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Biochemical Pharmacology, Vol. 32, No. 4, pp. 735–738, 1983.
Printed in Great Britain.

0006-2952/83/040735-04 $03.00/0

Metabolic activation of 2,4-xylidine and its mutagenic metabolite

(*Received* 19 *April* 1982; *accepted* 27 *August* 1982)

Xylidines (dimethylanilines) are metabolic products of certain food dyes, cosmetics and pharmaceuticals [1, 2], and their hepatotoxic effects in dogs, rats and mice [2, 3] have been reported. Among their isomers, 2,4-xylidine showed the most marked toxic effect on the liver of rats [4] and is known as a reductive product of the azo dye, Ponceau R, which is tumorigenic in rats and mice [5, 6]. Although the metabolism of 2,4-xylidine in rats has been studied earlier by Lindstrom [7], the relationship between the specific metabolite formation and the induction of toxicity has not been sufficiently established.

Recently, we and other workers found that 2,4-xylidine was mutagenic in *Salmonella typhimurium* TA100 after metabolic activation by polychlorinated biphenyl (PCB)-treated rat liver 9000 *g* supernatant fraction [8, *]. This finding suggests that 2,4-xylidine is converted to a mutagenic metabolite which is capable of inducing mutation without further activation. We isolated the metabolites of 2,4-xylidine by using high performance liquid chromatography (HPLC) and tested their mutagenicity with a bacterial mutation assay. It has been reported that the HPLC analysis is the most reliable method for separation of the metabolites that are thought to be present at low concentrations and are susceptible to air oxidation [9, 10]. Concomitantly, the bacterial mutation test provides a simple and very sensitive method for detecting such small amounts of metabolites [11]. In this paper, we report the structure of the mutagenic metabolite of 2,4-xylidine and discuss the relationship between the formation of mutagenic metabolite and the enzyme-mediated mutagenicity of 2,4-xylidine.

Materials and methods

Materials. 2,4-Xylidine was purchased from Wako Pure Chemicals, Osaka, Japan. 2,4-Xylidine was purified by distillation (b.p. 101°/20 mm Hg), and the purity was checked by HPLC with acetonitrile–water (3:7) as described in the legend to Fig. 1. Authentic samples of 2,4-dimethylphenylhydroxylamine and 2,4,2′,4′-tetra-

* K. Yoshikawa *et al.*, unpublished observations.

methylazoxybenzene were synthesized by the reduction of 2,4-dimethylnitrobenzene with zinc dust and by the condensation of 2,4-dimethylphenylhydroxylamine and 2,4-dimethylnitrosobenzene respectively [12, 13]. S. *typhimurium* TA100 was provided by Dr. B. N. Ames of the University of California (U.S.A.). Male Fischer rats were obtained from the Charles River Japan Co., Kanagawa, Japan.

Preparation of enzymes. Male Fischer rats (100–120 g) were pretreated with PCBs (Kanechlor KC-400, 500 mg/kg in olive oil), i.p., 5 days before being killed; controls received olive oil alone. All animals were starved for 18 hr before they were killed. S-9 fractions were prepared from PCB-treated and untreated rats as described elsewhere [14].

Detection of the mutagenic metabolite of 2,4-xylidine. The S-9 mixture (PCB-pretreated rat liver S-9 plus cofactors) contained 5 mM G-6-P, 4 mM NADPH, 4 mM NADH, 33 mM KCl, 8 mM $MgCl_2$, 100 mM phosphate buffer (pH 7.4) and 3.75 ml S-9 (129 mg protein) in a total volume of 12.5 ml [15]. The incubation was started immediately after the addition of 200 μmoles of 2,4-xylidine, shaking (100 strokes/min) at 37° for 30 min. After the incubation, the mixture was extracted twice with 25 ml of dichloromethane. The pooled extracts were evaporated to dryness under a stream of nitrogen gas, and the residue was dissolved into 100 μl of acetonitrile. An aliquot (20 μl) of the sample was analyzed by HPLC, as described in the legend to Fig. 1. For the mutagenicity test, each fraction separated by HPLC was placed in centrifuge tubes (50 ml) and extracted with 20 ml of dichloromethane. The extracts were blown dry with nitrogen gas. The residues were redissolved into 200 μl of dimethyl sulfoxide (DMSO) and 100-, 50- and 25-μl portions of the solution were used for the mutation assay with *S. typhimurium* TA100. The assay was carried out without metabolic activation according to the method of Ames *et al.* [11].

Quantitative analysis of 2,4-dimethylphenylhydroxylamine formed. Authentic 2,4-dimethylphenylhydroxylamine was quantitatively analyzed by preparing a fresh 4×10^{-3} M solution in deoxygenated acetonitrile followed by immediate dilution and chromatographic analysis. The method of HPLC analysis was the same as described in the legend to Fig. 1. A linear relationship was found between peak heights and concentrations of authentic 2,4-dimethylphenylhydroxylamine, at least in the concentration range of 1×10^{-4} to 4×10^{-3} M.

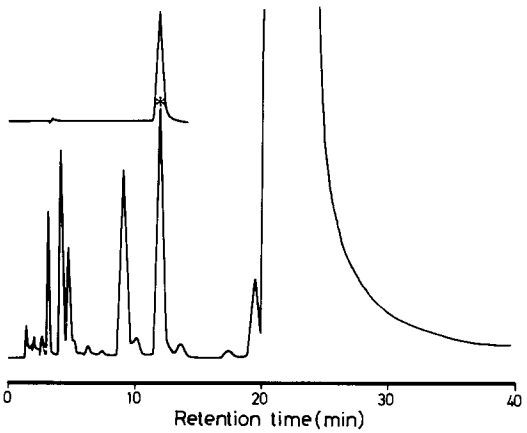

Fig. 1. Chromatogram of 2,4-dimethylphenylhydroxylamine (upper left) and 2,4-xylidine metabolites by reverse-phase chromatography. A high pressure liquid chromatogram (model HLC-803 A, Toyo Soda Manufacturing Co., Ltd., Tokyo, Japan) equipped with an ultraviolet absorbance detector operated at 240 nm, Altex model 420 microprocessor controller/programmer (Altex Scientific Inc., CA, U.S.A.) and a reverse-phase column, Toyo Soda TSK LS-410 (4 mm i.d. × 300 mm), were used. A mobile phase of acetonitrile–0.25 mM sodium phosphate (dibasic) solution (3:7) was used at a flow rate of 1.0 ml/min. The mobile phase solution was deoxygenated by sonications. The peak with an asterisk indicates the fraction which showed the mutagenicity toward *S. typhimurium* TA100 cells without activation.

The extraction efficiencies were also determined in this analytical system. An amount (50 nmoles) of 2,4-dimethylphenylhydroxylamine was added to 12.5 ml of S-9 mixture and immediately extracted with 25 ml of dichloromethane. The extracts were concentrated and dissolved in 100 μl of acetonitrile as described above. An aliquot (20 μl) of the sample was injected into HPLC, and the amount of the recovered 2,4-dimethylphenylhydroxylamine was determined. The average percent of recovery of 2,4-dimethylphenylhydroxylamine during extraction procedure was 60%.

The amount of 2,4-dimethylphenylhydroxylamine, the *N*-hydroxylated metabolite of 2,4-xylidine, formed was measured as follows. A reaction mixture containing S-9 mixture (12.5 ml) and 2,4-xylidine (200 μmoles) was incubated, extracted, and concentrated to dryness as described above. The residue was dissolved in 200 μl of acetonitrile, and 20 μl of the sample was analyzed by HPLC as described in the legend to Fig. 1. The amount of 2,4-dimethylphenylhydroxylamine formed was measured by comparing the peak height to that of standard amounts of the authentic sample. Two separate determinations were made, and the mean value was calculated after correction for a recovery of 60%.

Mass spectrometry. A reaction mixture containing S-9 mixture (100 ml) and 2,4-xylidine (1.6 mmoles) was incubated, extracted (twice with 200 ml of dichloromethane), and concentrated to dryness as described above. The residue was dissolved in 800 μl of acetonitrile, and 100-μl samples were analyzed by HPLC. The fraction showing a retention time of 12.2 min was obtained. After extracting with 20 ml of dichloromethane, the extract was concentrated and dried. The residue was dissolved into a small quantity of acetonitrile (5 μl). The liquid sample was analyzed by mass spectrometry (Nihondenshi o1SG-2 type, e.i. mode, electron energy 70 eV, ionizing current 250 μA) according to the method of Kanohta *et al.* [16]. Mass spectra of authentic samples of both 2,4-dimethylphenylhydroxylamine and 2,4,2',4'-tetramethylazoxybenzene were also obtained.

Quantitative mutation assay. An overnight culture of *S. typhimurium* TA100 was washed, resuspended in 1/15 M phosphate buffer, pH 7.4 ($3–5 \times 10^8$ cells/ml), and set aside for quantitative mutation assay (induced-mutation frequency method) [17]. An aliquot (0.1 ml) of bacterial suspension was added to the assay mixture containing 0.1 ml of the chemical solution and 1.0 ml of S-9 mixture (or the buffer) and incubated at 37° for 30 min with shaking. Both 2,4-xylidine and 2,4-dimethylphenylhydroxylamine were dissolved in DMSO. For the mutation assay, aliquots (1.0 ml) of the mixture were centrifuged, and the collected cells were mixed with 2 ml of soft agar (0.45% agar and 0.6% NaCl) and then poured onto minimal agar plates each containing L-histidine and D-biotin. The number of His^+ colonies (M) was scored after incubation at 37° for 2 days. To measure the number of surviving cells, aliquots (0.1 ml) of the mixtures were diluted with the buffer to various concentrations and then poured into the semi-enriched agar plates [18]. The number of surviving cells (N) was scored after overnight incubation at 37°. The induced mutation frequency was calculated as the value of $(M - M_0)/N$, where the symbols with and without the subscript "0" stand for the values in the untreated and the chemical-treated cell respectively.

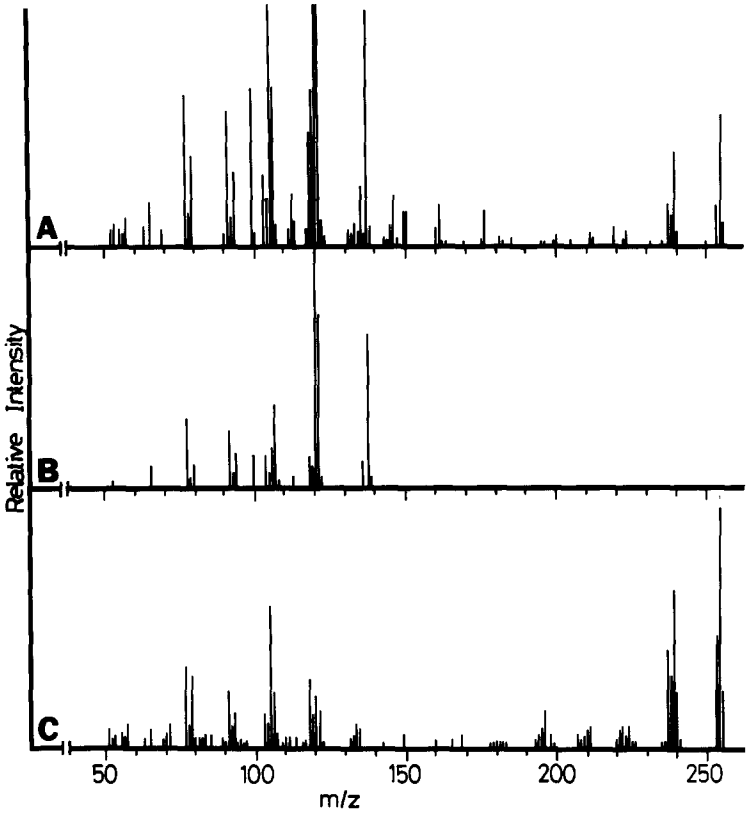

Fig. 2. Comparative mass spectra of mutagenic metabolite of 2,4-xylidine (A), authentic 2,4-dimethylphenylhydroxylamine (B), and authentic 2,4,2′,4′-tetramethylazoxybenzene (C).

Results and discussion

To determine which of the metabolites of 2,4-xylidine was mutagenic, we separated the metabolites by HPLC and tested their mutagenicity. Only one metabolite was found to be mutagenic in TA100 cells (530, 388, 207 and 99 His^+/plate at doses of 100, 50, 25 and 0 μl of the sample respectively) without metabolic activation. As shown in Fig. 1, under the conditions used, the retention times of 2,4-dimethylphenylhydroxylamine, 2,4-xylidine, 2,4-dimethylnitrobenzene and 2,4,2′,4′-tetramethylazoxybenzene were 12.2, 21.3, 90.9 and 231 min respectively. On the other hand, the retention time of the mutagenic metabolite was 12.2 min, which corresponded to that of the authentic 2,4-dimethylphenylhydroxylamine.

We determined the rate of metabolism, 2,4-xylidine to 2,4-dimethylphenylhydroxylamine, in this HPLC analytical system. The amount of 2,4-dimethylphenylhydroxylamine formed linearly increased with the incubation time and with the concentration of S-9 added (data not shown). By the incubation with S-9 mixture at 37° for 30 min, 0.57% of 2,4-xylidine was metabolized to 2,4-dimethylphenylhydroxylamine (1.1 μmoles per 30 min per 12.5 ml S-9 mixture).

The identity of the mutagenic metabolite, 2,4-dimethylphenylhydroxylamine, was further confirmed with mass spectrometry. The results are shown in Fig. 2. The peaks were observed at *m/z* 137 and *m/z* 120 (Fig. 2A), which are the molecular ion peak and the base peak of 2,4-dimethylphenylhydroxylamine respectively (Fig. 2B). In this mass spectrum, however, the peaks at *m/z* 254 and *m/z* 239 were also observed. As shown in Fig. 2C, they are the molecular ion peak and the fragment ion peak $[M - 15]^+$ of 2,4,2′,4′-tetramethylazoxybenzene respectively. This contaminant is supposed to be formed by the oxidation of 2,4-dimethylphenylhydroxylamine during the purification step.

Based on the results obtained by HPLC and mass spectrometric analysis, we concluded that the mutagenic metabolite of 2,4-xylidine is its *N*-hydroxylated metabolite, 2,4-dimethylphenylhydroxylamine.

To compare the mutagenicity of 2,4-dimethylphenylhydroxylamine with that of 2,4-xylidine, a quantitative mutation assay was performed [17]. As shown in Fig. 3, 2,4-dimethylphenylhydroxylamine was directly mutagenic in TA100 cells, whereas 2,4-xylidine was mutagenic only in the presence of S-9 mixture. For these two compounds, the TA100 strain was more sensitive than the TA98 strain (data not shown). Approximately 200-fold differences in the doses used were observed between these two compounds: equivalent mutation frequencies were obtained with 7.3 to 73 nmoles/ml of 2,4-dimethylphenylhydroxylamine and 1.65 to 16.5 μmoles/ml of 2,4-xylidine. Such a difference may reflect the rate of metabolism, that is, 0.57% of 2,4-xylidine was converted to 2,4-dimethylphenylhydroxylamine during the incubation.

N-Hydroxylation of aromatic amines is an initial and essential reaction in the formation of the metabolites that are responsible for toxicities such as mutagenicity, carcinogenicity, and ferrihemoglobin formation [19, 20]. Although this mechanism is well established, 2,4-dimethylphenylhydroxylamine has not been shown unequivocally to be a metabolite of 2,4-xylidine. Moreover, the direct-acting mutagenicity of the metabolite has not been reported. This may be due to the inherent instabilities of 2,4-dimethylphenylhydroxylamine and the lack of a reliable method for its analysis. In fact, a half-life of 2,4-dimethylphenylhydroxylamine in 0.1 M phosphate buffer, pH 7.4, at 37° is *ca.* 20 min (unpublished data). However,

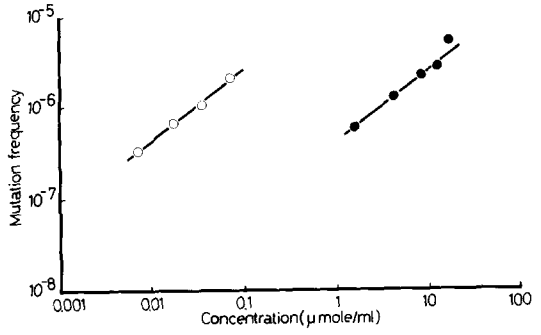

Fig. 3. Induced mutation frequencies versus dose of 2,4-xylidine and 2,4-dimethylphenylhydroxylamine in *S. typhimurium* TA100. Experiments were carried out in the presence of S-9 mixture for 2,4-xylidine (–●–) and in the absence of S-9 mixture for 2,4-dimethylphenylhydroxylamine (–○–). The mutagenicity test of 2,4-xylidine in the absence of S-9 mixture was also carried out, but the result is not shown because there were only negative responses.

with certain precautions, its ease of air oxidation is not a serious problem during the analytical process. Sternson and Dewitte [21] reported that a half-life of phenylhydroxylamine in 0.26 M ammonium acetate solution was *ca.* 20 min, but in the same solution, which was deoxygenated with argon, phenylhydroxylamine did not exhibit significant decomposition, even after 2 hr. The mobile phase in our chromatographic analysis was routinely deoxygenated by sonication; thus our method can accurately maintain the level of hydroxylamine. By using this method, we found that 0.57% of 2,4-xylidine was converted to its *N*-hydroxylated metabolite, which was responsible for the mutagenicity of the mother compound, 2,4-xylidine. The mutagenic potential of 2,4-dimethylphenylhydroxylamine was about the same as another carcinogenic hydroxylamine, β-naphthylhydroxylamine [22].

In summary, the mutagenic metabolite of 2,4-xylidine was identified as 2,4-dimethylphenylhydroxylamine. The metabolite proved to be potent direct mutagen, using *S. typhimurium* TA100. The mutagenicity and the amount of the *N*-hydroxylated metabolite formed could account for the enzyme-mediated mutagenicity of 2,4-xylidine.

Acknowledgements—We would like to acknowledge Dr. Kenzo Kanohta for his competence in analysis with mass spectrometry. A part of this research was supported by a scientific research fund from the Ministry of Education, Science and Culture, Japan.

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