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SEX DIFFERENCE IN THE *O*-DEALKYLATION ACTIVITY OF 7-HYDROXYCOUMARIN *O*-ALKYL DERIVATIVES IN LIVER MICROSOMES OF RATS

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Abstract—Sex differences in the *O*-dealkylation activities of *O*-alkyl derivatives of 7-hydroxycoumarin were compared using liver microsomes from male and female rats. The sex difference (male>female) in the *O*-depropylation activity was found to be greater than the sex differences in *O*-demethylation and *O*-deethylation activities. The magnitude of sex difference seen in the *O*-depropylation activity was diminished after pretreatment of rats with spironolactone, phenobarbital, isosafrole or 3-methylcholanthrene. The latter two inducers were much more effective than the former in enhancing the activity. The sex differences in the *O*-dealkylation activities were also seen when the activities were measured in the presence of cumene hydroperoxide instead of NADPH. The sex difference of the *O*-depropylation activity remained essentially unchanged by the fortification of male and female microsomes with purified NADPH-cytochrome *c* (P-450) reductase. The difference was also seen when reconstituted with cytochrome P-450 partially purified from both male and female rats by means of ω-amino-*n*-octyl Sepharose 4B and hydroxylapatite columns. The addition of 7,8-benzoflavone to the incubation mixture resulted in the increased magnitude of sex difference in the *O*-depropylation activity.

From these results, we confirm that one or more cytochrome P-450 species other than a cytochrome P-450 species sensitive to 7,8-benzoflavone are present in male microsomes in higher amounts than in female microsomes.

It has been well established that there are marked sex differences in the activities of drug metabolizing enzymes in liver microsomes of rats. Quite extensive studies made on the sex difference of drug metabolizing enzymes in the past two decades led Kato and his associates to classify drugs into two groups according to the response of each drug enzyme to androgen, as reviewed by Kato [1].

In view of the current studies on drug metabolizing enzymes, cytochrome P-450 has been confirmed to play the major role in drug oxidation reactions in liver microsomes. However, the amount of cytochrome P-450 present in male rats is only 20 – 40 per cent greater than that in female rats [2], suggesting that enzymatic properties rather than the amount of cytochrome P-450 differ between male and female microsomes. In support of the idea that catalytic properties of cytochrome P-450 differ between male and female rats, Schenkman *et al.* [3] demonstrated that the differences in the drug oxidizing activities could be accounted for by the difference in the affinity of the drugs to cytochrome P-450. Further, Gigon *et al.* [4, 5] and Davies *et al.* [6] proposed that the sex differences in the drug oxidation activities are due to the difference in the activity of NADPH-cytochrome P-450 reductase being enhanced by addition of the drug substrate. More recent studies by several laboratories have clarified that there are multiple species of cytochrome P-450 in liver microsomes of rats [7–10]. Thus it seems possible to assume that a particular species of cytochrome P-450 is involved in exhibiting the sex difference. However, no direct evidence has been reported in the literature.

To further clarify the mechanisms of sex difference in drug metabolism, we initiated this study using 7-hydroxycoumarin *O*-methyl-, *O*-ethyl- and *O*-propyl-derivatives as substrates and found that the magnitude of the sex difference on the *O*-dealkylation activities varied markedly with the substitution of the alkyl group. In addition, we would like to report on rather direct evidence that one or more species of cytochrome P-450 catalyzing 7-propoxycoumarin *O*-depropylation exists in higher amounts in male rats than in female rats.

MATERIALS AND METHODS

Male and female rats of Wistar strain were used throughout this study. The age of the animals ranged from 6 to 10 weeks, but were used in the same age for comparison of the male and female rats. The animals were maintained on a commercial rat chow (CE-2, Nippon Clea, Japan) and were given tap water *ad lib*. Isosafrole and 3-methylcholanthrene, dissolved in corn oil, were injected intraperitoneally once a day for 3 consecutive days at daily doses of 150 and 40 mg/kg, respectively. Spironolactone and phenobarbital, dissolved in saline, were given intraperitoneally twice a day for 4 consecutive days at doses of 100 and 40 mg/kg, respectively. The animals were killed 24 hr after the last injection. Liver microsomes were prepared as described previously [11], and the microsomes suspended with distilled

water at a concentration of about 30 mg/ml were stored under an atmosphere of nitrogen at −80°. The microsomal preparations were used for experiments within a month, in which period no significant decrease in the drug metabolizing activities was observed. A typical incubation mixture for the assay of *O*-dealkylase activities consisted of microsomes (about 0.5 mg), Na,K-phosphate (pH 7.4, 50 mM), EDTA (0.1 mM), an NADPH-generating system (0.5 mM NADP, 5 mM glucose 6-phosphate, 15 mM $MgCl_2$ and 1 unit of glucose 6-phosphate dehydrogenase) and a substrate (0.5 mM) in a final volume of 0.5 ml. When benzphetamine *N*-demethylase activity was assayed, the amount of the incubation mixture was doubled and the substrate concentration was increased to 1.0 mM. These mixtures were incubated aerobically at 37° for 10 min. The reaction was started by addition of the NADPH-generating system previously incubated at 37° for 3 min to generate NADPH. The incubation mixture for the assay of cumene hydroperoxide-dependent *O*-dealkylation activities contained microsomes (0.5 mg), Na,K-phosphate (pH 7.4, 50 mM), $MgCl_2$ (15 mM), EDTA (0.1 mM), substrate (0.5 mM) and cumene hydroperoxide (3.3 mM) in a final volume of 0.5 ml. The mixtures were preincubated at 37° for 2 min, then the reaction was started by addition of cumene hydroperoxide. Incubations were carried out aerobically at 37° for 5 min. The linearity of the cumene hydroperoxide-dependent reaction was lost within 5 min when the formation of 7-hydroxycoumarin was monitored by recording the fluorescence increase by the method of Ullrich and Weber [12]. However, because of the limitation on the analytical sensitivity, incubations were terminated at 5 min. The incubation mixture for the assay of *O*-depropylation activity of partially purified cytochrome P-450 contained 50 μl of the cytochrome P-450 fraction eluted from the hydroxylapatite column, 1.45 unit of NADPH-cytochrome *c* reductase having the specific activity of 50.0 units per mg of protein purified as reported previously [13] with some modifications, 50 mM HEPES (pH 7.4), 15 mM $MgCl_2$, 0.5 mM 7-propoxycoumarin and 0.1 mM NADPH. The activity of cytochrome P-450 to depropylate 7-propoxycoumarin in the reconstituted system was measured by recording the fluorescence increase due to the formation of 7-hydroxycoumarin as reported by Ullrich and Weber [12]. The microsomal *O*-dealkylation activities were estimated by determining the 7-hydroxycoumarin according to the method of Aitio [14]. The method of Ullrich and Weber [12] could not be utilized for the microsomal experiments since smaller amounts of 7-hydroxycoumarin could not be detected accurately, probably due to the quench of the fluorescence of 7-hydroxycoumarin by microsomes. Oxidative *N*-demethylation activity of benzphetamine was measured by determining the formaldehyde by the method of Nash [15]. Cytochrome P-450 and cytochrome b_5 were determined by the method of Omura and Sato [16, 17]. The activity of NADPH-cytochrome *c* reductase was determined by the method reported by Phillips and Langdon [18]. The reductase activity was defined as the unit which reduced one μmole of cytochrome *c* per min. The spectrophotometric measurements were carried out using a Hitachi recording spectrophotometer, Model 556. Microsomal protein was determined by the method of Lowry *et al.* [19], using bovine serum albumin as the standard.

7-Hydroxycoumarin *O*-methyl-, *O*-ethyl- and *O*-propyl-derivatives were synthesized using the corresponding alkyl iodides essentially as described previously [12]. These compounds were further purified by means of a silica gel column using ether as an eluant. ω-Amino-*n*-octyl Sepharose 4B was synthesized from cyanogen bromide-activated Sepharose 4B and 1,8-diaminooctane as described by Cuatrecasas [20]. Hydroxylapatite was purchased from Bio Rad, Richmond, CA, U.S.A., isosafrole from Tokyo Chemicals Inc., Tokyo, Japan, and 3-methylcholanthrene and 7,8-benzoflavone from Wako Pure Chemicals, Osaka, Japan. Commercial cholic acid was twice recrystallized from hot 50% ethanol. Emulgen 913, a non-ionic detergent, and benzphetamine were generous gifts from Kao-Atlas Co., Tokyo, Japan and Upjohn, Kalamazoo, MI, U.S.A. respectively. NADP, NADPH and glucose 6-phosphate were purchased from Oriental Yeast Co., Osaka, Japan, glucose 6-phosphate dehydrogenase from Boehringer Mannheim, Germany, cumene hydroperoxide from Nakarai Chemicals, Osaka, Japan, and HEPES (*N*-2-hydroxyethylpiperazine N′-2-ethane sulfonic acid) from Sigma, Chemical Co., St. Louis, MO, U.S.A.

RESULTS

The sex differences in the microsomal dealkylation activities of 7-hydroxycoumarin *O*-alkyl derivatives and benzphetamine are shown in Table 1. We found that the sex differences in the dealkylation activities varied with the substitution of the alkyl group of 7-hydroxycoumarin *O*-alkyl derivatives. The sex differences in the *O*-demethylation, *O*-deethylation and *O*-depropylation activities were 2.49, 4.04 and 20.67, respectively, when calculated as male/female activities. The magnitude of the sex difference in the *O*-depropylation activity was, to our knowledge, greater than those of any other drug metabolizing activities reported. The sex differences in the *O*-demethylation and *O*-deethylation activities were comparable to that of benzphetamine *N*-demethylation activity. The effects of treatment with phenobarbital or spironolactone on the activities are also shown in Table 1. The pretreatment of rats with phenobarbital increased the microsomal activities measured. The increase in the activities was more pronounced in females than males, consequently resulting in the decrease of the sex differences. The changes in the activities and in the magnitude of the sex differences were similar to those observed in the benzphetamine *N*-demethylation activity measured as the control. The treatment with spironolactone did not appreciably change the activities in male rats, while it stimulated the activities in female rats. Thus the sex differences were decreased by the spironolactone treatment to a lesser degree than by the phenobarbital treatment. The results showing that the induction of drug oxidizing activities were seen

Table 1. Effects of treatment of rats with phenobarbital or spironolactone on dealkylation activities for 7-hydroxycoumarin *O*-alkyl derivatives and benzphetamine*

Treatment	Sex	7-Methoxycoumarin	7-Ethoxycoumarin	7-Propoxycoumarin	Benzphetamine
		(nmoles product formed/mg/min)			
Saline	♂	0.778 ± 0.184(1.0)	0.951 ± 0.150(1.0)	1.075 ± 0.228(1.0)	7.10 ± 1.02(1.0)
	♀	0.312 ± 0.071(1.0)	0.235 ± 0.040(1.0)	0.052 ± 0.017(1.0)	2.11 ± 0.53(1.0)
	♂/♀	2.49	4.04	20.67	3.36
Phenobarbital	♂	2.100 ± 0.511(2.7)	3.180 ± 0.735(3.3)	2.261 ± 0.697(2.1)	20.38 ± 3.29(2.9)
	♀	1.085 ± 0.173(3.5)	1.457 ± 0.319(6.2)	0.467 ± 0.111(9.0)	10.48 ± 2.61(5.0)
	♂/♀	1.94	2.18	4.84	1.94
Spironolactone	♂	0.702 ± 0.089(0.9)	1.055 ± 0.125(1.1)	0.784 ± 0.149(0.7)	6.88 ± 1.11(1.0)
	♀	0.535 ± 0.081(1.7)	0.538 ± 0.102(2.3)	0.264 ± 0.059(5.1)	4.50 ± 1.00(2.1)
	♂/♀	1.31	1.96	2.96	1.53

* Values are means ± S.D. (N = 5). The number in the bracket is the ratio of the activity to the corresponding control. See Materials and Methods for the experimental details.

only in female rats are in good agreement with the finding reported by Stripp *et al.* [21]. They also observed a significant increase of the activity of NADPH-cytochrome *c* reductase in both male and female rats. In our experiment, we found that the increase in the reductase activity was greater in females than in males (Table 2). The effects of treatments of rats with 3-methylcholanthrene or isosafrole on the dealkylation activities are shown in Table 3. When corn oil was injected as the control, the sex differences in the *O*-deethylation and *O*-depropylation activities were decreased, whereas those in the *O*-demethylation and *N*-demethylation activities remained unchanged. The decrease in the extent of the sex differences in the *O*-deethylation and *O*-depropylation activities is probably due to the increased rates of the dealkylations in female rats. The pretreatment with 3-methylcholanthrene or with isosafrole was essentially without effects on benzphetamine *N*-demethylation activity. The treatments did not produce a marked change in the *O*-demethylation activity in the male rats, but produced a slight increase of the activity in female rats. Thus, the magnitude of the sex difference was decreased by either of these treatments. On the other hand, the treatments with 3-methylcholanthrene and isosafrole stimulated the *O*-deethylation and *O*-depropylation activities in male rats, and in addition, the activities were dramatically increased in female rats. Consequently, the sex differences were completely abolished by these treatments. The effects of treatment with 3-methylcholanthrene or with isosafrole on the contents of cytochrome P-450 and cytochrome b_5 and the activity of NADPH-cytochrome *c* reductase were also examined (Table 4). The treatment with 3-methylcholanthrene increased the cytochrome P-450 content in both sexes; the extent of the increase in the female was slightly higher than that in the male. The treatment, however, did not produce marked changes in the content of cytochrome b_5 and the activity of NADPH-cytochrome *c* reductase. The treatment with isosafrole did not produce apparent increases in the contents of cytochrome P-450 and cytochrome b_5 and the activity of NADPH-cyto-

Table 2. Effects of treatment of rats with phenobarbital or spironolactone on the contents of cytochromes P-450 and b_5 and the activity of NADPH-cytochrome *c* reductase*

Treatment	Sex	Cytochrome P-450 (nmoles/mg protein)	Cytochrome b_5 (nmoles/mg protein)	NADPH-cyt. *c* red. (units/mg protein)
Saline	♂	0.871 ± 0.095(1.0)	0.327 ± 0.073†(1.0)	0.247 ± 0.023(1.0)
	♀	0.647 ± 0.086(1.0)	0.357 ± 0.052(1.0)	0.203 ± 0.072(1.0)
	♂/♀	1.35	0.92	1.22
Phenobarbital	♂	1.586 ± 0.149(1.8)	0.367 ± 0.062(1.1)	0.492 ± 0.037(2.0)
	♀	0.744 ± 0.086(1.1)	0.346 ± 0.032(1.0)	0.281 ± 0.043(1.4)
	♂/♀	2.13	1.06	1.75
Spironolactone	♂	0.558 ± 0.105(0.6)	0.260 ± 0.062(0.8)	0.391 ± 0.041(1.6)
	♀	0.544 ± 0.062(0.8)	0.365 ± 0.028(1.0)	0.556 ± 0.060(2.7)
	♂/♀	1.03	0.71	0.70

* Values are means ± S.D. (N = 5). The number in the bracket is the ratio of the activity to the corresponding control. See Materials and Methods for the experimental details.
† N = 4.

Table 3. Effect of treatment of rats with 3-methylcholanthrene or isosafrole on dealkylation activities for 7-hydroxycoumarin *O*-alkyl derivatives and benzphetamine*

Treatment	Sex	7-Methoxycoumarin	7-Ethoxycoumarin	7-Propoxycoumarin	Benzphetamine
		(nmoles product formed/mg/min)			
Corn oil	♂	0.962 ± 0.206 (1.0)	1.074 ± 0.196 (1.0)	0.946 ± 0.192 (1.0)	9.14 ± 1.75 (1.0)
	♀	0.388 ± 0.031†(1.0)	0.383 ± 0.055†(1.0)	0.125 ± 0.037†(1.0)	2.70 ± 0.68 (1.0)
	♂/♀	2.47	2.80	7.57	3.39
3-Methylcholanthrene	♂	0.905 ± 0.082 (0.9)	8.560 ± 2.047 (8.0)	7.178 ± 1.541 (7.6)	8.53 ± 2.31 (0.9)
	♀	0.560 ± 0.042 (1.4)	8.772 ± 0.800(22.9)	7.166 ± 0.609(57.4)	2.55 ± 0.31 (0.9)
	♂/♀	1.62	0.98	1.00	3.35
Isosafrole	♂	0.802 ± 0.063 (0.8)	6.560 ± 0.971 (6.1)	5.456 ± 0.581 (5.8)	10.27 ± 1.36 (1.1)
	♀	0.595 ± 0.056 (1.5)	8.192 ± 1.175(21.4)	5.618 ± 0.609(45.0)	3.64 ± 0.69 (1.3)
	♂/♀	1.35	0.80	0.97	2.82

* Values are means ± S.D. (N = 5). The number in the bracket is the ratio of the activity to the corresponding control. The experimental details are described in Materials and Methods.
† N = 4.

chrome *c* reductase. Recent studies by Dickens *et al.* [22] have demonstrated that a metabolite of isosafrole present in microsomes after the administration binds to the reduced cytochrome P-450 and interferes with the binding of carbon monoxide to the reduced cytochrome P-450. They also reported that the interference was eliminated by the preincubation of the isosafrole treated microsomes with some compounds, including *p*-nitroanisole and 7-ethoxycoumarin. In accordance with the reported results, we also observed that the apparent content of cytochrome P-450 was increased by the preincubation of microsomes with *p*-nitroanisole (not shown). Therefore, it can be assumed that isosafrole increased the contents of cytochrome P-450 greater than those shown in Table 4.

Estabrook and his co-workers [23, 24] first noted that in phenobarbital-treated rat liver microsomes there is about 20 times more cytochrome P-450 than NADPH-cytochrome *c* reductase on the basis of molecular number. Because of the presence of only a limited amount of the reductase in liver microsomes compared to the amount of cytochrome P-450, it seems possible to assume that NADPH-cytochrome *c* reductase limits the activity of cytochrome P-450. In accordance with this idea, Kamataki *et al.* [25] proposed that the activity of cytochrome P-450 to *N*-demethylate benzphetamine increased markedly on increasing the ratio of the reductase (units) to cytochrome P-450 (nmoles) using the reconstituted system. In support of the idea, Miwa *et al.* [26] have shown clearly that in microsomes the activity of cytochrome P-450 is limited by the NADPH-cytochrome *c* reductase. Studies by Kitada *et al.* [27] further supported the idea. They showed that the fortification of microsomes with the purified NADPH-cytochrome *c* reductase produced the enhancement of the drug metabolizing activity and that the enhancement of the activity was dependent upon the substrate employed. To examine the possibility of whether or not the sex differences in the *O*-dealkylation activities are due at least in part to the limited amount of the reductase in male and female rats, the effects of fortification of the male and female microsomes with the purified NADPH-cytochrome *c* reductase on the activities of *O*-dealkylases were studied. As can be seen in Fig. 1, the fortification of microsomes with the purified reductase resulted in the increases in the *O*-dealkylation activities in male and female microsomes, while the magnitude of the sex difference in the activities remained essentially unchanged. Therefore, it can be confirmed that the sex differences in the *O*-dealkylation activities are not caused by the presence of limited amounts of NADPH-cytochrome *c* reductase.

Table 4. Effects of treatment of rats with 3-methylcholanthrene or isosafrole on the contents of cytochromes P-450 and b_5 and the activity of NADPH-cytochrome *c* reductase

Treatment	Sex	Cytochrome P-450 (nmoles/mg protein)	Cytochrome b_5 (nmoles/mg protein)	NADPH-cytochrome *c* red. (unit/mg protein)
Corn oil	♂	1.004 ± 0.166†(1.0)	0.506 ± 0.053 (1.0)	0.248 ± 0.036 (1.0)
	♀	0.726 ± 0.119 (1.0)	0.524 ± 0.167 (1.0)	0.252 ± 0.058 (1.0)
	♂/♀	1.38	0.97	0.98
3-Methylcholanthrene	♂	1.436 ± 0.134 (1.4)	0.543 ± 0.055 (1.1)	0.237 ± 0.050 (1.0)
	♀	1.141 ± 0.113 (1.6)	0.469 ± 0.038 (0.9)	0.290 ± 0.047 (1.2)
	♂/♀	1.26	1.16	0.82
Isosafrole	♂	1.096 ± 0.075 (1.1)	0.604 ± 0.080 (1.2)	0.281 ± 0.057 (1.1)
	♀	0.976 ± 0.075 (1.3)	0.564 ± 0.066 (1.1)	0.321 ± 0.060 (1.3)
	♂/♀	1.12	1.07	0.88

* Values are means ± S.D. (N = 5). The number in the bracket is the ratio of the activity to the corresponding control. For experimental details, see Materials and Methods.
† N = 4.

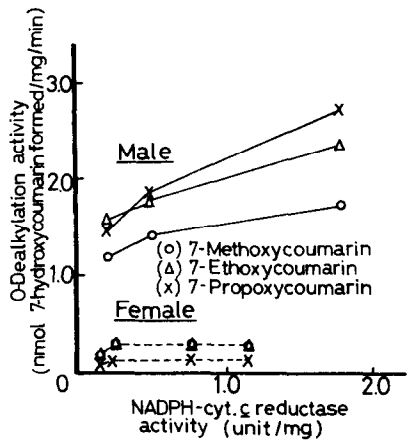

Fig. 1. The increase in the *O*-dealkylation activities by the fortification of microsomes with purified NADPH-cytochrome *c* reductase. To a tube containing 5 mg protein of liver microsomes was added a desired amount of the purified NADPH-cytochrome *c* reductase and potassium phosphate (pH 7.4) to give a final concentration of 10 mM. The amounts of the purified NADPH-cytochrome *c* reductase added were 0, 12.5, 25.0 and 50.0 units for female microsomes and 0, 12.5 and 50.0 units for male microsomes, respectively. The mixture was incubated at 37° for 20 min, then the mixture was diluted with 7.0 ml of cold 10 mM potassium phosphate (pH 7.4). The microsomes fortified with the purified NADPH-cytochrome *c* reductase was obtained by centrifugation at 105,000 *g* for 30 min. The resulting microsomal pellet was washed once by homogenization with 10 mM potassium phosphate (pH 7.4) and centrifugation at 105,000 *g* for 30 min. The microsomes thus obtained were used for the assay of *O*-dealkylation activities.

Kadlubar *et al.* [28] and Rahimtula *et al.* [29, 30] reported that liver microsomes are able to oxidize drugs without addition of NADPH if certain peroxides such as cumene hydroperoxide are present. The peroxidase activity of cytochrome P-450 has been confirmed using the purified cytochrome P-450 [31]. To support the idea that the sex differences in the *O*-dealkylation activities are caused by cytochrome P-450, the sex differences in the cumene hydroperoxide-dependent *O*-dealkylation activities were examined (Table 5). The sex differences in the *O*-dealkylation activities were also found in the cumene hydroperoxide-dependent reactions, although the magnitude of the sex differences were somewhat less than those obtained using NADPH.

In order to confirm that the marked sex difference seen in the *O*-depropylation activity is due to the difference between male and female rats in the amounts of one or more species of cytochrome P-450 responsible for the *O*-depropylation reaction, microsomes from intact male and female rats were solubilized and cytochrome P-450 was partially purified by means of ω-amino-*n*-octyl Sepharose 4B and hydroxylapatite columns. The elution profiles from hydroxylapatite columns of cytochrome P-450 and the *O*-depropylation activity obtained by the reconstitution with purified NADPH-cytochrome *c* reductase are shown in Fig. 2. Although the recovery of the total activities were low (roughly 8.5 and 38 per cent of the activity was recovered from male and female microsomes, respectively), a clear sex difference in the elution profile of the *O*-depropylation activity was observed. These results provide direct evidence that sex difference is caused by cytochrome

Table 5. Sex differences in the cumene hydroperoxide-dependent dealkylation activities*

Treatment	Sex	7-Methoxy coumarin	7-Ethoxy coumarin	7-Propoxy coumarin
		(nmoles 7-hydroxycoumarin formed/mg protein/5 min)		
Corn oil	♂	2.68 (1.0)	1.94 (1.0)	1.32 (1.0)
	♀	1.49 (1.0)	1.03 (1.0)	0.40 (1.0)
	♂/♀	1.8	1.9	3.3
3-Methylcholanthrene	♂	2.75 (1.0)	37.31(19.2)	43.08(32.7)
	♀	1.35 (0.9)	31.20(30.4)	35.60(89.7)
	♂/♀	2.0	1.2	1.2
Isosafrole	♂	2.42 (0.9)	14.82 (7.6)	15.19(11.5)
	♀	2.60 (1.8)	16.00(15.6)	15.80(39.8)
	♂/♀	0.9	0.9	1.0
Saline	♂	2.03 (1.0)	1.68 (1.0)	1.35 (1.0)
	♀	1.35 (1.0)	0.92 (1.0)	0.53 (1.0)
	♂/♀	1.5	1.8	2.6
Spironolactone	♂	1.09 (0.5)	1.25 (0.7)	0.98 (0.7)
	♀	0.80 (0.6)	0.98 (1.1)	0.76 (1.5)
	♂/♀	1.4	1.3	1.3
Phenobarbital	♂	3.84 (1.9)	3.87 (2.1)	2.79 (2.1)
	♀	1.29 (1.0)	1.29 (1.4)	0.84 (1.6)
	♂/♀	3.0	3.0	3.3

* Pooled microsomes were used for the assay. The values given in the table are the means of duplicate determinations. The number in the bracket is the ratio of the activity to the corresponding control. Other experimental details are described in Materials and Methods.

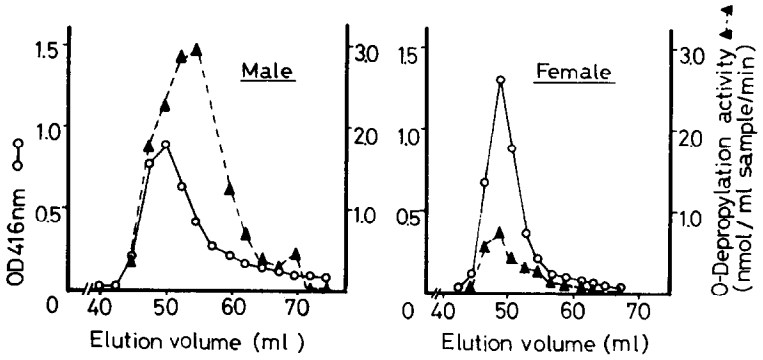

Fig. 2. Elution profiles of cytochrome P-450 and the *O*-depropylation activity of 7-propoxycoumarin from hydroxylapatite columns. Cytochrome P-450 was partially purified from intact male and female rat liver microsomes by the method essentially as described by Imai and Sato [32] with some minor modifications as reported by Kamataki *et al.* [33]. The method was further modified in that the concentration of microsomal protein in the solubilization medium was 2.0 mg per ml rather than 4.0 mg per ml, that 0.2 per cent rather than 0.08 per cent Emulgen 913 was used for the elution of cytochrome P-450 from ω-amino-*n*-octyl Sepharose 4B columns, and that 150 mM rather than 100 mM potassium phosphate (pH 7.25) was used for the elution from hydroxylapatite columns. The concentration of cytochrome p-450 was represented as the absorbance at 416 nm, since cytochrome P-450 eluted from the hydroxylapatite columns seemed to be apparently low spin state as judged by the absolute spectra. The *O*-depropylation activity was reconstituted as described in Materials and Methods.

P-450; a higher amount of a particular species of cytochrome P-450 which catalyzes the *O*-depropylation is present in male microsomes than in female microsomes.

Diamond and Gelboin [34] found that 7,8-benzoflavone inhibits aryl hydrocarbon hydroxylase activity in hamster embryo cell cultures specifically. Thereafter, Ullrich *et. al.* [35] reported that *in vitro* addition of 7,8-benzoflavone resulted in the specific inhibition of the 3-methylcholanthrene-induced *O*-deethylation activity of 7-ethoxycoumarin. The inhibitor has been widely used as the specific inhibitor of a drug metabolizing enzyme inducible by polycyclic aromatic hydrocarbons such as 3-methylcholanthrene and benzo[a]pyrene. As shown in Table 3, the *O*-depropylation activity was markedly

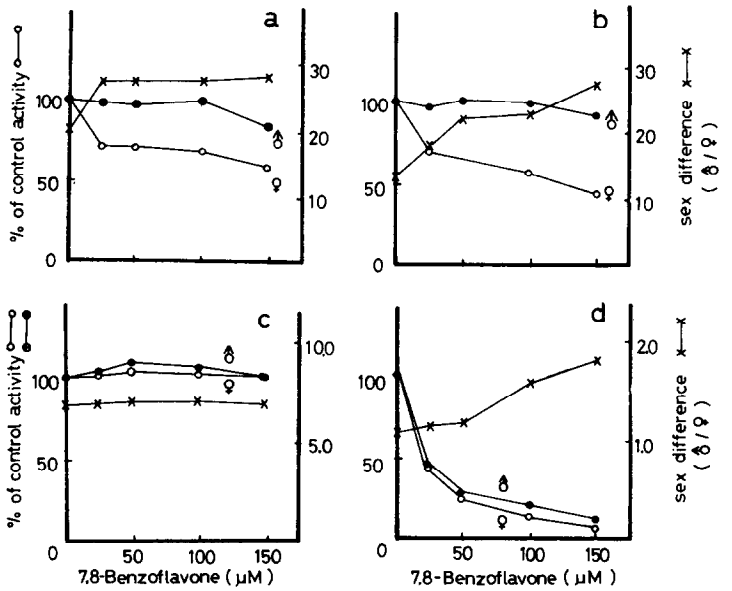

Fig. 3. Inhibition of 7-propoxycoumarin *O*-depropylation activity by 7,8-benzoflavone. Pooled microsomes from saline-treated (a), corn oil-treated (b), phenobarbital-treated (c) and 3-methylcholanthrene-treated (d) male and female rats were used. The microsomal *O*-depropylation activities were measured as described in Materials and Methods except that the indicated amounts of 7,8-benzoflavone dissolved in 5 μl of acetone were added to the incubation mixture. The *O*-depropylation activities of saline-treated, corn oil-treated, phenobarbital-treated and 3-methylcholanthrene-treated male rats in the absence of 7,8-benzoflavone were 1.58, 1.78, 3.85 and 11.2 nmoles per mg protein per min, and those of female rats were 0.09, 0.13, 0.56 and 10.4 nmoles per mg protein per min, respectively. The activities were represented as per cent of control activities which was obtained in the absence of 7,8-benzoflavone.

induced by treatment of rats with 3-methylcholanthrene. Thus, it appeared possible to assume that the activities seen in the non-induced male and female rats are due to a cytochrome P-450 species capable of being inhibited by 7,8-benzo[*a*]flavone. As can be seen from Figs. 3(a) and (b), the addition of 7,8-benzoflavone partly inhibited the *O*-depropylation activities in saline and corn oil treated male and female rats. The inhibition in female rats was more pronounced than in male rats, resulting in the increased magnitude of the sex difference. The sex difference in the presence of 150 μM 7,8-benzoflavone thus became about 29. From these results, it was suggested that the cytochrome P-450 species sensitive to 7,8-benzoflavone is not involved in the occurrence of the sex difference. The effects of 7,8-benzoflavone on the *O*-depropylation activity was further examined using microsomes from either 3-methylcholanthrene- or phenobarbital-induced rats (Figs. 3c and d). The addition of 7,8-benzoflavone was without effect on the activity in the phenobarbital-treated male and female microsomes, while it inhibited the *O*-depropylation activity of 3-methylcholanthrene-treated rat liver microsomes. In 3-methylcholanthrene-treated rats, the sex difference in the activity was increased from 1.1 to 1.8 by the presence of 150 μM 7,8-benzoflavone.

DISCUSSION

Kato and Gillette [36] demonstrated that the magnitude of sex difference in the activities of drug metabolizing enzymes of rat liver depends largely on the substrate used. Thus hexobarbital hydroxylation, aminopyrine *N*-demethylation and pentobarbital oxidation are markedly sex-dependent metabolic pathways, whereas aniline hydroxylation and zoxazolamine hydroxylation are less so or not at all sex-dependent. Among the 7-hydroxycoumarin *O*-alkyl derivatives employed in these experiments, 7-propoxycoumarin was found to be the unique substrate which showed a marked sex difference in the metabolism in the rat.

Based on numerous evidence, drugs have been classified into two groups according to the response of the enzyme to androgen [1]. In addition to the response of the enzyme to androgen, the effects of inducers were also shown to be apparently different between the enzymes catalyzing these two groups of drugs [37]. For example, hexobarbital hydroxylation and aminopyrine *N*-demethylation activities are induced by treatment of rats with phenobarbital but not with 3-methylcholanthrene, whereas aniline hydroxylation and zoxazolamine hydroxylation activities are induced by either of these pretreatments. Therefore, 7-methoxycoumarin and benzphetamine seem to be similar to hexobarbital and aminopyrine. In contrast to this concept, Wiebel and Gelboin [38] reported that there was a 3 – 4-fold sex difference in the benzo[*a*]pyrene hydroxylase activity which is able to be induced by 3-methylcholanthrene. Thus, 7-propoxycoumarin appears to be involved in this substrate category.

It is of interest to determine which one of multiple cytochrome P-450 species is involved in the occurrence of the sex difference. Our results shown in Fig. 3 indicate that a cytochrome P-450 species sensitive to 7,8-benzoflavone is probably not involved in the occurrence of sex difference in the 7-propoxycoumarin *O*-depropylation, since the sex difference in the non-induced rats and 3-methylcholanthrene-induced rats were increased by the addition of 7,8-benzoflavone.

As shown in Fig. 2, we have partially purified cytochrome P-450 from untreated male and female rat liver microsomes. The activity of the cytochrome P-450 fractions to *O*-depropylate 7-propoxycoumarin was also measured. Although the recovery of the total activity was not high enough, it was shown that the *O*-depropylation activity could be reconstituted by cytochrome P-450 fractions when the purified NADPH-cytochrome *c* reductase was added, and that one or more of the cytochrome P-450 species eluted from hydroxylapatite columns by washing with 150 mM potassium phosphate containing 0.2% Emulgen 913 and 20% glycerol is responsible for the sex difference. Further purification of the cytochrome P-450 species responsible for the sex difference is now in progress.

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