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Metabolism of 4-Ethoxy-2-methyl-5-morpholino-3(2*H*)-pyridazinone (Emorfazone). V.[1] Effect of Inducer Pretreatment on Oxygenation of the Morpholino Moiety in Guinea Pigs

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The *in vivo* and *in vitro* metabolism and the cytochrome P-450 substrate-binding difference spectrum of emorfazone, 4-ethoxy-2-methyl-5-morpholino-3(2*H*)-pyridazinone, were studied in non-pretreated and in phenobarbital (PB)- and 3-methylcholanthrene(MC)-pretreated male guinea pigs.

In non-pretreated animals, emorfazone was primarily metabolized to 5-(*N*-carboxymethyl-*N*-2-hydroxyethylamino)-4-ethoxy-2-methyl-3(2*H*)-pyridazinone (M-8) and 5-[2-(carboxymethyloxy)ethylamino]-4-ethoxy-2-methyl-3(2*H*)-pyridazinone (M-9), produced by oxidative cleavage of O–C or N–C bonds in the morpholino moiety. The M-8: M-9 ratios were 10.1, 2.0 and 0.3 at doses of 20, 100 and 500 mg/kg emorfazone, respectively. In PB-pretreated animals, these ratios were 1.6, 0.7 and 0.1; in MC-pretreated animals they were >100, 16.9 and 5.8 at doses of 20, 100 and 500 mg/kg, respectively, indicating that PB pretreatment increased the production of M-9, whereas MC-pretreatment lead to the production of greater amounts of M-8.

In *in vitro* experiments, the morpholino moiety was oxidatively metabolized by microsomes in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH); metabolism involving reduced nicotinamide adenine dinucleotide (NADH) was slight. In microsomes from non-pretreated, PB- and MC-pretreated animals, respectively, the K_m values for the oxygenation of the carbon atom adjacent to the N atom were 1.1×10^{-2}, 3.9×10^{-3} and 1.2×10^{-2} M; V_{max} values were 0.483, 0.419 and 0.225 μmol/4 nmol cyt. P-450/20 min; these values for the oxygenation of the carbon atom adjacent to the O atom were 1.5×10^{-5}, 1.9×10^{-4} and 1.8×10^{-4} M and 0.065, 0.075 and 0.338 μmol/4 nmol cyt. P-450/20 min.

With increasing substrate concentrations, the apparent substrate-binding difference spectrum in microsomes from the non-pretreated group gradually changed from a reverse type I to a type I spectrum. Each binding difference spectral pattern with inducer-pretreated microsomes was different, *i.e.* the proportions of type I and reverse type I spectrum were increased in PB- and MC-pretreated microsomes, respectively.

Based on these results, it may be said that the two kinds of carbon atoms of the morpholino moiety are oxidized by two species of microsomal cytochrome P-450-dependent monooxygenation systems with different affinity and capacity, and that these oxygenation mechanisms are correlated with the substrate-binding difference spectra.

Keywords——cytochrome P-450; liver microsomes; guinea pigs; metabolism of morpholino group; substrate-binding spectrum; emorfazone; anti-inflammatory drug

Introduction

Several kinds of cytochrome P-450 have been found in liver microsomes. Some of them can be specifically induced by treatment with inducers such as phenobarbital (PB), 3-methylcholanthrene (MC) and polychlorinated biphenyls (PCBs) and a few of them have been isolated and their substrate specificities for some model compounds have been examined.[2] However, what role each cytochrome P-450 plays in the oxygenation of various compounds remains to be determined, and major questions to be answered in elucidating the mechanisms of drug metabolism by cytochrome P-450 relate to the heterogeneity and specificity of the cytochrome.

We found that emorfazone (4-ethoxy-2-methyl-5-morpholino-3(2*H*)-pyridazinone), a

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basic anti-inflammatory agent, was metabolized mainly at the morpholino moiety in six animal species and in man, and there were species differences in the metabolic patterns.[3] Furthermore, we demonstrated that in guinea pigs, the sites of oxidative cleavage of the morpholino moiety changed dose-dependently; at low doses, the O–C bond was degraded primarily, while at high doses, it was the N–C bond.[3] If these two oxidative cleavage reactions of the morpholino moiety were initiated by the microsomal production of intermediates such as carbinolamine or hemiacetal (Fig. 1), it may be speculated that different cytochrome P-450 species are involved in the two types of oxygenation.

In the present investigation, we tried to elucidate the oxygenation mechanism of the morpholino moiety of emorfazone by studying the *in vivo* and *in vitro* metabolism and drug-enzyme interaction, and by examining their correlation.

Experimental

Chemicals——Emorfazone and its metabolites were synthesized in our laboratories.[4] Emorfazone (mp 89—92°C) is highly soluble both in water and organic solvents (*e.g.* methanol, ether and chloroform). Reduced nicotinamide adenine dinucleotide phosphate (NADPH), reduced nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide phosphate (NADP), nicotinamide adenine dinucleotide (NAD) and glucose-6-phosphate (G-6-P) were purchased from Sigma Chemical Co.; PB and MC from Sankyo Pharmaceutical Co. Ltd. and Wako Chemical Industries Ltd., respectively.

Animals and Pretreatment——Male Hartley guinea pigs, weighing 400—550 g, were purchased from Shizuoka Agricultural Cooperative Association for Laboratory Animals. They received a commercial chow (CE-II, Nippon Clea) and were allowed water *ad lib.* For inducer administration, PB was dissolved in saline and injected (80 mg/kg) intraperitoneally (*i.p.*) once a day for 3 consecutive. Emorfazone treatment and subsequent experiments were started 24 h after the last injection. MC was dissolved in corn oil and injected *i.p.* once (30 mg/kg) 48 h before sacrifice. The animals were fasted overnight before the experiments.

Determination of Biliary Metabolites——The guinea pigs were anesthetized with pentobarbital (20 mg/kg; Somnopentyl®) and the common bile duct was canulated. They were given emorfazone orally (*p.o.*) (20, 100 or 500 mg/kg) and the bile was collected for the next 8 h. Determination of the metabolites has been described elsewhere.[5] In brief, neutral metabolites were extracted twice with 2 volumes of chloroform, and the residual water layer was acidified to pH 2 with 1 N HCl and concentrated. The acidic metabolites containing the carboxyl group were esterified with a diazoethane ethereal solution. Metabolites contained in each fraction were determined by gas chromatography (2% OV-17 on Chromosorb W).

Preparation of Microsomes——The animals were decapitated, and their livers were removed immediately, placed in cold 1.15% KCl solution, and homogenized in three volumes of cold 1.15% KCl solution in a Potter-Elvehjem homogenizer with a Teflon pestle. The homogenates were centrifuged at 10000 ***g*** for 20 min, and the supernatant was centrifuged twice at 105000 ***g*** for 60 min, in a Hitachi 65P preparative centrifuge. Microsome pellets were resuspended in 0.1 M potassium phosphate buffer (pH 7.4) and centrifuged at 10000 ***g*** for 20 min. All procedures were carried out below 4°C. Cytochrome P-450 was measured according to the method described by Omura and Sato,[6] using a molecular extinction coefficient of 91 $mM^{-1}\cdot cm^{-1}$; microsomal protein was determined by the method of Lowry *et al.*[7]

In Vitro* Metabolic Experiments**——Incubation mixtures contained various amounts of substrate (1×10^{-4}—1×10^{-2} M); the complete systems (final volume 2 ml) consisted of NADPH-generating system [NADP (0.65 μmol), G-6-P (10 μmol) and $MgCl_2$ (25 μmol)], NAD (2 μmol), microsomes (4 nmol of cytochrome P-450), 105000 ***g supernatant (corresponding to 125 mg of wet liver) and 0.1 M potassium phosphate buffer (pH 7.4). The mixtures were incubated aerobically at 37°C for 20 min. The reaction was stopped by adding 1 ml of 20% $ZnSO_4$ and saturated $Ba(OH)_2$ solution. A mixture consisting of 2 ml of supernatant from deproteinized solution, 5 ml of chloroform and excess sodium chloride was shaken, and 4 ml of the organic phase was taken to represent the neutral metabolite fraction. Then 1.5 ml of the water phase was acidified with 0.2 ml of 1 N HCl, and extracted as in the case of the neutral metabolite fraction. Metabolites contained in each fraction were determined by high-performance liquid chromatography (HPLC). The acidic fraction, dissolved in methanol, was directly applied to HPLC; the neutral fraction was applied after overnight reduction with $NaBH_4$ in methanol for the conversion of carbinolamine and hemiacetal intermediates to M-3 and M-2, respectively, while the carbinolamine intermediate was not present in the mixture from the complete system. An internal standard substance (4-chloro-2-methyl-5-morpholino-3(2*H*)-pyridazinone) was added and aliquots of the methanol solution were subjected to HPLC, using a μBondapak C_{18} (Waters Associates, Inc., USA) column on a Shimadzu LC 3A (Japan) machine with methanol–1% acetic acid (35: 65, v/v) as the mobile phase. The eluted metabolites were detected by absorbance measurement at 254 nm.

Difference Spectra——All difference spectra were determined at room temperature, using a Shimadzu UV 300 double-beam spectrometer. Freshly prepared microsomal suspensions (2 nmol of cytochrome

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P-450 in 1 ml) were divided into 2.5 ml portions, and 0.1 ml of emorfazone in aqueous solution was added to the microsomal suspension in the sample cuvette; an equal volume of 0.1 M phosphate buffer (pH 7.4) was added to the reference cuvette.

Results

In Vivo Metabolism

In these experiments we studied the biliary excretion of emorfazone and its major metabolites within 8 h of the *p.o.* administration of various doses to non-pretreated and PB- or MC-pretreated guinea pigs. As shown in Table I, the major metabolites were 5-(*N*-carboxymethyl-*N*-2-hydroxyethylamino)-4-ethoxy-2-methyl-3(2*H*)-pyridazinone (M-8) and 5-[2-(carboxymethyloxy)ethylamino]-4-ethoxy-2-methyl-3(2*H*)-pyridazinone (M-9). A few other metabolites were also excreted, but the total amounts were small (less than 3%).

In non-pretreated guinea pigs, there was a marked dose-dependent difference in the metabolic patterns; at 20, 100 and 500 mg/kg emorfazone, M-8 accounted for 40.2, 24.5 and 5.8%, respectively, of the dose administered. At 500 mg/kg emorfazone (high dose), M-9

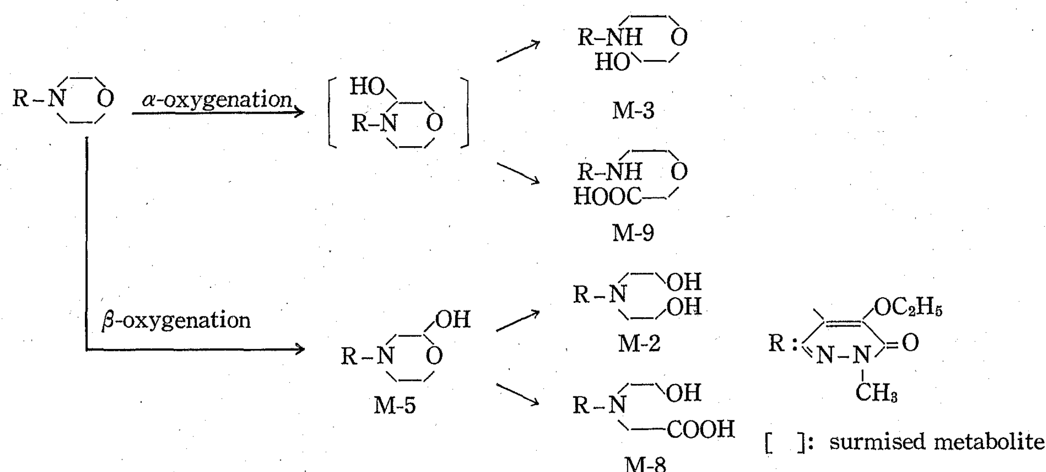

Fig. 1. Metabolism of the Morpholino Moiety of Emorfazone in Guinea Pigs

TABLE I. Biliary Excretion of Emorfazone and Major Metabolites after the Oral Administration of Various Doses of Emorfazone to Guinea Pigs

Dose (mg/kg)	20	100	500
Non-pretreated			
Unchanged	Trace	0.8±0.0	1.5±0.2
M-8	40.2±6.7	24.5±1.7	5.8±1.9
M-9	4.0±1.8	12.3±0.5	17.4±0.4
Ratio (M-8/M-9)	10.1	2.0	0.3
PB-pretreated			
Unchanged	Trace	0.6±0.1	2.0±0.3
M-8	29.7±7.2	20.9±4.2	2.2±0.2
M-9	18.7±1.7	31.5±4.2	37.4±2.6
Ratio (M-8/M-9)	1.6	0.7	0.1
MC-pretreated			
Unchanged	Trace	0.2±0.1	0.7±0.2
M-8	55.8±1.0	48.9±8.4	38.1±0.7
M-9	0.4±0.1	2.9±0.3	6.6±0.5
Ratio (M-8/M-9)	139.5	16.9	5.8

Each value represents the % excretion (mean ± S.E.) of emorfazone and its major metabolites in three to five animals within 8 h of emorfazone administration.

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excretion, 17.4%, was 3 times higher than that of M-8. In PB-pretreated animals there was a dose-dependent increase of M-9 excretion; the M-8: M-9 ratio was 1.6, 0.7 and 0.1 at 20, 100 and 500 mg/kg emorfazone, respectively. In MC-pretreated guinea pigs, M-8 was the major metabolite excreted, irrespective of the emorfazone dose. In corn oil-pretreated controls, the metabolic patterns were similar to those of non-pretreated animals.

In Vitro Metabolism

The metabolic degradation of emorfazone in *in vitro* experiments occurred mainly at the morpholino moiety; oxygenation of the carbon atom adjacent to the N atom of the morpholino moiety (α-oxygenation) and of that adjacent to the O atom (β-oxygenation) were the main metabolic pathways.

A fair amount of the initial key intermediate in β-oxygenation, 4-ethoxy-5-(2-hydroxy-tetrahydro-1,4-oxazin-4-yl)-2-methyl-3(2*H*)-pyridazinone (M-5), was produced in the presence of NADPH, but this was not the case when NADH was the coenzyme (Table II). In the presence of NADPH and supernatant, M-3, M-9 and M-8 were produced; the amount of the

Table II. Requirement for Cofactors in the Metabolism of the Morpholino Moiety of Emorfazone

Preparation	Cofactor[a]	M-5	M-8	M-3	M-9
Ms[b]	NADPH	52.7±10.7	n.d.[c]	n.d.	n.d.
Ms	NADH	3.4±1.6	n.d.	n.d.	n.d.
Ms+Supernatant[c]	NADPH	63.4±4.2	n.d.	80.7±8.3	39.7±4.0
Ms+Supernatant	NADPH+NAD	65.5±11.4	6.7±0.6	17.2±3.7	97.7±16.2

Each value represents the amount of metabolites (nmol/4 nmol of cytochrome P-450/20 min) from three experiments (mean ± S.E.).
a) 2 μmol of each cofactor was added.
b) Ms(microsomes) were prepared so as to contain 4 nmol of cytochrome P-450.
c) Supernatant (105000 *g* supernatant) was obtained from 125 mg of wet liver.
d) Not detected.

reductive cleavage product (M-3) was higher than that of oxidative cleavage product (M-9). The further addition of NAD to this mixture resulted in an increase in M-9 production and a decrease in M-3. The formation of 5-[bis(2-hydroxyethyl)amino]-4-ethoxy-2-methyl-3(2*H*)-pyridazinone (M-2) and M-8, which are generated by β-oxygenation, was small or below the detection limit under all conditions tested.

Based on these results, we performed experiments using the complete system, including the NADPH-generating system plus NAD as a cofactor, and microsomes plus 105000 ***g*** supernatant as the enzyme. Under these conditions, the amounts of M-9, M-5, M-3 and M-8 increased with prolonged incubation (Fig. 2). Other metabolites such as 2-hydroxyethylamino or carboxymethylamino derivatives resulting from biodegradation of the morpholino moiety, as well as the product demethylated at the N-2 position, were also detected, but in amounts too small for quantification.

Double reciprocal plots for microsomes from non-pretreated, PB- or MC-pretreated guinea pigs are shown in Fig. 3. K_m and V_{max} values for two oxidative enzymes were calculated in order to obtain more

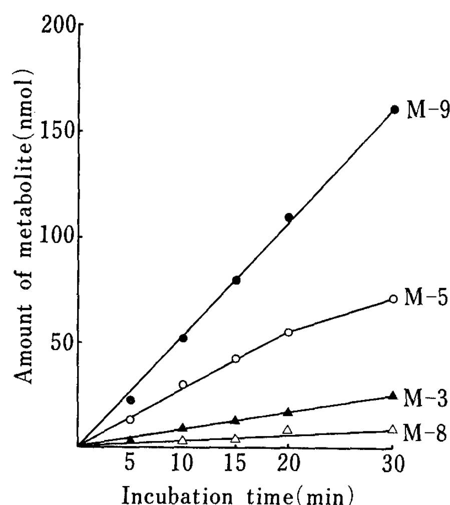

Fig. 2. Time Course of the Formation of Emorfazone Metabolites in Guinea Pigs

The incubation mixture contained microsomes (4 nmol of cytochrome P-450, 3.78 mg of protein), 105000 *g* supernatant (obtained from 125 mg of wet liver), cofactors [NADP (0.65 μmol), G-6-P (10 μmol), $MgCl_2$ (25 μmol), NAD (2 μmol)] and substrate (10 μmol) in 2 ml of 0.1 M phosphate buffer (pH 7.4). The mixture was incubated aerobically at 37 °C.

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detailed profiles of the two different oxidative reactions (Table III). K_m and V_{max} values of the two oxidative reactions were calculated from the sum of M-9 and M-3 for α-oxygenation and the sum of M-5 and M-8 for β-oxygenation.

In microsomes from non-pretreated animals, α-oxygenation predominated at high substrate concentration (*e.g.* $\beta/\alpha = 0.48$ at 1×10^{-2} M), while at concentrations below 1×10^{-3} M, β-oxygenation was predominant. After PB pretreatment, the amount of metabolites produced by β-oxygenation was similar to that generated by microsomes from non-pretreated animals; however, the amount of metabolites produced by α-oxygenation was more than twice as high. MC pretreatment produced a decrease in α-oxygenation and an approximately 5-fold increase in β-oxygenation; therefore, irrespective of the substrate concentration used, β-oxygenation was always higher in microsomes from MC-pretreated animals.

As shown in Table III, the apparent K_m values for β-oxygenation in microsomes from the 3 test groups were very similar. On the other hand, the K_m value for α-oxygenation in microsomes from PB-pretreated animals was one-third of that of non- or MC-pretreated

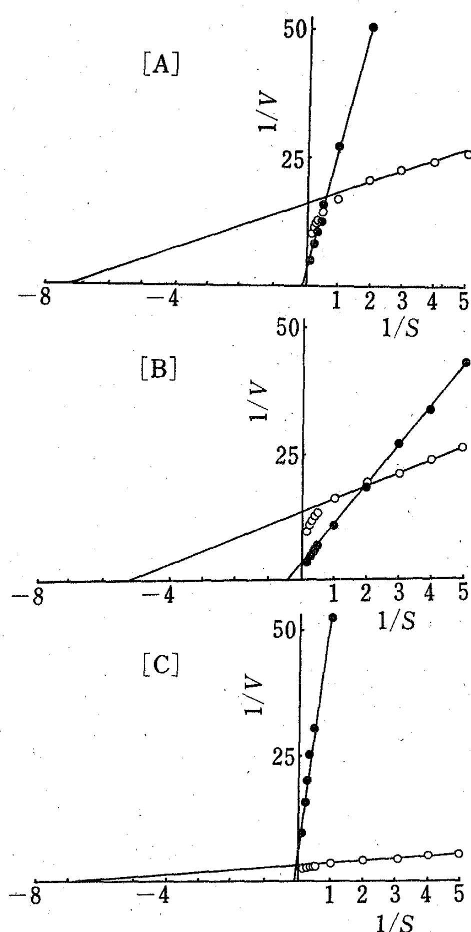

Fig. 3. Lineweaver–Burk Plots of Oxygenation Activity in the Metabolism of the Morpholino Moiety of Emorfazone by Guinea Pig Liver Microsomes

●—● : α-oxygenation,
○—○ : β-oxygenation.
Abscissa, substrate concentration (S) in mM; ordinate, metabolic activity (V) in μmol/4 nmol of cytochrome P-450/20 min. Values are the mean of four determinations. [A]: non-pretreated, [B]: phenobarbital-pretreated, [C]: 3-methylcholanthrene-pretreated.

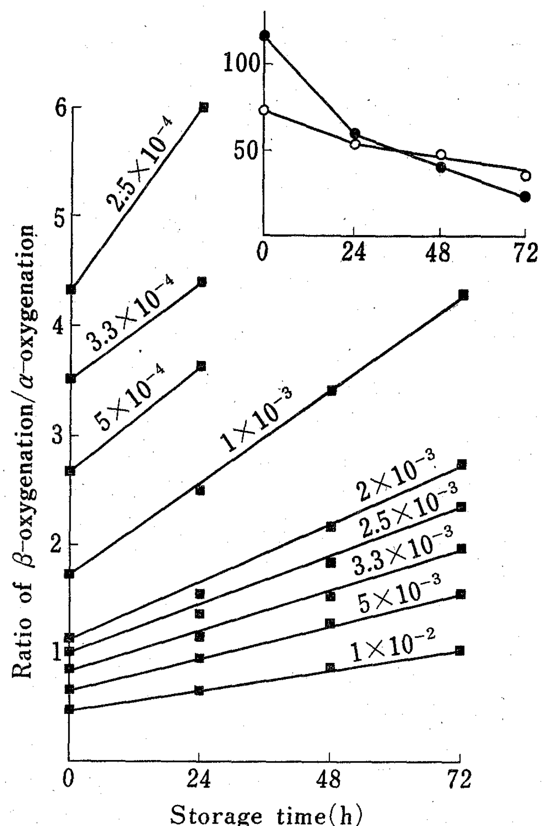

Fig. 4. Changes in the Ratio of the Two Types of Oxygenation Reaction with Storage Time of Microsomes at Various Substrate Concentrations

The insert shows the actual metabolic activity (nmol/4 nmol of cytochrome P-450/20 min) at 5×10^{-3} M substrate concentration. The incubation mixture contained 3.28 mg of microsomal protein and 4.0, 2.8, 1.7 and 1.1 nmol of cytochrome P-450 at 0, 24, 48 and 72 h, respectively. Incubation was carried out under the conditions described in the legend to Fig. 2.

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guinea pigs. This indicates that the β-oxygenation enzymes in the latter 2 groups had approximately 70 times higher affinity for substrate than the α-oxygenation enzymes; after PB pretreatment, the α/β ratio was 21. V_{max} values of α-oxygenation in non- and PB-pretreated animals were 7.4 and 5.6 times higher, respectively, than those of β-oxygenation. This indicates that the α-oxygenation enzymes were much more capable of processing substrate than the β-oxygenation enzymes in microsomes from non- and PB-pretreated animals. Conversely, in microsomes from MC-pretreated guinea pigs, β-oxygenation enzymes were more capable of substrate processing.

Figure 4 shows the correlation between the ratio of metabolic activity (β-oxygenation/α-oxygenation) at different substrate concentrations and the storage period of microsomes at 4°C. The β/α ratio increased with passage of time, irrespective of substrate concentrations.

The effects of some representative cytochrome P-450 inhibitors on the oxygenation are shown in Table IV. Although all tested agents suppressed both oxygenation reactions depending on their concentrations, the degrees of inhibition of the α- and β-oxygenation were fairly different. That is, SKF-525A and metyrapone preferentially repressed the α-oxygenation, while 7,8-benzoflavone preferentially repressed the β-oxygenation.

TABLE III. Parameters of the Oxygenation of the Morpholino Moiety of Emorfazone by Liver Microsomes from Non-pretreated, Phenobarbital-pretreated or 3-Methylcholanthrene-pretreated Guinea Pigs

	K_m (M)			V_{max} (μmol/4 nmol cyt. P-450/20 min)		
	α	β	α/β	α	β	α/β
Non	1.1×10^{-2}	1.5×10^{-4}	73	0.483	0.065	7.4
PB	3.9×10^{-3}	1.9×10^{-4}	21	0.419	0.075	5.6
MC	1.2×10^{-2}	1.8×10^{-4}	67	0.225	0.338	0.7

Each value represents the mean of three to five experiments.
α: α-oxygenation, β: β-oxygenation.

TABLE IV. Effect of Inhibitors on Oxygenation of the Morpholino Moiety of Emorfazone

Inhibitor		Oxygenation system	
		α	β
SKF-525A	0	100	100
	10^{-6} M	91 ± 3	95 ± 3
	10^{-5} M	75 ± 3	99 ± 3
	10^{-4} M	60 ± 1	90 ± 1
	10^{-3} M	28 ± 6	45 ± 6
Metyrapone	0	100	100
	10^{-6} M	69 ± 11	88 ± 8
	10^{-5} M	38 ± 4	83 ± 8
	10^{-4} M	26 ± 2	78 ± 6
7,8-Benzoflavone	0	100	100
	10^{-6} M	100 ± 3	93 ± 3
	10^{-5} M	88 ± 3	76 ± 8
	10^{-4} M	70 ± 6	38 ± 14

Each value represents the mean percent of control $\pm$ S.E. and was obtained by triplicate or quadruplicate determinations. Incubation was carried out under the conditions described in the legend to Fig. 2.

Substrate-binding Difference Spectra

Changes in difference spectra induced by the addition of emorfazone to oxidized cytochrome P-450 are illustrated in Fig. 5. Microsomes from non-pretreated animals elicited a typical

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reverse type I (RI) spectrum (λ_{max} about 317 nm) at the low substrate concentration (1×10^{-4} M), but some red shift was noted upon increasing the concentration; positive absorption was greatest at a substrate concentration of approximately 1×10^{-3} M. A further increase in substrate concentration brought about a gradual change in the spectral pattern; at 1×10^{-2} M, negative absorbance difference was seen with λ_{min} of around 425 nm. Below 400 nm it was impossible to observe the UV absorption difference peculiar to enzyme-substrate binding because of overlapping absorption by emorfazone itself.

On the other hand, the difference spectrum produced by microsomes from PB-pretreated animals could not be classified as either type I, II or RI. The spectrum showed a peak at about 415 nm and a trough at about 425 nm. The spectral pattern changed depending on the substrate concentration; the change was more pronounced at the trough than at the peak. In preliminary experiments, we had noted that the addition of sodium hexobarbital altered this strange spectrum to a typical RI spectrum. In the case of microsomes from MC-pretreated animals, the negative absorption difference seen in the microsomes from the other two test groups vanished completely and only strong positive absorbance difference at λ_{max} was observed. The spectrum showed the strongest absorbance at 1×10^{-3} M substrate concentration; when the substrate concentration was increased, the absorbance decreased and a slight blue shift was noted, while below 1×10^{-3} M, the absorbance decreased with the substrate concentration.

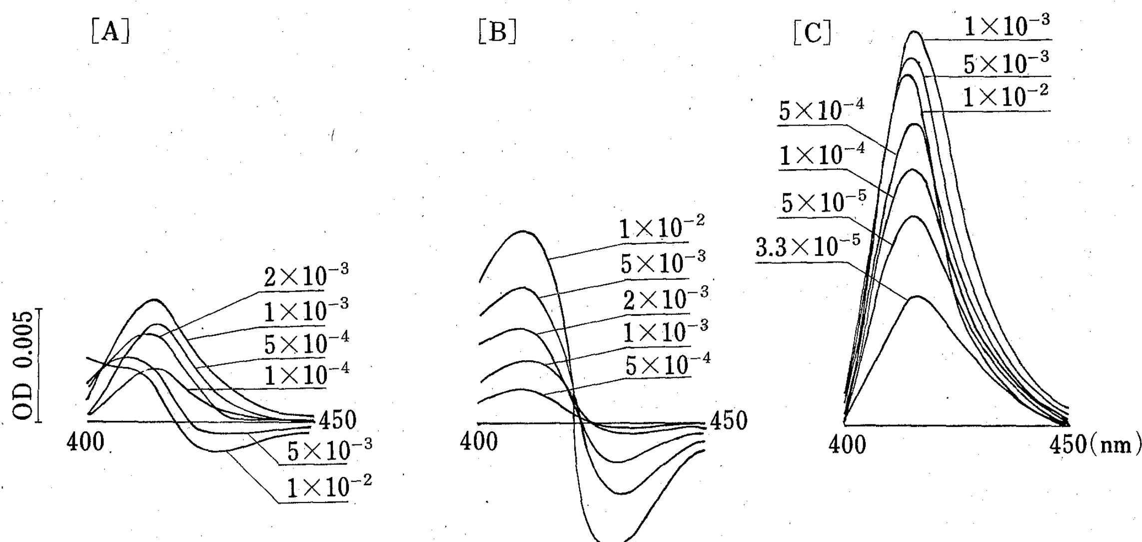

Fig. 5. Substrate Binding Difference Spectra of Emorfazone with Liver Microsomes from Non-pretreated [A], Phenobarbital-pretreated[B] and 3-Methylcholanthrene-pretreated[C] Guinea Pigs

Discussion

Recent advances in the separation, purification and characterization of various kinds of cytochrome P-450 have confirmed that cytochrome P-450 shows multiplicity, heterogeneity and various patterns of specificity toward foreign compounds. For example, Ryan[2a)] and Guengerich[2b)] have shown that purified cytochrome P-450 enzymes differ from each other in their activity to oxidize a particular compound. This suggests that different metabolic patterns are possibly due to various types of cytochrome P-450 enzymes rather than the broad spectrum of one. Therefore, the metabolic activities are not necessarily proportional to the total amount of cytochrome P-450, but more than one cytochrome P-450 species may participate in one oxygenation reaction.

In our experiments on the *in vivo* and *in vitro* metabolism of emorfazone in guinea pigs, we found that differences in emorfazone dosage and inducer pretreatment affected the metabolic pattern of the morpholino moiety of the drug. The difference in oxygenation of two kinds of

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carbon atoms in the morpholine ring suggested that, although the distance between them is small, the oxidation enzymes recognized them clearly as different sites. This phenomenon is explicable in terms of the participation of different cytochrome P-450 species.

In this study, the *in vivo* and *in vitro* metabolic oxidation patterns were very similar in non-pretreated, PB- or MC-pretreated guinea pigs. We noted that the carbon atom species (N–C or O–C) in the morpholino moiety that was oxidized depended on the kind of cytochrome P-450 involved, and differed after induction by PB or MC. Furthermore, there was a difference in the stability of these enzymes during microsome storage; the enzymes which catalyzed α-oxygenation were less stable than those which catalyzed β-oxygenation, suggesting that different cytochrome P-450 species were involved in the two types of oxygenation. We also noted that the enzymatic kinetics of the two oxygenation systems were clearly different; β-oxygenation was approximately 70 times faster than α-oxygenation in microsomes from non-pretreated and MC-pretreated animals; it was approximately 20 times faster than after PB pretreatment. The V_{max} values of α-oxygenation were larger in microsomes from non- and PB-pretreated guinea pigs while MC pretreatment produced higher values for β-oxygenation. The K_m and V_{max} values obtained in our *in vitro* experiments may explain the dose-dependent and the PB- or MC- induced changes seen in the *in vivo* metabolic patterns.

However, more detailed study of the enzymatic kinetics of β-oxygenation revealed a change in microsomal activity at high substrate concentration, and although the K_m values of the microsomes from the 3 animal groups were equal, the plots at high substrate concentrations deviated from linearity. Upon PB pretreatment, α-oxygenation was higher than in non-pretreated or MC-pretreated animals. While the mechanisms underlying this phenomenon remain to be elucidated, information regarding the cytochrome P-450 species may shed some light on the matter.

If different kinds of cytochrome P-450 are indeed involved for the two different types of oxygenation, then there may be a difference in the physicochemical properties of different cytochrome P-450 species. Others[8] have already reported that the CO binding difference spectrum of reduced cytochrome P-450 showed an absorption maximum at 450 nm in microsomes from non- and PB-pretreated guinea pigs; it was at 449.5 nm following MC pretreatment. Our results are in agreement with these data. Electoron spin resonance (ESR) data of Jefcoate *et al.*[9] indicated that low- and high-spin state cytochrome P-450 predominate in microsomes from PB- and MC-pretreated rats, respectively. Based on our *in vitro* study, it is suggested that the metabolic reactions at the two different carbon atom species in the morpholino moiety were catalyzed by different cytochrome P-450 species. If the cytochrome P-450 was in different spin states, this would be reflected in the emorfazone binding difference spectrum. The difference spectra we obtained showed different patterns, depending on the pretreatment. However, they may be composed of type I and RI spectra, because a typical pure type RI spectrum was obtained in the presence of sodium hexobarbital (2×10^{-3} M) at various substrate concentrations and in microsomes from non- or MC-pretreated animals when the substrate concentration was low. We suggest that the difference in the shape of the spectra (Fig. 5) may be attributable to differences in the contents and properties of the cytochrome P-450 involved. In microsomes from non-pretreated animals, the gradual change in the spectrum pattern may be ascribable to the clear difference in the dissociation constants (K_s) corresponding to type I and type RI spectra. Although we did not calculate the constants for each spectrum, we would expect that the dissociation constant in the case of type RI spectrum is very small while that in the case of type I spectrum is much larger.

Gradual changes in the spectral pattern with increasing substrate concentrations have been reported for four compounds, agroclavine,[10] lysergic acid-diethylamide (LSD),[11] allylneopentyl barbituric acid[12] and pleuromutilin.[13] These patterns changed from type I to type RI; the change was in a direction opposite to that observed with emorfazone.

When compared with that of microsomes from non-pretreated animals, the spectral

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change we observed after MC pretreatment can be regarded as quantitative rather than qualitative. In other words, the spectral change may be due, not to a change in K_s values, but to a change in the relative quantity ratio between type RI and I spectra, *i.e.* type RI spectrum became dominant. The strange spectral change observed upon PB pretreatment may be ascribable to the K_s value of type I spectrum being smaller than in microsomes from non- or MC-pretreated guinea pigs.

Regarding the relationship between the *in vivo* and *in vitro* metabolism and the difference spectra of emorfazone, based on the following evidence, we hypothesize that the induction of type I and type RI spectra reflects α- and β-oxygenation, respectively.

1) The increase in type I spectrum accompanied an increase in α-oxygenation, as seen in microsomes from PB-pretreated animals and in the presence of high substrate concentrations.

2) The increase in type RI spectrum accompanied an increase in β-oxygenation, as seen in microsomes from MC-pretreated animals and in the presence of low substrate concentrations.

Yoshida and Kumaoka[14] proposed that type I spectrum appears upon combination of a relative large hydrocarbon residue of the substrate with the protein part of low-spin state cytochrome P-450, and that type RI spectrum reflects a change which occurs when a hydroxyl group combines with the heme iron of high-spin state cytochrome P-450. Kumaki and Nevert[15] presented a hypothetical scheme of these binding modes. In addition, low-spin state cytochrome P-450 is induced by PB and is inhibited preferentially by metyrapone, while high-spin state cytochrome P-450 is induced by MC or 3,4-benzpyrene and is inhibited selectively by 7,8-benzoflavone.[16] These informations also support the relationship between metabolism and binding manner in this compound.

As described above, the two kinds of oxygenation in the morpholino moiety of emorfazone involve different species of cytochrome P-450 which can be differentiated clearly from the kinetic parameters, and binding of the substrate to oxidized cytochrome P-450 results in a composite curve of type I and RI spectra. Our results demonstrate the multiplicity and/or heterogeneity of cytochrome P-450 and therefore suggest that complex sites and various rates are involved in the metabolism of foreign compounds.

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