It is not certain what physiological mechanisms decrease the tempo of 5-hydroxytryptamine production in raphe neurones when the level of brain 5-hydroxytryptamine rises. The findings in this study support the hypothesis[12,13] that receptors relatively specific for 5-hydroxytryptamine are situated distal to raphe nerve endings; these receptors' neurones control, by feedback inhibition, an excessive rate of 5-hydroxytryptamine production by the raphe neurones as evidenced by moderately raised levels of 5-hydroxytryptamine at the receptors. This study further suggests that, at very high levels of 5-hydroxytryptamine, these receptors become saturated and inactivated; in this situation the inhibitory feedback control is eliminated. Experiments now in progress are designed to test the validity of this hypothesis.

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Species differences in lipid peroxidation and their effects on ethylmorphine *N*-demethylase activity in liver microsomes

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WE HAVE recently demonstrated the close inverse relationship between lipid peroxidation and activities of drug-metabolizing enzymes in liver microsomes of rats.[1] Regarding the changes in ethylmorphine *N*-demethylating activity, the activity was decreased when microsomes were incubated in the presence of ferrous ion, and was apparently increased by incubation of microsomes with inhibitors of lipid peroxidation such as EDTA, *o*-phenanthroline, α,α'-dipyridyl and Co^{2+}. The present study was initiated in order to examine further the effects of EDTA and ferrous ion on lipid peroxidation and activity of ethylmorphine *N*-demethylase in liver microsomes from various species.

Thiobarbituric acid (TBA) and ethylmorphine were purchased from commercial sources and used without further purification. Thiobarbituric acid solution (0·67%) was prepared as previously described.[1] NADP, glucose 6-phosphate-Na and glucose 6-phosphate dehydrogenase (EC 1.1.1.49, gradc I, Kontroll-Nr. 7291111) were purchased from Boehringer Mannheim (Japan) Co. Ltd.

Male rats (Sprague–Dawley strain) weighing 70–90 g, male mice of dd strain weighing 23–27·5 g, male guinea pigs weighing 320–340 g, and male albino rabbits weighing 2·25–2·61 kg were maintained on commercial chow, and were starved for about 18 hr prior to sacrifice. Three livers of rats and 12 livers of mice were pooled and used for each respective experiment. Liver microsomes of the animals were prepared by a method previously described.[1]

TABLE 1. SPECIES DIFFERENCES IN LIPID PEROXIDATION, ACTIVITY OF ETHYLMORPHINE *N*-DEMETHYLASE, CONTENT OF CYTOCHROME P-450 AND ACTIVITIES OF NADPH-LINKED CYTOCHROME C REDUCTASE AND NEOTETRAZOLIUM DIAPHORASE*

Activity	Ethylmorphine	Inhibitor or stimulator	Rat (n = 5)	Mouse (n = 5)	Guinea pig (n = 5)	Rabbit (n = 3)
Lipid peroxidation (TBA value/10 min × 10^3)	Control	Control	44·1 ± 1·4	20·6 ± 1·5	34·1 ± 2·1	3·0 ± 0·3
		+EDTA	1·6 ± 0·2†	0·9 ± 0·1†	0·6 ± 0†	0·8 ± 0·2†
		+Fe^{2+}	69·4 ± 1·7†	51·1 ± 0·9†	57·0 ± 2·0†	20·9 ± 2·1†
	+ Ethylmorphine	Control	21·9 ± 0·8	11·8 ± 0·6	16·0 ± 0·9	2·5 ± 0·4
		+EDTA	1·5 ± 0·2†	0·6 ± 0·1†	0·6 ± 0·2†	0·7 ± 0·1†
		+Fe^{2+}	63·1 ± 0·8†	40·5 ± 0·8†	32·5 ± 2·0†	14·2 ± 0·7†
Ethylmorphine *N*-demethylation (nmoles/mg protein/10 min)		Control	48·4 ± 2·6	24·4 ± 0·5	37·3 ± 2·3	21·5 ± 2·1
		+EDTA	74·9 ± 3·4†	33·5 ± 1·0†	38·4 ± 1·8	22·2 ± 2·3
		+Fe^{2+}	9·7 ± 0·6†	7·8 ± 0·1†	33·0 ± 1·4	17·9 ± 2·0
Cytochrome P-450 (nmoles/mg protein)			0·908 ± 0·016	0·694 ± 0·032	1·685 ± 0·046	1·272 ± 0·001
NADPH-cytochrome c reductase (nmoles/mg protein/min)			33·75 ± 2·04	40·50 ± 1·53	37·35 ± 2·61	31·05 ± 2·07
NADPH-neotetrazolium diaphorase (units/mg protein × 10^3)			32·0 ± 0·5	22·5 ± 0·5	18·0 ± 1·5	20·5 ± 2·0

* Experimental details are presented in the text. Values represent mean ± S.E.

† Differs significantly ($P < 0·05$) from corresponding control value.

The incubation mixture consisted of microsomes, NADPH-generating system (0·33 mM NADP, 8 mM glucose 6-phosphate, 6 mM $MgCl_2$ and 0·113 unit glucose 6-phosphate dehydrogenase) and 0·8 ml of 0·2 M Na-K phosphate buffer, pH 7·4, in a final volume of 2·5 ml. When examining the effects of ferrous ion and EDTA, ferrous ion [20 μM, as $Fe(NH_4)_2(SO_4)_2$-6 H_2O] or EDTA (0·1 mM) was added to the incubation mixture. Incubations were carried out at 37° for 30 min (in the rat, mouse and rabbit) or 20 min (in the guinea pig) aerobically. Activity of ethylmorphine *N*-demethylase was measured, using ethylmorphine (1 mM), by determining the amount of formaldehyde formed with the method of Nash.[2] Lipid peroxide was determined as previously described.[1] The formation of lipid peroxides was represented as TBA values, which were obtained by calculating $O.D._{532\ nm}$ per mg of microsomal protein added to the incubation mixture. Cytochrome P-450 was assayed essentially by the method of Omura and Sato.[3] An extinction coefficient increment of 91 $mM^{-1}\ cm^{-1}$ was used for calculation of cytochrome P-450 content from the absorbance difference between 450 and 490 nm. Enzyme activities of NADPH-linked cytochrome c reductase and neotetrazolium diaphorase were measured at 25° by the method of Williams and Kamin.[4] The microsomal protein was determined according to the method of Lowry *et al.*[5]

All experimental results are presented in Table 1. There was a marked species difference in lipid peroxidation. The most lipid peroxidation occurred in rats, followed by guinea pigs, mice and rabbits. Rabbit liver microsomes had little ability for lipid peroxidation. Addition of EDTA (0·1 mM) inhibited lipid peroxidation almost completely in all animal species. On the other hand, ferrous ion produced a significant increment in the formation of lipid peroxides. The formation of lipid peroxides in rabbit liver microsomes was increased about 7-fold, but the amount of lipid peroxides was lower than that in other animal species. Gram and Fouts[6] have demonstrated that there was little ability to form lipid peroxides in rabbit liver 9000 *g* supernatant fraction. Our results confirm that rabbit liver microsomes have an ability to form lipid peroxides, though to a lesser degree than in other animal species. Pederson and Aust[7] have recently reported that the lipid peroxidation reaction is catalyzed by NADPH-cytochrome c reductase, while Hrycay and O'Brien[8,9] have suggested that lipid peroxidation can be mediated by cytochrome P-450 or cytochrome P-420. We measured content of cytochrome P-450 and activities of NADPH-cytochrome c reductase and NADPH-neotetrazolium diaphorase, and looked for correlations between lipid peroxidation and content of cytochrome P-450, or activities of NADPH-linked cytochrome c reductase and neotetrazolium diaphorase. As shown in Table 1, we found no correlation between them. This suggests that there might be another enzyme catalyzing lipid peroxidation other than cytochrome P-450, NADPH-cytochrome c reductase and NADPH-neotetrazolium diaphorase.

As reported previously,[1,10,11] activities of drug-metabolizing enzymes are strongly affected by lipid peroxidation in liver microsomes. In the present study, we tested further the effects of lipid peroxidation on activity of ethylmorphine *N*-demethylase in various animal species. Activity of ethylmorphine *N*-demethylase was significantly increased by addition of EDTA and decreased by ferrous ion, with an inverse relation to changes in lipid peroxidation in rat and mouse liver microsomes. There were no significant effects of EDTA and ferrous ion on activity of ethylmorphine *N*-demethylase in guinea pig and rabbit liver microsomes. In rabbit liver microsomes, rather small amounts of lipid peroxides were formed even in the presence of ferrous ion; it seems reasonable that significant changes in ethylmorphine *N*-demethylase did not occur. It is difficult to explain the data in guinea pig liver microsomes, in which lipid peroxidation occurred to the same extent as in the mouse. Therefore, ethylmorphine *N*-demethylase in guinea pig liver microsomes might be stable against the lipid peroxidation-induced alterations in the microsomal membranes. Furthermore, we could observe another interesting species difference in the values of the activity of ethylmorphine *N*-demethylase per content of cytochrome P-450. The value was highest in rats, followed by mice, guinea pigs and rabbits. The tendency was observed most clearly when ethylmorphine *N*-demethylase was assayed in the presence of EDTA.

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Enzymic demethylation of a carcinogenic tryptophan metabolite, 8-methoxykynurenic acid

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8-METHOXYKYNURENIC acid is a urinary metabolite of tryptophan in humans,[1] swine[2] and monkeys.[3] Bryan *et al.*[4,5] reported that it was carcinogenic for the mouse bladder when implanted in this organ in cholesterol pellets. Systemic administration of 8-methoxykynurenic acid has also been shown to produce a significant incidence of malignant tumors of the mouse lymphoreticular system.[6] Recently, Lower *et al.*[7] investigated the fate of 8-methoxykynurenic acid *in vivo* to find out whether this compound need be metabolized to display carcinogenic activity, but its demethylation was not examined. The present paper describes some properties of this demethylating system.

Male Donryu strain rats (150–200 g) were used in all experiments. The liver or kidney was immediately removed and homogenized with 4 vol. of isotonic KCl. Microsomes were separated from the soluble fraction by centrifuging the 24,000 *g* supernatant at 100,000 *g* for 60 min. The 24,000 *g* supernatant fraction consisting of the microsomes plus soluble fraction was used mainly in this study.

Demethylase activity was determined by estimating the amount of xanthurenic acid and formaldehyde formed. The assay medium contained 15 μmoles of 8-methoxykynurenic acid, 50 μmoles of $MgCl_2$, 50 μmoles of nicotinamide, 2·4 μmoles of NADPH, 0·5 m-mole of potassium phosphate buffer, pH 7·5 and 24,000 *g* supernatant obtained from 800 mg of liver or kidney in a total volume of 8 ml. Enzyme was omitted in the blank. The incubation was carried out at 37° for 1 h with shaking. Formaldehyde was assayed with the Nash reagent.[8] Known amounts of formaldehyde carried through the incubation and assay procedures served as standards. To estimate the amount of xanthurenic acid, the reaction was stopped by adding 0·5 ml of 50% trichloroacetic acid and the mixture was filtered. Seven ml of the filtrate was adjusted to pH 8 with a 4 N KOH and applied to a column (1 × 5 cm) of Dowex 1 (formate form), which was washed with 50 ml of distilled water, 30 ml of 2 N HCOOH, 30 ml of 4 N HCOOH and eluted with 70 ml of 6 N HCOOH. The eluate with 6 N HCOOH was evaporated in vacuo and the residue was dissolved in a small volume of aqueous ammonia. The total volume was spotted on a thin layer plate (0·5 mm thick) of Avicel SF cellulose powder (Merck) and the chromatogram was developed with a mixture of ethylacetate–isopropanol–28% aqueous ammonia (9:6:4 by vol.). The region corresponding to xanthurenic acid was scrapped from the plate with a razor blade into a glass tube and extracted with 50 ml of methanol. The extract was evaporated to dryness and the residue was dissolved in 4·0 ml of 0·1 M potassium phosphate buffer, pH 7·5; the optical density at 342 nm was measured against that of the blank. For calculation of the amount of xanthurenic acid formed, the molecular extinction coefficient was taken as 6500. Known amounts of xanthurenic acid in 0·1 M potassium phosphate buffer, pH 8 were simultaneously subjected to column and thin layer chromatography.

8-Methoxykynurenic acid was incubated with the 24,000 *g* supernatant fraction obtained from 800 mg of rat liver and the reaction product was analyzed as described above. An additional spot with yellow fluorescence under ammonia gas appeared on a thin layer chromatogram besides the light blue spot of 8-methoxykynurenic acid. The product was chromatographically identical with authentic xanthurenic acid [cf. reference 2, Table 2]. If the supernatant was boiled or the reaction stopped at zero time xanthurenic acid was not detected. Negligible amounts of xanthurenic acid were formed if the microsomal or soluble fractions were used alone and if the 24,000 *g* supernatant fraction was dialyzed against 0·005 M potassium phosphate buffer, pH 7·5, no xanthurenic acid was formed. When the dialyzed microsomal and