Biochemical Pharmacology, Vol. 34, No. 7, pp. 919–924, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

RELATION BETWEEN HEPATIC MICROSOMAL METABOLISM OF N-NITROSAMINES AND CYTOCHROME P-450 SPECIES

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(*Received* 11 *May* 1984; *accepted* 13 *August* 1984)

Abstract—Effects of SKF 525A (0.1 mM), metyrapone (0.1 mM), α-naphthoflavone (ANF)‡ (0.5 mM) and pyrazole (1.0 mM) on N-nitrosodimethylamine (NDMA), N-nitrosomethylbutylamine (NMBuA) and N-nitrosomethylbenzylamine (NMBeA) metabolism by hepatic microsomes from rats pretreated with inducers were investigated. NDMA demethylation was weakly increased by phenobarbital (PB) treatment. The demethylation was inhibited by SKF 525A and enhanced by metyrapone in non-treated and PB-treated microsomes, and weakly inhibited by ANF in 3-methylcholanthrene(MC)-treated microsomes. NMBuA demethylation was increased by PB treatment and inhibited by SKF 525A in all microsomes. Metyrapone inhibited the demethylation in PB-treated microsomes. NMBuA debutylation was increased by PB and MC treatments, and inhibited by metyrapone in all microsomes. The strongest inhibition by metyrapone was observed in PB-treated microsomes. The debutylation was inhibited by SKF 525A in non-treated and PB-treated microsomes and by ANF in MC-treated microsomes. NMBeA demethylation was decreased by MC treatment and weakly inhibited by SKF 525A in all microsomes. The effects of the inducers and inhibitors on NMBeA debenzylation were almost the same as those on NMBuA debutylation except that the increasing effect of MC was small. Pyrazole was a relatively selective inhibitor of NDMA demethylation. These results suggest the following:
1. NDMA demethylation is catalyzed by PB-induced cytochrome P-450 species (P450-PB) and MC-induced cytochrome P-450 species (P448-MC). But their specific activity is low and the other cytochrome P-450 species demethylate NDMA.
2. NMBuA demethylation is catalyzed by P450-PB. But the specific activity is not high and the other cytochrome P-450 species also demethylate NMBuA.
3. NMBuA debutylation is catalyzed by P450-PB and P448-MC.
4. Almost all of NMBeA demethylation is catalyzed by cytochrome P-450 species other than P450-PB and P448-MC.
5. NMBeA debenzylation is catalyzed by P450-PB and P448-MC, but the specific activity of P448-MC is not high.

The oxidative dealkylations of N-nitrosodialkylamines by tissue-specific microsomal oxygenases are important because very active alkyl cations are formed and bind to DNA, or the other nucleophilic molecules in a body. It is considered that the dealkylations result from hydroxylations of the α-carbons by mixed-function oxidase [1]. However, the enzymology of nitrosamine metabolism is not clearly elucidated except in N-nitrosodimethylamine (NDMA) demethylation. Chau *et al.* [2] reported a kinetic study on the metabolism of NDMA, N-nitrosomethylethylamine and N-nitrosodiethylamine and Farrelly *et al.* [3–5] carried out the kinetic analysis of the metabolism of methylalkylnitrosamines. Suzuki *et al.* [6, 7] also reported the effect of inducers and inhibitors on dialkylnitrosamine metabolism and mutagenicity. But they did not discuss the relation to cytochrome P-450 species. The group of Yang investigated the enzymology of metabolism of methylalkylnitrosamines and demonstrated that rat liver microsomes contain a cytochrome P-450 isozyme, which has a high affinity for NDMA [8–11]. They also suggested that different P-450 isozymes have different specificity toward different nitrosamine substrates [12]. However, they estimated only the demethylation. Recently we estimated the dealkylations of NDMA, N-nitrosomethylbutylamine (NMBuA) and N-nitrosomethylbenzylamine (NMBeA), and found that phenobarbital (PB) and 3-methylcholanthrene (MC) treatments influence the dealkylations differently [13].

It was reported that SKF 525A and metyrapone selectively inhibits PB-induced drug metabolizing activities, and α-naphthoflavone (ANF) inhibits MC-induced drug metabolizing activities [14–19]. It

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‡ Abbreviations: PB, phenobarbital; MC, 3-methylcholanthrene; ANF, α-naphthoflavone; P450-PB, cytochrome P-450 species induced by phenobarbital; P448-MC, cytochrome P-450 species induced by 3-methylcholanthrene; NDMA, N-nitrosodimethylamine; NMBuA, N-nitrosomethylbutylamine; NMBeA, N-nitrosomethylbenzylamine.

was also reported that pyrazole is a selective inhibitor of NDMA demethylase [20]. In this report, the effects of SKF 525A, metyrapone, ANF and pyrazole on the dealkylations of NDMA, NMBuA and NMBeA in hepatic microsomes from rats treated with PB and MC were investigated, and relationships between the dealkylations and cytochrome P-450 species were discussed.

MATERIALS AND METHODS

Animals and preparation of microsomes. Male Wistar rats (10–12 weeks of age) were purchased from Shizuoka Laboratory Animal Center, Shizuoka, fed on a standard diet and received water *ad lib*. PB was dissolved in saline and given i.p. at the dose of 0.3 mmole/kg body weight at 48 and 24 hr prior to killing. MC was dissolved in corn oil and given i.p. at the dose of 0.075 mmole/kg body weight at 48 and 24 hr prior to killing. Hepatic microsomes were prepared as described previously [13].

Enzyme assays. The dealkylations of nitrosamines were assayed by previous procedures [13] with some modifications. The incubation mixture contained 0.5 mM NADP, 5.0 mM glucose 6-phosphate, 5.0 mM $MgCl_2$, 0.5 unit/ml glucose 6-phosphate dehydrogenase, 80 mM potassium phosphate buffer (pH 7.4) and 5.0 mM nitrosamines as substrates in a total volume of 1.5 ml (in the assay of NDMA and NMBeA metabolism) or 2.0 ml (in the assay of NMBuA metabolism). Semicarbazide (5.0 mM) was added in the assay of NMBuA metabolism, because butyraldehyde was further metabolized without semicarbazide. The amount of microsomal protein was about 2 mg/ml. Protein concentration in microsomes was determined by the method of Lowry *et al.* [21].

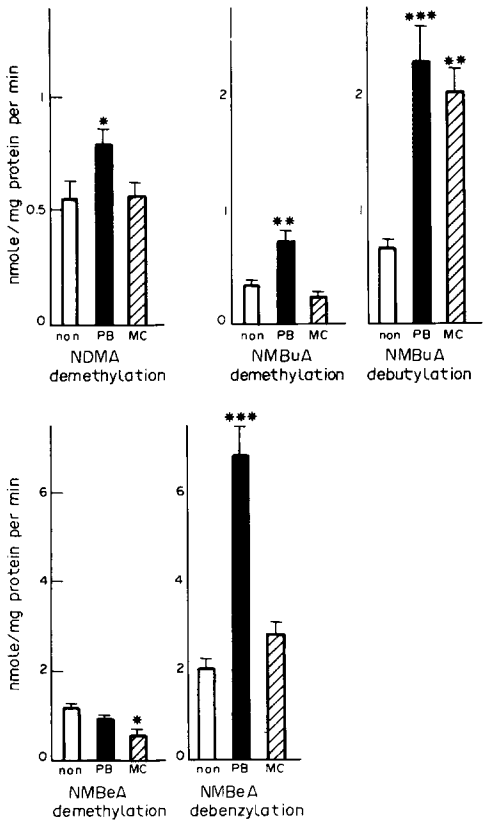

Fig. 1. Effects of PB and MC on NDMA, NMBuA and NMBeA dealkylations. Each dealkylation was calculated on the basis of mg microsomal protein. non: non-treated rats; PB: PB-treated rats; MC: MC-treated rats. Each bar represents the mean from 4 to 6 rats, and the standard errors are indicated. *Significantly different from non ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.001$).

In the assay of the demethylation of NDMA and NMBeA, the reaction was stopped by the addition of 1.5 ml of 10% trichloroacetic acid. Background samples were incubated without nitrosamines. Formaldehyde formation was estimated by the method of Nash [22].

In the assay of the dealkylation of NMBuA the reaction was stopped by the addition of 2.0 ml of 10% perchloric acid, and cyclopentanone (200 nmole) was added as an internal standard. Background samples were incubated without NMBuA. The supernatants were assayed for the presence of formaldehyde, butyraldehyde and cyclopentanone by the method of Farrelly [3] with modifications. 2,4-Dinitrophenylhydrazine (DNPH) reagent (0.9 ml of 250 mg in 100 ml 6N HCl) and isooctane (3.0 ml) were added to 3.5 ml of the supernatants. The mixtures were shaken for 30 min. The organic layers were removed and 3.0 ml of isooctane was added again. After shaking for 10 min the organic layers were removed. DNPH derivatives of formaldehyde, butyraldehyde and cyclopentanone contained in both organic layers were extracted with 2.0 ml of acetonitrile, which was evaporated to dryness. The residue was redissolved in ethyl acetate and analyzed by gas chromatography as previously described [13].

The assay of the debenzylation of NMBeA was carried out as previously described [13]. As reported in [13], part of benzaldehyde formed is reduced to benzyl alcohol by microsomal enzyme(s), and benzyl alcohol is also formed by NMBeA demethylation. Thus debenzylation activity was calculated by subtracting the formaldehyde formation from the sum of the benzaldehyde and benzyl alcohol formations.

ANF was suspended in 0.6% Tween 80, and 0.1 ml of the suspension was added. Tween 80 (0.1 ml of 0.6%) had no significant effect on the dealkylations. SKF 525A, metyrapone, ANF, and pyrazole had no effect on the recovery of formaldehyde, butyraldehyde and benzyl alcohol. They had no effect on the recovery of benzaldehyde as benzaldehyde plus benzyl alcohol, either.

Chemicals. NMBuA and NMBeA were synthesized by the method of Druckrey *et al.* [23]. NDMA, MC, pyrazole and ANF were purchased from Tokyo Kasei Co. (Tokyo, Japan). PB and metyrapone were purchased from Dai-ichi Seiyaku Co. (Tokyo, Japan) and Aldrich Chem. Co. (Milwaukee, WI), respectively. SKF 525A was generously donated by Smith Kline and French Labs (Philadelphia, PH).

Statistical analysis. For evaluation of effects of inducers, Student's *t*-test was used. The differences

between activities in the absence and presence of inhibitors were evaluated by paired *t*-test.

RESULTS

Effects of inducers

PB treatment of rats increased microsomal NDMA demethylation by 43% and MC treatment didn't change the reaction. However, the demethylation per mol cytochrome P-450 was decreased by both treatments significantly (Figs. 1 and 2). NMBuA demethylation was also increased 2.0-fold by PB treatment and not by MC treatment. However, when the activity was calculated on the basis of mol cytochrome P-450, the demethylation was not changed by PB treatment but decreased by MC treatment. NMBuA debutylation was increased 3.5- and 3.0-fold by PB and MC treatments, respectively. The debutylation was significantly increased even on the basis of mol cytochrome P-450. NMBeA demethylation was not changed by PB treatment and decreased to 50% by MC treatment. On the basis of mol cytochrome P-450 both treatments decreased the demethylation to below 50%. On the other hand, NMBeA debenzylation was increased 3.4-fold by PB treatment. The increase was significant even on the basis of mol cytochrome P-450. MC treatment also increased the debenzylation by 35%, but not significantly.

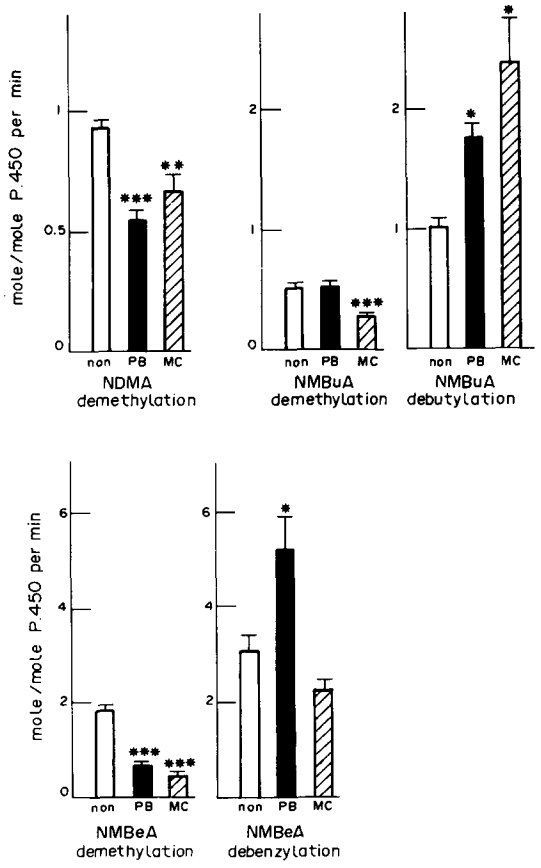

Fig. 2. Effects of PB and MC on NDMA, NMBuA and NMBeA dealkylations per mol cytochrome P-450. (See the legend to Fig. 1.)

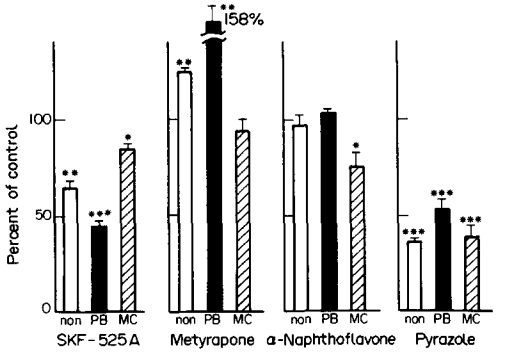

Fig. 3. Effects of inhibitors on NDMA demethylation. SKF 525A: 0.1 mM; Metyrapone: 0.1 mM; α-naphthoflavone: 0.5 mM; pyrazole 1.0 mM. Each activity was calculated as a percentage of the activity in the absence of inhibitors (controls). Each bar represents the mean from 3 to 6 rats and the standard errors are indicated. * Significant different from controls ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.001$). (See also the legend to Fig. 1 and Materials and Methods.)

Effects of inhibitors on NDMA demethylation

SKF 525A (0.1 mM) inhibited the demethylation in all microsomes. The inhibition was strong (55%) in PB-treated microsomes and weak (15%) in MC-treated microsomes. Metyrapone (0.1 mM) weakly enhanced the demethylation in non-treated microsomes (24%) as reported by Lake *et al.* [24]. The larger enhancement by metyrapone was observed in PB-treated microsomes (58%), but the enhancement was not observed in MC-treated microsomes (Fig. 3). On the other hand, the significant inhibition by ANF (0.5 mM) was observed only in MC-treated microsomes though it was weak (25%). Pyrazole (1.0 mM) inhibited the demethylation by about 50% or more in all microsomes.

Effects of inhibitors on NMBuA metabolism

SKF 525A inhibited NMBuA demethylation by about 50% in all microsomes. Metyrapone inhibited the demethylation only in PB-treated microsomes. On the other hand, the inhibition by ANF was observed in MC-treated microsomes (29%), but not significant. The inhibition by pyrazole was also observed in MC-treated microsomes (Fig. 4).

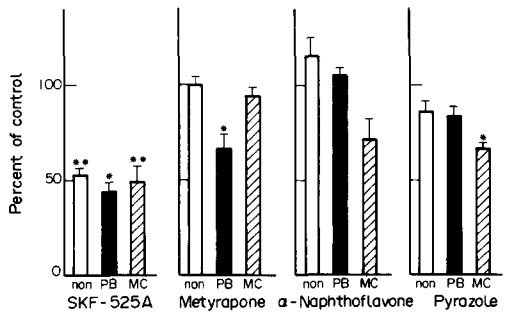

Fig. 4. Effects of inhibitors on NMBuA demethylation. Each bar represents the mean from 4 to 6 rats and the standard errors are indicated. (See also the legend to Fig. 3.)

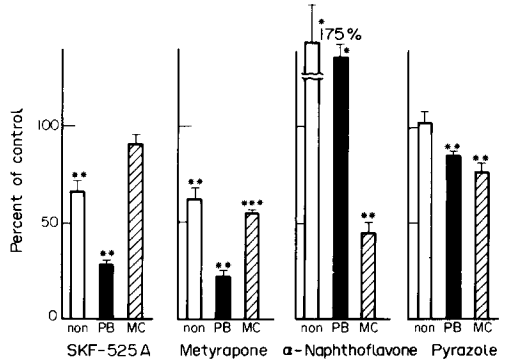

Fig. 5. Effects of inhibitors on NMBuA debutylation. (See the legend to Fig. 4.)

SKF 525A inhibited NMBuA debutylation in non-treated and PB-treated microsomes. The inhibition was especially strong (72%) in PB-treated microsomes and not observed in MC-treated microsomes. Metyrapone inhibited the debutylation in all microsomes, and the strongest inhibition was observed in PB-treated microsomes. ANF inhibited the debutylation in MC-treated microsomes (55%) and enhanced in non-treated and PB-treated microsomes. Pyrazole weakly inhibited the debutylation in PB-treated and MC-treated microsomes (Fig. 5).

Effects of inhibitors on NMBeA metabolism

No significant effect of inhibitors on NMBeA demethylation was observed, except that SKF 525A weakly inhibited the demethylation (Fig. 6).

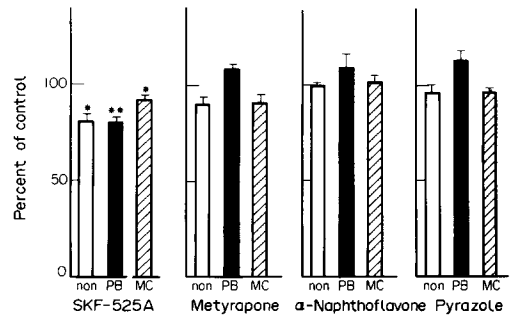

Fig. 6. Effects of inhibitors on NMBeA demethylation. (See the legend to Fig. 3.)

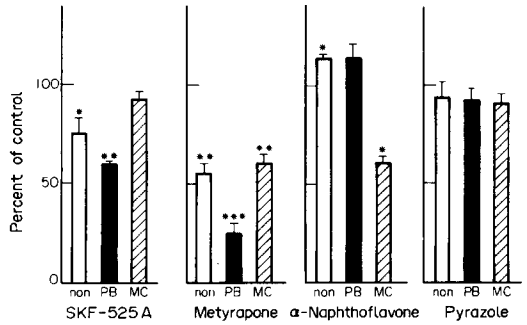

Fig. 7. Effects of inhibitors on NMBeA debenzylation. (See the legend to Fig. 3).

Table 1. Summary of results

		non	PB	MC
			+	±
NDMA demethylation	SKF 525A	↓	↓ ↓	↓
	metyrapone	↑	↑ ↑	
	ANF			↓
	pyrazole	↓ ↓	↓ ↓	↓ ↓
			+	±
NMBuA demethylation	SKF 525A	↓ ↓	↓ ↓	↓ ↓
	metyrapone		↓	
	ANF			↓ (?)
	pyrazole			↓
			++	++
NMBuA debutylation	SKF 525A	↓	↓ ↓ ↓	
	metyrapone	↓ ↓	↓ ↓ ↓	↓ ↓
	ANF	↑ ↑ ↑	↑ ↑	↓ ↓
	pyrazole		↓	↓
			±	−
NMBeA demethylation	SKF 525A	↓	↓	↓
	metyrapone			
	ANF			
	pyrazole			
			++	+(?)
NMBeA debenzylation	SKF 525A	↓	↓ ↓	
	metyrapone	↓ ↓	↓ ↓ ↓	↓ ↓
	ANF	↑	↑ (?)	↓ ↓
	pyrazole			

Effects of inducers: ++, much increased; +, increased; ±, not changed; −, decreased.

Effects of inhibitors: ↓ ↓ ↓, inhibited more than 65%; ↓ ↓, inhibited more than 35%; ↓, inhibited; ↑, enhanced; ↑ ↑, enhanced more than 35%; ↑ ↑ ↑, enhanced more than 65%.

The effects of inhibitors on NMBeA debenzylation was very similar to those on NMBuA debutylation. SKF 525A inhibited the debenzylation in non-treated and PB-treated microsomes. Metyrapone inhibited the debenzylation in all microsomes, and the strongest inhibition was observed in PB-treated microsomes. On the other hand, ANF inhibited the debenzylation by about 40% in MC-treated microsomes and very weakly enhanced in non-treated and PB-treated microsomes, though not significantly in PB-treated microsomes. Pyrazole had no effect on the debenzylation (Fig. 7).

DISCUSSION

It has been confirmed that multiple species of cytochrome P-450 are present in microsomes. Today it is reported that these cytochrome P-450 isozymes have substrate specificity though it is broad [25, 26]. Our results are summarized in Table 1, which suggests that dialkylnitrosamine dealkylations are catalyzed by several cytochrome P-450 isozymes.

Cytochrome P-450 species induced by PB (P450-PB) is assumed to be active in debutylating NMBuA

and debenzylating NMBeA because of the increase by PB treatment and the strong inhibition by SKF 525A and metyrapone in PB-treated microsomes. It is probable that cytochrome P-450 species induced by MC (P448-MC) is also active in debutylating NMBuA and debenzylating NMBeA because of the inhibition by ANF in MC-treated microsomes. However, the specific activity of P448-MC in debenzylating NMBeA does not seem to be high, because the increase by MC treatment was small. Unexpectedly, ANF enhanced NMBuA debutylation and NMBeA debenzylation in non-treated and PB-treated microsomes. The enhancing and inhibiting effects of ANF on NMBuA debutylation and NMBeA debenzylation may depend on cytochrome P-450 species as shown in benzo[*a*]pyrene hydroxylation by Huang *et al.* [27].

In this report PB treatment increased NDMA demethylase activity by 43%, but in our previous report [13] PB increased the activity by only 10%. It is probable that the poor reproducibility in experiments was caused by the multiplicity of the demethylase. It was reported that two or more enzymic forms underlie NDMA demethylase activity [1], and the kinetic analysis showed the presence of two or more Km values for the demethylation. The high Km (more than 20 mM) demethylation was reported to be induced by PB and MC treatments [28, 29], and P450-PB and P448-MB were very active in demethylating high concentrations of NDMA [30, 31]. On the other hand, the low Km (below 2 mM) demethylation was considered to be dependent on cytochrome P-450 species other than P450-PB and P448-MC [8–12, 32], though the participation of a monoamine oxidase [EC 1.4.3.4] type enzyme was suggested [33, 34]. The low Km demethylase activity was not changed or reduced by PB and MC treatments [28, 29, 32]. It was inhibited by pyrazole and enhanced by metyrapone [24, 32, 35]. Our present results suggest that 5 mM NDMA is demethylated by both the high Km demethylase, which is at least in part dependent on P450-PB, and the low Km demethylase. In addition, ANF inhibited the NDMA demethylation in MC-treated microsomes, though MC treatment did not increase it. Thus there is a possibility that the low Km demethylase are decreased by MC and in MC-treated microsomes P448-MC partly demethylates NDMA as suggested in our previous paper [32]. But the specific activities of P450-PB and P448-MC seem to be low, because the demethylation per mol cytochrome P-450 was decreased by PB and MC treatments.

It is probable that P450-PB is also active in demethylating NMBuA because of the increase by PB treatment and the inhibition by SKF 525A. However, the specific activity seems to be low and NMBuA demethylation may be also dependent on cytochrome P-450 species other than P450-PB, because the demethylation per mol cytochrome P-450 wasn't increased by PB treatment and metyrapone had no effect on the demethylation in non-treated microsomes. In addition, ANF inhibited NMBuA demethylation in MC-treated microsomes though not significantly. Thus P450-MC might be also active in demethylating NMBuA, though the specific activity is very low.

P450-PB and P448-MC seem to take only a small part in NMBeA demethylation, because the effects of the inhibitors were very small. We cannot deny the participation of enzyme(s) not depending on cytochrome P-450, but it is considered that NMBeA demethylation mainly depends on cytochrome P-450 from the work of the group of Yang [8, 10–12]. Thus it is probable that almost all of the demethylation is catalyzed by cytochrome P-450 species other than P450-PB and P448-MC. However, Tu *et al.* [12] reported that purified cytochrome P-450 from PB-treated rats had high NMBeA demethylase activity. Because their study was carried out using Sprague-Dawley rats, the difference between our results and theirs might be due to the strain difference. Further studies will be required to resolve the discrepancy.

Pyrazole was reported to be an inhibitor of NDMA demethylation [20, 29, 35, 36]. From our results that pyrazole was not a potent inhibitor of NMBuA and NMBeA metabolism, the compound is assumed to be a relatively selective inhibitor of NDMA metabolism, though the mechanism of inhibition is unknown.

Carcinogenic N-nitrosamines exhibit tissue specificity [23, 37]: NDMA is a potent hepatic carcinogen; NMBuA is a bladder, esophageal and hepatic carcinogen; NMBeA is a potent esophageal carcinogen. It is considered that their organ specificity is caused in part by the metabolic activation in target organs. Schweinsberg and Kourus [38] and Labuc and Archer [39] found that rat esophageal mucosa has the high NMBeA debenzylase activity, and suggested that the enzyme(s) play(s) a role in determining the specificity. Our results suggest that several cytochrome P-450 species are active in dealkylating nitrosamines and support each dealkylase activity. Thus there is a possibility that organ specific carcinogenicity of these nitrosamines is changed by various inducers.

Acknowledgements—We are indebted to Dr. M. Nakadate in our institute for his help in synthesizing NMBuA and NMBeA. We also thank Miss R. Konno for her skillful technical assistance. This research was in part supported by a Grant-in-Aid for Research from the Ministry of Education, Science and Culture of Japan.

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