanthrene was a product of Eastman Organic Chemical Co., Rochester, New York, U.S.A.

Methods

Administration of safrole or isosafrole for experiments in vivo. For studies on metabolites of 2-acetamidofluorene in the urine, rats weighing about 60 or 120g were injected intraperitoneally with either safrole or isosafrole (100mg/kg body wt.) in 0.2ml of corn oil. Control animals were injected with corn oil. Injections were given each day for 3 consecutive days. On the third day, 4h after the safrole or isosafrole injections, all animals received intraperitoneal injections of a freshly prepared suspension of 2-acetamidofluorene (3.0mg/100g body wt.) in 1.75% gum acacia and 0.15M-NaCl. Before injections, all animals were given food and water *ad libitum*. After injection all animals were housed in individual metal metabolism cages; only drinking water was available during urine collection. Faeces were separated by a wire screen, and the 24h urine samples were collected in containers under solid CO_2.

Determination of the metabolites of 2-acetamidofluorene in urine. Urine samples were thawed, filtered through glass-wool and after adjustment to pH6.0 were diluted. A portion of urine sample equivalent to about one-third of the total sample was incubated with Takadiastase and β-glucuronidase as described previously (Miller *et al.*, 1960; Weisburger *et al.*, 1956). After incubation for 18h at 37°C hydrolysed fluorene derivatives were extracted with diethyl ether. The acidic metabolites were extracted from the ether extract into 0.5M-NaOH; finally the alkali extract was neutralized to pH6.0 and the metabolites were extracted in diethyl ether. Acidic metabolites were chromatographed on Whatman no. 1 paper with the solvent system cyclohexane–2-methylpropan-2-ol–acetic acid–water (16:4:2:1, by vol.) (Weisburger *et al.*, 1956). After chromatography the strips were air-dried and then viewed under u.v. light. Appropriate absorption zones were cut out and eluted in 3ml of aq. 95% (v/v) ethanol. The metabolites were determined quantitatively by u.v. absorption in the range 350–270nm by using a Coleman–Hitachi model 124 spectrophotometer with a recorder. The molar extinction coefficient and percentage recovery for these metabolites were used as described by Lotlikar (1970*a*). The amounts of 1-hydroxy-2-acetamidofluorene were generally too low to be measured adequately and the values for this metabolite have therefore been omitted.

Administration of safrole, isosafrole or 3-methylcholanthrene for studies in vitro. For some experiments, safrole or isosafrole (100mg/kg body wt.) in corn oil was injected intraperitoneally in male rats weighing about 70–80g and 150–220g once per day for 3 consecutive days. Another group of rats was injected with corn oil for 2 days and on the third day was given an intraperitoneal injection of 3-methylcholanthrene (100mg/kg body wt.) suspended in corn oil. Control animals were injected with corn oil. All animals were killed 24h after their third injection.

In other experiments, safrole or isosafrole (100mg or 300mg/kg body wt.) was injected once, 24h before the rats were killed. The dose of 3-methylcholanthrene was the same as above. A similar study was done with male hamsters (100g body wt.), with a dose of safrole or isosafrole of 200mg/kg body wt.; the dose of 3-methylcholanthrene was the same as above. A control group was injected with corn oil.

Preparation of liver microsomal material and cytosol. After the animals were decapitated and exsanguinated, the livers were immediately removed and chilled in ice-cold 0.25M-sucrose solution. A 25% (w/v) liver homogenate prepared in 0.25M-sucrose soln. was centrifuged at 10000*g* for 15min to remove nuclei and mitochondria. The microsomal pellet was obtained by centrifuging the mitochondrial supernatant at 105000*g* for 60min. The microsomal supernatant designated as cytosol was used for assay of enzymic esterification of *N*-hydroxy-2-acetamidofluorene. The surface of the microsomal pellet was washed twice with 0.25M-sucrose before it was resuspended in the same medium.

Assay for ring- and N-hydroxylation of 2-acetamidofluorene. Unless otherwise indicated, the incubation medium was as described by Lotlikar (1970*a*). After incubation in air for 20min at 37°C, 8ml of ice-cold 1M-sodium acetate buffer, pH6.0, was added per flask. Contents of 9–18 flasks were combined for each analysis and extracted immediately with diethyl ether. The acidic metabolites were extracted from the ether extract into 0.5M-NaOH; finally the alkali extract was neutralized to pH6.0 and the metabolites were extracted in diethyl ether. The acidic metabolites were chromatographed on Whatman no. 1 paper

with cyclohexane–2-methylpropan-2-ol–acetic acid–water [16:4:2:1, by vol. (solvent system A) or 18:2:2:1 (solvent system B)]. Elution and quantitation of the metabolites were as described by Lotlikar (1970*a*), except for 5- and 7-hydroxy-2-acetamidofluorene. These two metabolites could not be separated in the solvent system B; these were eluted together (R_F 0–0.27) and calculated as 7-hydroxy-2-acetamidofluorene.

Assay for enzymic esterification of N-hydroxy-2-acetamidofluorene. The incubation medium and assay procedure were the same as described by Lotlikar (1970*b*) except that liver cytosol equivalent to 50 instead of 100mg wet wt. of tissue was employed in the present studies.

Results

The effects of safrole and isosafrole pretreatment of rats on the urinary excretion of 2-acetamidofluorene metabolites are summarized in Table 1. Isosafrole treatment did not have any appreciable effect on the urinary excretion of hydroxy metabolites of 2-acetamidofluorene by either young or adult male rats. However, safrole treatment increased excretion of 3- and 5-hydroxy-2-acetamidofluorene by both young and adult animals. In addition, it caused an increased urinary excretion of *N*-hydroxy-2-acetamidofluorene by adult males in comparison with the control group.

Because safrole and isosafrole induce biphenyl hydroxylase (Parke & Rahman, 1970, 1971), it was of interest to study the effects of pretreatment of these compounds on metabolism of 2-acetamidofluorene by rat liver microsomal preparations. Such studies are summarized in Table 2. Results of pretreatment with 3-methylcholanthrene are presented for comparison. In agreement with the results of Lotlikar *et al.* (1967), pretreatment with this compound caused severalfold increase in both *N*- and ring-hydroxylation of 2-acetamidofluorene in both young and adult male rats compared with their respective controls. Safrole or isosafrole (100mg/day per kg body wt.) pretreatment for 3 days also caused increases in ring-hydroxylation of 2-acetamidofluorene in both groups. Increases in *N*-hydroxylation were much less than those in ring-hydroxylation. However, increases in ring-hydroxylation by isosafrole treatment were twice as much in comparison with those of safrole-treated animals. Among the three compounds tested, 3-methylcholanthrene exerted the most pronounced effect on hydroxylation of 2-acetamidofluorene by rat liver microsomal preparations.

Whereas 3-methylcholanthrene (100mg/kg body wt.) was given as a single dose, safrole or isosafrole was given to rats at the same dosage (100mg/day per kg body wt.) for 3 consecutive days (Tables 1 and 2). It was of interest to study the effects of administration of a single dose of these compounds. A single injection of safrole or isosafrole (100mg/kg body wt.) to adult rats increased formation only of 3-hydroxy-2-acetamidofluorene; *N*-, 7- and 5-hydroxy-2-acetamidofluorene were not affected. However, increasing the dosage to 300mg/kg body wt. caused a severalfold increase in formation of 3-, 5- and 7-hydroxy-2-acetamidofluorene formation, like that presented in Table 2. Increases in amounts of *N*-hydroxy-2-acetamidofluorene were variable and not pronounced.

The increased hepatic 2-acetamidofluorene hydroxylation activity after 3-methylcholanthrene

Table 1. *Urinary excretion of metabolites of 2-acetamidofluorene by male rats after administration of safrole and isosafrole*

Four young (60g body wt.) or adult (160g body wt.) male rats were used per group. All injections to animals were given intraperitoneally. Control animals were injected with corn oil. At 4h after the third injection of safrole or isosafrole (100mg/day per kg body wt.), all animals received injections of 2-acetamidofluorene (30mg/kg body wt.) and 24h urine samples were collected. One rat was used for each urine collection. All other details are described in the Experimental section. Results are given as means ± S.E.M. of determinations of urine samples from four animals. * Statistical comparisons between control and safrole- or isosafrole-treated groups with P values <0.05 are considered significant.

Animals	Treatment	2-Acetamidofluorene derivative ...	Urinary excretion (% of dose)			
			N-Hydroxy	3-Hydroxy	5-Hydroxy	7-Hydroxy
Young	Control		2.5±0.6	4.9±0.9	7.3±1.4	20±5.7
	Safrole		3.3±0.5	6.9±0.5*	12±2.5*	27±6.4
	Isosafrole		1.8±0.3	5.4±0.8	10±1.6	21±5.6
Adult	Control		1.1±0.4	1.8±0.4	4.2±1.5	12±5.5
	Safrole		1.9±0.1*	4.2±0.6*	11±1.7*	19±1.1
	Isosafrole		1.2±0.3	2.6±0.5	7.9±2.7	18±3.8

Table 2. *Hydroxylation of 2-acetamidofluorene by liver microsomal preparations from rats pretreated with 3-methylcholanthrene, safrole and isosafrole*

Young (70–80g body wt.) and adult (150–220g body wt.) male rats were used for these experiments. All injections were given intraperitoneally. Safrole or isosafrole (100mg/day per kg body wt.) in corn oil was injected for 3 days. One group of rats was injected with corn oil for 2 days and 3-methylcholanthrene (100mg/kg body wt., suspended in corn oil) on the third day. Control animals were injected with corn oil for 3 days. All animals were killed 24h after their third injection. Hydroxylation of 2-acetamidofluorene by liver microsomal preparations was assayed as described in the Experimental section. The acidic metabolites were chromatographed on Whatman no. 1 paper with solvent system B. In this solvent system, 5- and 7-hydroxy-2-acetamidofluorene could not be separated; these were eluted together and determined quantitatively as 7-hydroxy-2-acetamidofluorene. Liver from one rat was used for each analysis. Results are given as means ± S.E.M.
* Statistical comparisons between control and treated groups with P values < 0.05 are considered significant.

Animals	Treatment	No. of analyses	2-Acetamidofluorene derivative ...	Hydroxylation (nmol formed/20min per g wet wt. of liver)		
				N-Hydroxy	3-Hydroxy	7-Hydroxy
Young	Control	2		15 ± 2	21 ± 2	207 ± 5
	3-Methylcholanthrene	2		70 ± 3*	185 ± 7*	1228 ± 281*
	Safrole	2		20 ± 0*	50 ± 0*	392 ± 22*
	Isosafrole	3		22 ± 1.0*	100 ± 13*	829 ± 35*
Adult	Control	9		14 ± 8	10 ± 7	174 ± 54
	3-Methylcholanthrene	9		114 ± 53*	115 ± 23*	1117 ± 249*
	Safrole	5		26 ± 8*	46 ± 21*	373 ± 63*
	Isosafrole	3		18 ± 5	88 ± 24*	704 ± 153*

Table 3. *Effects of administration of ethionine on increases in hepatic hydroxylation activity after treatment of rats with 3-methylcholanthrene, safrole and isosafrole*

Adult (180–220g body wt.) male rats were used for this study. All injections were given intraperitoneally. DL-Ethionine (85mg/100g body wt.) or DL-methionine (85mg/100g body wt.) or both were dissolved in 0.9% NaCl soln. for injections. Safrole (30mg/100g body wt.), isosafrole (30mg/100g body wt.) or 3-methylcholanthrene (10mg/100g body wt.) was injected 15min after the administration of the amino acids. Control animals were injected with 0.9% NaCl soln. and corn oil. All animals were killed 24h after injection of the compounds. The assay for hydroxylation of 2-acetamidofluorene by liver microsomal preparations, and other details, are described in the Experimental section. The acidic metabolites were separated on Whatman no. 1 paper by using solvent system A. Livers from two rats were used for each analysis. Results are expressed as percentages of the control calculated on 1g wet wt. of liver. Results are averages of two analyses.

Treatment	Position of hydroxylation ...	Formation of hydroxy-2-acetamidofluorene (% of control liver)			
		N-	3-	5-	7-
Control		100	100	100	100
3-Methylcholanthrene		350	1390	850	650
3-Methylcholanthrene+ethionine		140	278	388	124
3-Methylcholanthrene+ethionine+methionine		320	344	950	228
Safrole		112	200	285	162
Safrole+ethionine		87	186	157	96
Safrole+ethionine+methionine		100	428	700	323
Isosafrole		183	811	350	362
Isosafrole+ethionine		95	155	113	60
Isosafrole+ethionine+methionine		74	566	662	266

administration was due to the production of new enzymes. Thus administration of puromycin, actinomycin D (Lotlikar *et al.*, 1967) or DL-ethionine (Cramer *et al.*, 1960) simultaneously with 3-methylcholanthrene strongly inhibited the increase in hydroxylation. Ethionine inhibition was completely

prevented by the simultaneous administration of methionine (Cramer *et al.*, 1960). The effects of ethionine on increases in 2-acetamidofluorene hydroxylation by 3-methylcholanthrene, safrole or isosafrole are presented in Table 3. In the present experiments increases in hydroxylation of 2-acetamidofluorene by 3-methylcholanthrene were partly inhibited by ethionine. Inhibition of formation of *N*- and 5-hydroxy-2-acetamidofluorene by ethionine was almost completely restored by methionine. However, inhibition of formation of 3- and 7-hydroxy-2-acetamidofluorene by ethionine was partly restored by methionine. Similar results were also obtained with safrole or isosafrole pretreatment. Thus increase in hydroxylation activity due to safrole or isosafrole pretreatment was inhibited by simultaneous administration of ethionine. Similarly, ethionine inhibition was almost completely reversed by the simultaneous administration of methionine. Parke & Rahman (1970) have also shown that increased activity of biphenyl-4-hydroxylase due to safrole treatment was completely inhibited by actinomycin D.

Results of addition of safrole or isosafrole to liver microsomal preparations *in vitro* are summarized in Table 4. With the exception of formation of 7-hydroxy-2-acetamidofluorene, *N*- and ring-hydroxylation of 2-acetamidofluorene by microsomal preparations from livers of control rats was low.

Safrole or isosafrole at both concentrations (0.1 mM and 1 mM) inhibited the formation of 7-hydroxy-2-acetamidofluorene to a large extent. Hydroxylation of 2-acetamidofluorene by liver microsomal preparations from rats treated with 3-methylcholanthrene was not inhibited by 0.1 mM-safrole. Safrole (1 mM) inhibited formation of both 5- and 7-hydroxy-2-acetamidofluorene. Unlike safrole, 0.1 mM-isosafrole inhibited 5- and 7-hydroxylation of 2-acetamidofluorene to a large extent; at 1 mM inhibition was even more pronounced. *N*-Hydroxylation by rats treated with 3-methylcholanthrene was decreased by 1 mM-safrole or 1 mM-isosafrole. However, *P* values were higher than 0.05 ($P > 0.1$ and < 0.2 and hence not significant). 3-Hydroxylation (*O*-hydroxylation) of 2-acetamidofluorene was not inhibited at either of the two concentrations of the compounds.

We have found (Lotlikar *et al.*, 1967) that the livers from rats and hamsters treated with 3-methylcholanthrene gave different responses in *N*- and ring-hydroxylation of 2-acetamidofluorene. Thus treatment of rats with 3-methylcholanthrene caused an appreciably greater increase in ring-hydroxylation than in *N*-hydroxylation of 2-acetamidofluorene. However, treatment of hamsters with 3-methylcholanthrene caused a severalfold increase in *N*-hydroxylation of 2-acetamidofluorene only. The effects of safrole, isosafrole and 3-methylcholanthrene were therefore tested in hamsters (Table 5). Pretreatment with 3-methylcholanthrene gave a several-

Table 4. *Effects of safrole and isosafrole on hydroxylation of 2-acetamidofluorene by liver microsomal preparations from control rats and rats pretreated with 3-methylcholanthrene*

Four adult (160–180g body wt.) male rats were used per group. 3-Methylcholanthrene (10mg/100g body wt., suspended in corn oil) was injected intraperitoneally. Control animals were injected with corn oil. Animals were killed 24h after the injections. Livers from four rats were pooled for the study. The incubation medium, except for the amount of safrole and isosafrole added, is described in the Experimental section. Metabolites *N*-, 3-, 5- and 7-hydroxy-2-acetamidofluorene were separated on Whatman no. 1 paper with solvent system A. Values are given as means±S.E.M. of two determinations. * Statistical comparisons between control and safrole- or isosafrole-treated groups with *P* values < 0.05 are considered significant.

Pretreatment	Other addition (mM)	2-Acetamidofluorene derivative ...	Hydroxylation (nmol formed/20min per g wet wt. of liver)			
			N-Hydroxy	3-Hydroxy	5-Hydroxy	7-Hydroxy
Control	—		7±2	8±1	3±1	138±1
	Safrole (0.1)		9±1	7±0	2±0	53±12*
	Safrole (1)		4±1	3±0*	2±0	13±0*
	Isosafrole (0.1)		7±1	5±0	4±2	43±6*
	Isosafrole (1)		7±0	3±1	2±0	10±0*
3-Methylcholanthrene	—		41±9	194±16	275±7	557±4
	Safrole (0.1)		44±9	208±11	234±21	449±44
	Safrole (1)		16±0	192±9	144±3*	178±5*
	Isosafrole (0.1)		55±2	196±5	202±9*	340±25*
	Isosafrole (1)		20±2	164±5	51±1*	69±0*

Table 5. *Hydroxylation of 2-acetamidofluorene by liver microsomal preparations from hamsters pretreated with 3-methylcholanthrene, safrole and isosafrole*

All male hamsters (100–150g body wt.) were killed 24h after a single intraperitoneal injection of 3-methylcholanthrene, safrole or isosafrole. The incubation medium and other details are described in the Experimental section. Solvent system A was used for the separation of metabolites. Liver from one hamster was used for each analysis. Values are given as means ± S.E.M. * Statistical comparisons between control and treated groups with P values <0.05 are considered significant.

Treatment	Dose (mg/kg)	No. of analyses	2-Acetamidofluorene derivative ...	Hydroxylation (nmol formed/20min per g wet wt. of liver)			
				N-Hydroxy	3-Hydroxy	5-Hydroxy	7-Hydroxy
Control	—	7		133 ± 46	20 ± 4	87 ± 12	478 ± 111
3-Methylcholanthrene	100	7		938 ± 172*	57 ± 10*	126 ± 34*	487 ± 144
Safrole	50	4		174 ± 54	32 ± 16	121 ± 73	525 ± 125
	100	5		80 ± 66	20 ± 11	81 ± 45	362 ± 121
	200	3		70 ± 37	15 ± 2	71 ± 20	508 ± 19
Isosafrole	50	2		51 ± 3	23 ± 2	83 ± 15	358 ± 74
	100	3		72 ± 38	19 ± 1	69 ± 12	408 ± 97
	200	3		29 ± 8*	14 ± 1*	43 ± 4*	475 ± 26

fold increase in *N*-hydroxylation of 2-acetamidofluorene by liver microsomal material. These results are in agreement with previous studies (Lotlikar *et al.*, 1967). A single injection of safrole at any of three doses tested did not have any appreciable effect on either *N*- or ring-hydroxylation of 2-acetamidofluorene by liver microsomal preparations. Isosafrole (200mg/kg body wt.) inhibited the formation of *N*-, 3- and 5-hydroxy-2-acetamidofluorene; it did not have much effect on formation of 7-hydroxy-2-acetamidofluorene.

Safrole or isosafrole (100mg/day per kg body wt.) pretreatment for 3 days did not have any appreciable effect on enzymic esterification of *N*-hydroxy-2-acetamidofluorene by liver cytosols from male rats.

Discussion

The results presented in this paper indicate that safrole and isosafrole pretreatment of rats increased the activity of liver microsomal material for *N*- and ring-hydroxylation of 2-acetamidofluorene. Parke & Rahman (1970) have also demonstrated increased activities of liver biphenyl-4- and 2-hydroxylase after treatment of rats with these two chemicals. However, in recent studies, safrole or piperonyl butoxide pretreatment was found to inhibit *p*-hydroxylation and stimulate *o*-hydroxylation of biphenyl by mouse liver microsomal preparations (Friedman *et al.*, 1971; Jaffe *et al.*, 1969). In the present experiments, safrole and isosafrole pretreatment of rats stimulated both *o*- and *p*-hydroxylation of 2-acetamidofluorene by liver microsomal material. In these studies increases in *N*-hydroxylation of 2-acetamidofluorene due to treatment by these two chemicals were not pronounced.

Many microsomal drug-metabolizing enzyme-inducers such as 3-methylcholanthrene and phenobarbital are also metabolized by the same enzyme systems (Conney, 1967). Safrole and isosafrole also seem to be in this category. Safrole is oxidized by mouse liver microsomal preparations in the presence of NADPH and air (Casida *et al.*, 1966). The glucuronide of 1-hydroxysafrole has been isolated as a metabolite from the urine of rats pretreated with safrole (Borchert *et al.*, 1971). It is most likely that isosafrole is oxidized similarly by the liver microsomal preparation.

Parke & Rahman (1970) showed that the increase in biphenyl-4-hydroxylase activity due to safrole treatment was completely inhibited by actinomycin D. In the present studies, increases in hydroxylation of 2-acetamidofluorene by safrole or isosafrole treatment of rats was inhibited to a large extent by ethionine and this inhibition was reversed by the simultaneous administration of methionine. These studies suggest that pretreatment of rats with safrole

and isosafrole produces new synthesis of liver microsomal hydroxylases.

Pretreatment with 3-methylcholanthrene gives different responses in rats and hamsters (Lotlikar *et al.*, 1967). The present study now indicates that safrole or isosafrole pretreatment also causes a different response in hamsters and rats.

Safrole is a weaker hepatocarcinogen than 2-acetamidofluorene in the rat (Homburger *et al.*, 1961; Long *et al.*, 1963; Miller, 1970). The present studies indicate that both safrole and isosafrole are weaker hydroxylase inducers than 3-methylcholanthrene. However, it is possible that, like 3-methylcholanthrene (Miyaji *et al.*, 1953; Miller *et al.*, 1958), both of these chemicals inhibit liver carcinogenesis from 2-acetamidofluorene in the rat.

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