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SHORT COMMUNICATIONS

Comparative study on the metabolism of *N*-methyl-4-aminoazobenzene by two forms of cytochrome P-488*

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From the intimate correlation between the rate of the N-hydroxylation of *N*-methyl-4-aminoazobenzene (MAB) derivatives and their carcinogenic activities in rats, the N-hydroxylation of MAB is regarded as the most important step in carcinogenesis [1]. Concerning the enzyme involved in the N-hydroxylation of MAB, Kadlubar *et al.* [2] reported that, with microsomes from non-treated rats, flavin-containing monooxygenase (FMO) is involved in the N-hydroxylation of MAB. Recently, we found that this is true for microsomes from non-treated rats and phenobarbital-treated rats, but not for microsomes from 3-methylcholanthrene (MC)-treated rats (MC microsomes) [3, 4]. In the latter case, both FMO and cytochrome P-448 (cyt. P-488) are involved in the N-hydroxylation of MAB. It has been reported that at least two forms of cyt. P-488 are induced by MC treatment, i.e. P-488-H and P-448-L [5], which are largely in high and low spin states respectively. The cyt. P-448 used in our previous experiments is assumed to be the same as that described as P-448-L by Kamataki *et al.* [5] and cyt. P-448$_c$ by Thomas *et al.* [6]. On the other hand, P-448-H is the same as cyt. P-450$_d$ [5, 6].

It has been reported that the N-hydroxylation of primary amines such as 2-aminofluorene and aminobiphenyl is catalyzed mainly by P-448-H [5]. On the other hand, it has remained obscure which one of these forms of cyt. P-448 (P-448-H and P-448-L) is involved mainly in the N-hydroxylation of secondary amines. Using reconstituted cyt. P-448, we attempted to clarify the contribution of each form of cyt. P-448 in the N-hydroxylation of MAB and found that the N-hydroxylation of MAB was catalyzed mainly by P-448-H. This is the first report indicating that the N-hydroxylation of secondary amines is catalyzed by P-448-H.

Materials and methods

Preparation of microsomes. Male Sprague–Dawley rats were injected intraperitoneally with MC (20 mg/kg) 24 hr before being killed or with PCB (Kaneclor 500; 500 mg/kg) 7 days before being killed. Liver microsomes were obtained by differential centrifugation. Purification of P-488-L and P-448-H was reported previously [5].

Assay methods. The incubation mixture in a total volume of 1 ml, containing 0.2 nmole cyt. P-448, 0.6 units NADPH-cyt. P-450 reductase, 75 nmoles dilauroyl phosphatidylcholine, 1 mM NADPH, 0.2 mM [^{14}C]MAB and 50 mM Tris–HCl buffer (pH 7.5), was incubated at 37° for 10 min. The metabolites of MAB were extracted three times with benzene, and the extract was quickly evaporated under nitrogen. The residue was dissolved in 0.1 ml of methanol, and 90 μl of the methanol solution of the metabolites was applied to a reverse phase column (Nucleosil 5C$_{18}$, 4.8 × 250 mm). After being held for 1 min in 55% methanol in water, the metabolites were eluted with a linear gradient from 55% methanol to 90% methanol over a sweep time of 32 min at a flow rate of 0.8 ml/min. The eluate was monitored at 254 nm, and the fractions were collected directly into scintillation vials at 30-sec intervals. All the solvents for high performance liquid chromatography (HPLC) were completely deaerated before use. Fractions of HPLC were analyzed by means of a gas chromatograph (Varian model 3700)–electron–impact mass spectrometer (Varian MAT 44S) combination (GC-MS), equipped with a capillary column (25 m) coated with SE 54. Samples were preheated at 100–150° and examined at either 60° or 90°. Peaks d, e, g and h in HPLC were identified by the cochromatography of standard samples as well as by their mass spectra. The remaining peak, a, b, or c, was tentatively assigned by mass spectra (M^+ as well as fragmentation pattern) as one of reported metabolites of MAB, e.g. 4′-OH-MAB, 4′-OH-AB and 3-OH-AB [7]. The assignment of peak f is discussed in the text.

Results and discussion

In Fig. 1, the HPLC elution pattern of the metabolites of MAB is depicted. We previously could not identify peak f (retention time 30.5 min), because of the low content of the metabolite [4]. However, by using P-448-H the amount of the metabolite increased enough to identify it. Thus, by means of mass spectrometry, the molecular ion peaks at *m/z* 225, 148, 120, 105 and 77 were detected at a mass temperature of 60° (Fig. 2). At higher mass temperature (90°), small amounts of the molecular ion peaks at *m/z* 227, 150, 122, 105 and 77 were recognized. These values correspond well to the standard molecular fragments of MAB and AB as formed by cleavage in Fig. 3. In the metabolic pathways of MAB (Fig. 3) established by Kadlubar *et al.* [2], nitron was considered as too labile to detect directly. However, more direct evidence has been presented by Degawa and Hashimoto [8]. In the alkaline treatment of N-OH-MAB, they observed the formation of 4-nitroazobenzene and 4,4-bisphenylazo-azoxybenzene at a ratio of 2:1. The formation of the latter compound predominated when N-OH-AB was used as a starting material, while the formation of 4-nitroazobenzene predominated when formaldehyde and N-OH-AB were treated. This is best explained by the reaction scheme via nitron, consistent with the well-established case of phenylhydroxylamine [8]. In their study, the mass spectrum of N-OH-MAB (M^+ 227) showed a peak (M^+-2, 225) which corresponded to the molecular weight of nitron. Thus, it is reasonable to assume that a major part of the metabolite of peak f derives from nitron. The small amount of the molecular ion peaks at 90° cannot be identified unambiguously. One possible candidate is 4-nitroazobenzene, an oxidation product of nitron, and another is 3-OH-MAB, which has never been detected in HPLC among ring-hydroxylated metabolites of MAB hitherto reported.

With P-448-H, all the metabolites, 4′-hydroxy-4-aminoazobenzene (4′-OH-AB), 3-hydroxy-4-aminoazobenzene (3-OH-AB), 4′-hydroxy-*N*-methyl-4-aminoazobenzene (4′-OH-MAB), *N*-hydroxy-4-aminoazobenzene (N-OH-AB), 4-aminoazobenzene (AB), *N*-hydroxy-*N*-methyl-4-aminoazobenzene (N-OH-MAB) and nitron were detected (Fig. 1A), whereas N-OH-AB was scarcely detected with P-448-L (Fig. 1B). This may be due to the far smaller rate of N-hydroxylation of primary amines with P-448-L [5]. The rates of formation of the metabolites with two forms

* Cytochrome P-448 is one of the species of cytochrome which is induced by MC-treatment and has the absorption maximum at 448 nm.

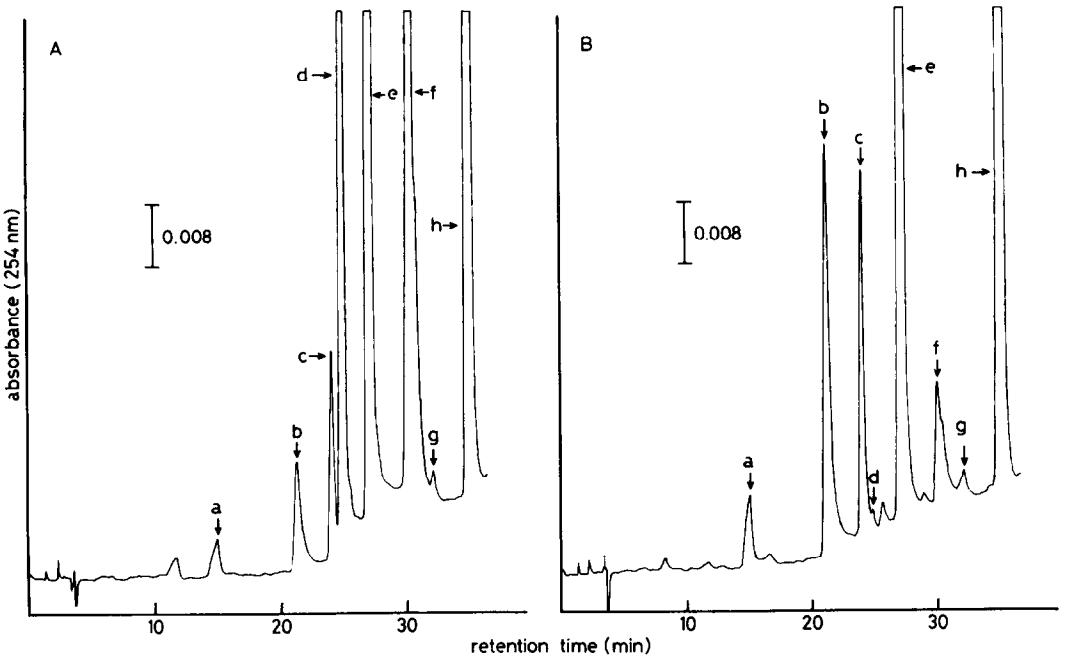

Fig. 1. HPLC elution patterns of the metabolites with P-448-H (A) and P-448-L (B). Key: (a) 4′-OH-AB, (b) 3-OH-AB, (c) 4′-OH-MAB, (d) N-OH-AB, (e) AB, (f) nitron, (g) N-OH-MAB, and (h) MAB.

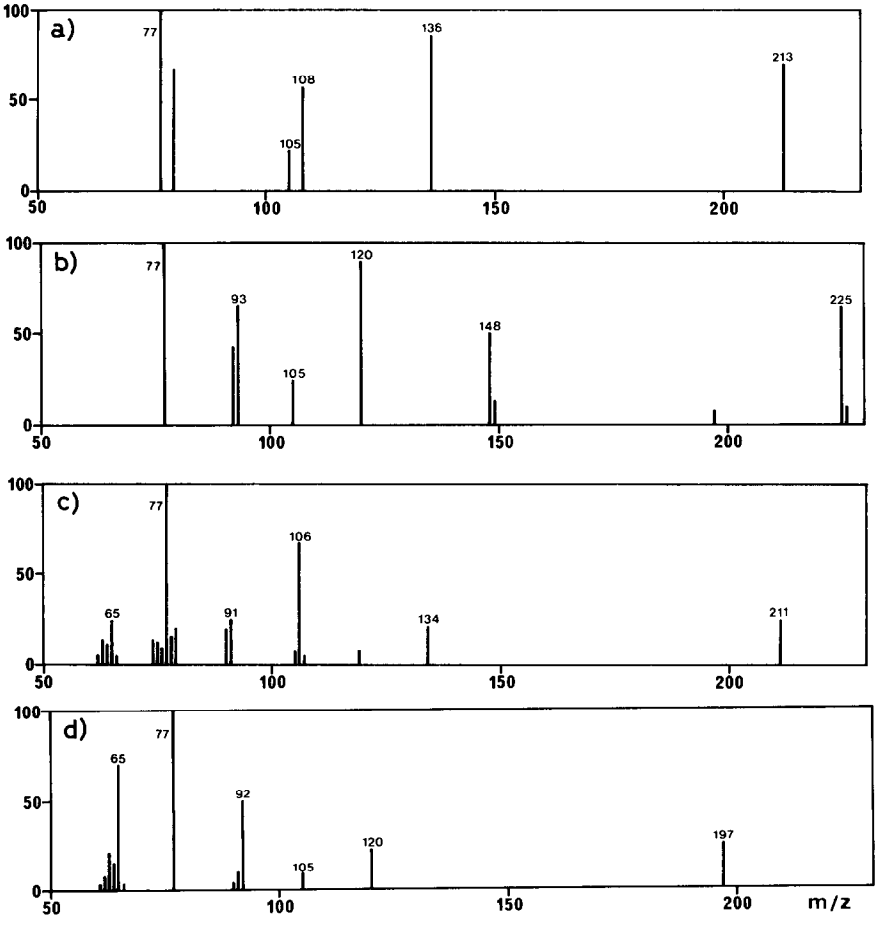

Fig. 2. Mass spectra of peak b (a), peak f (b), MAB (c), and AB (d).

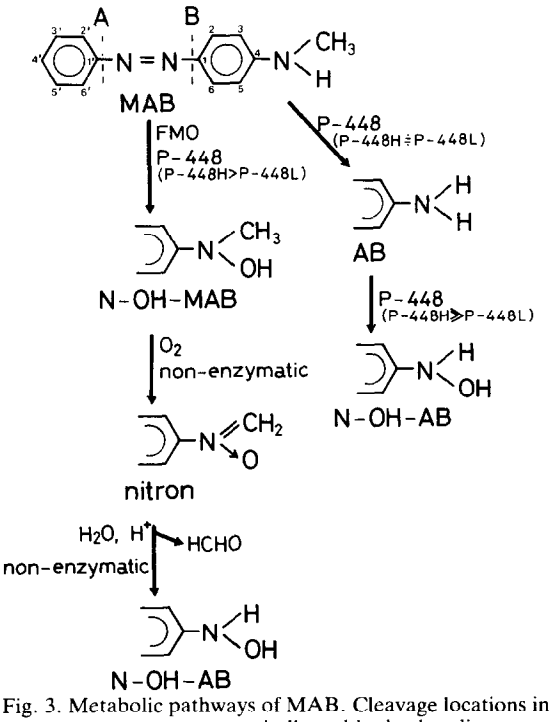

Fig. 3. Metabolic pathways of MAB. Cleavage locations in mass spectrometry are indicated by broken lines.

of cyt. P-448 are given in Table 1. The formation of N-OH-MAB and the increased formation of nitron, a degradation product of N-OH-MAB, were remarkable in the case of P-448-H compared with that of P-448-L, reflecting their N-hydroxylating activities. In the MC microsomes, at the protein level $P\text{-}450_d$ (the same as P-448-H) was only one-third of $P\text{-}450_c$ (the same as P-448-L) [6], whereas N-hydroxylating activity with P-448-H was several times as large as that with P-448-L. Thus, it can be concluded that in the N-hydroxylation of MAB the contribution of P-448-H is larger than that of P-448-L. N-OH-AB was detected specifically with P-448-H, while the rate of formation of AB from MAB was similar with the two forms of cyt. P-448. The ring hydroxylations, i.e. the formations of 4′-OH-MAB, 4′-OH-AB and 3-OH-AB, were 1.7 to 3 times higher with P-448-L than with P-448-H.

In summary, the contribution of P-448-H was large in the N-hydroxylation of MAB in contrast to the ring hydroxylation which was more efficiently catalyzed by P-448-L. These results suggest the important role of P-448-H in the metabolic activation of MAB in MC microsomes, although MC treatment *in vivo* not only enhances N-hydroxylation to initiate carcinogenesis but also accelerates other detoxification processes to inhibit carcinogenesis.

Table 1. Rate of formation of the metabolites of MAB with P-448-H and P-448-L

	Metabolites formed (nmoles/nmole cyt. P-448/min)	
	P-448-H	P-448-L
4′-OH-AB	0.201 ± 0.21	0.337 ± 0.025
3-OH-AB	0.641 ± 0.066	2.075 ± 0.109
4′-OH-MAB	0.735 ± 0.033	1.267 ± 0.061
N-OH-AB	7.065 ± 1.148	< 0.020
AB	17.532 ± 0.801	18.053 ± 0.444
Nitron	6.702 ± 0.433	1.059 ± 0.063
N-OH-MAB	0.830 ± 0.180	0.382 ± 0.039

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REFERENCES

1. T. Kimura, M. Kodama and C. Nagata, *Carcinogenesis* **3**, 1393 (1982).
2. F. F. Kadlubar, J. A. Miller and E. C. Miller, *Cancer Res.* **36**, 1196 (1976).
3. T. Kimura, M. Kodama and C. Nagata, *Gann* **73**, 55 (1982).
4. T. Kimura, M. Kodama and C. Nagata, *Gann* **75**, 895 (1984).
5. T. Kamataki, K. Maeda, Y. Yamazoe, N. Matsuda, K. Ishii and R. Kato, *Molec. Pharmac.* **24**, 146 (1983).
6. P. E. Thomas, L. M. Reik, D. E. Ryan and W. Levin, *J. biol. Chem.* **258**, 4590 (1983).
7. J. C. Arcos and M. F. Argus, in *Chemical Induction of Cancer*, Vol. IIB, pp. 233–7. Academic Press, New York (1974).
8. M. Degawa and Y. Hashimoto, *Chem. pharm. Bull. Tokyo* **24**, 1485 (1976).

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Transport of $^{42}K^+$, $^{201}Tl^+$ and $[^{99m}Tc(dmpe)_2 \cdot Cl_2]^+$ by neonatal rat myocyte cultures

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Interest in the mechanism of transport of monovalent cations has increased due to the widespread use of $^{201}Tl^+$ for the detection of myocardial infarction and ischemia by myocardial perfusion imaging [1]. Utilizing human erythrocytes, Skulskii *et al.* [2] indicated that the (Na^+, K^+)ATPase (EC 3.6.1.3) recognizes both K^+ and Tl^+ and that ion transport differed only in a greater affinity for Tl^+ than K^+. It has also been shown that Tl^+ is interchangeable with K^+ in stimulating Na^+ efflux from erythrocytes [2]. Based on these types of results, it is generally accepted that Tl^+ acts physiologically as a K^+ analog [3]. The utility of $^{201}Tl^+$ for myocardial imaging is limited by its high cost,