EFFECTS OF 7, 8-BENZOFLAVONE AND SKF 525-A ON THE ENZYME-MEDIATED MUTAGENICITY OF PHENYLENEDIAMINES

Takehiko NOHMI, Rumiko MIYATA, Kunie YOSHIKAWA and Motoi ISHIDATE Jr.

Division of Mutagenesis, Biological Safety Research Center,
National Institute of Hygienic Sciences, 1-18-1 Kamiyoga,
Setagaya-ku, Tokyo 158 (Japan)

Accepted February 1, 1982

Abstract—The effects of microsomal enzyme inhibitors (7, 8-BF and SKF 525-A) on the S-9-mediated mutagenicity of o-, m- and p-phenylenediamine were investigated using Salmonella typhimurium TA98. SKF 525-A did not affect the enzyme-mediated mutagenicity of m- and p-phenylenediamine, while 7, 8-BF reduced significantly the mutagenicity of all three isomers of phenylenediamine. When the enzyme reactions in the agar overlay were stopped successively by adding 7, 8-BF directly onto the plate, the number of revertants increased linearly with time at least for 6 hours.

These data suggest that cytochrome P-448 takes a main role in the activation of phenylenediamines and that in the agar layer this microsomal enzyme remain active for a period as long as 6 hours at 37°C.

Key words: Mutagenicity, phenylenediamine, Cytochrome P-448, 7, 8-Benzoflavone, SKF 525-A, Salmonella typhimurium TA98.

INTRODUCTION

It has been reported that about 80% of commercial hair dyes in Japan were mutagenic against Salmonella typhimurium TA98 (Yoshikawa et al., 1976). This value agrees well with that on American specimens reported by Ames et al. (1975). Among various ingredients, aromatic amines have been identified as mutagenic principles of the hair dyes (Ames et al., 1975).

Phenylenediamines are known as main raw materials of hair dyes and one of the

Abbreviations 3-MC=3-methylcholanthrene; PB=phenobarbital; PCBs=polychlorinated biphenyls; 7,8-BF=7, 8-benzoflavone; SKF 525-A=β-dimethylaminoethyl-diphenylpropylacetate; G-6-P=glucose-6-phosphate; G-6-PDH=glucose-6-phosphate dehydrogenase; NADH=nicotinamide adenine dinucleotide, reduced form; NADPH=nicotinamide adenine dinucleotide phosphate, reduced form; S-9=liver 9,000×g supernatant fraction
mutagenic aromatic amines. Their isomers, o-, m- and p-phenylenediamine, have different mutagenic activity and require activation with S-9 for mutagenesis (Garner and Nutman, 1977). Our previous report revealed that the mutagenicity of phenylenediamines and triaminobenzenes was more efficiently detected with the S-9 from the rats pretreated with 3-MC rather than PB (Yoshikawa et al., 1979).

It is well established that inducers of hepatic monoxygenase can be categorized into two main groups (Conney, 1967); 3-MC and PB. 3-MC induces the synthesis of cytochrome P-448, a hemeprotein that differs in spectral and catalytic properties from cytochrome P-450 present in uninduced rats or in rats pretreated from PB (Ryan et al., 1975). PCBs are unique in that they induce both types of cytochromes and thus hemeproteins induced by PCBs are a mixture of both cytochromes (Alvares et al., 1973). On the other hand, various compounds have been known as a inhibitor of these two cytochromes. 7, 8-BF is known to act as a specific inhibitor of the cytochrome P-448-dependent monoxygenase. Conversely, inhibition of drug metabolism by SKF 525-A has been interpreted as evidence for the involvement of cytochrome P-450 (Razzouk et al., 1978; Hales and Jain, 1980; Diamond and Gelboin, 1969).

The present experiment deals with a study on the involvement of these cytochromes in the metabolic activation of phenylenediamines using such inhibitors, 7, 8-BF and SKF 525-A, in the system of Salmonella/microsome mutation assay. Preliminary report of this paper has already been published elsewhere (Nohmi et al., 1981).

MATERIALS AND METHODS

Chemicals

o-, m-, p-Phenylenediamine and 7, 8-BF were purchased from Wako Pure Chemical Co., Tokyo and were kept dark in the freezer. NADH, NADPH, G-6-P and G-6-P DH were purchased from Oriental Yeast Co., Tokyo. SKF 525-A was kindly supplied by Dr. M. Uchiyama in National Institute of Hygienic Sciences. 3-Hydroxybenzo(a)-pyrene was supplied by Dr. H. Yagi in National Institute of Health, U. S. A.

Preparation of enzyme fractions

Male wistar rats (100-130 g) were used. PCB mixture, KC-400 (500 mg/kg in olive oil), was injected i. p. 5 days before sacrifice. Control animals were received only olive oil 1 ml/kg. The S-9 was prepared according to the method of Ames et al. (1975-2). Microsomal fractions were prepared from the S-9 by further centrifugation at 105,000X g for 60 min. S-9, microsome and soluble supernatant fractions were stored at -80°C until use.

Analytical method

The content of cytochrome P-450 was determined by the method of Omura and Sato (1964) using an extinction coefficient of 91 mM⁻¹ cm⁻¹ for A₄₈₀₋₄₉₀. Cytochrome b₅ was determined with NADH using an extinction coefficient of 185 mM⁻¹ cm⁻¹ for A₄₃₄₋₄₃₉. NADPH cytochrome c reductase activity was determined by the method of Manzel et al. (1971). Aniline hydroxylase activity was determined by the method of Imai et al. (1966). Aryl hydrocarbon hydroxylase (AHH) activity was determined by the method of
Mutagenicity of phenylenediamines

Nebert and Gelboin (1968) with 3-hydroxybenzo(a)pyrene as a fluorometric standard. Dimethylnitrosamine demethylase activity was determined by the method of Frantz and Malling (1975). Protein content was determined by the method of Lowry et al. (1951) with bovine serum albumine as the standard.

Testing for the bacterial mutagenicity

The method was basically that of Ames et al. (1975-2). The test chemical in 0.1 ml dimethylsulfoxide (DMSO) and 0.1 ml overnight culture of S. typhimurium TA98, which was kindly supplied by Dr. B. N. Ames (Dept. of Biochemistry, Univ. of California) were placed in a small test tube and mixed with 0.5 ml S-9 mix (150 μl of S-9 = 5.2 mg protein, unless otherwise indicated, 2 μ moles NADH, 2 μ moles NADPH, 2.5 μ moles G-6-P, 4 μ moles MgCl₂, 16.5 μ moles KCl and 40 μ moles sodium phosphate buffer, pH 7.4). This mixture was mixed with 2 ml top agar (0.6% agar and 0.6% NaCl) at 45°C and spread on a minimal agar plate containing each L-histidine and D-biotin. Plates were incubated at 37°C for two days and the number of His⁺ colonies was counted. In experiments where microsomes were used instead of S-9, the reaction mixture also contained 0.5 units of G-6-P DH. For inhibition studies, SKF 525-A was previously incubated with S-9 mix for 15 min before the addition of phenylenediamines. 7, 8-BF was added into S-9 mix just before the addition of phenylenediamines.

The effect of incubation time

The following method depends on the inhibitory effect of 7, 8-BF on the enzyme-mediated mutagenicity of m-phenylenediamine. The reaction mixture without 7, 8-BF was mixed with 2 ml top agar and poured onto the plate. Plates were kept in a dark incubator at 37°C. After 0, 1, 2, 3, 4 and 6 hours, each group of plates was taken out. 7, 8-BF (500 nmoles) in 10 μl DMSO solution was mixed well with 2 ml soft agar and spread over the top agar layer. After additional incubation up to 48 hours, the number of revertants was counted. In the control experiments, 2 ml soft agar, which contained no inhibitor, was also poured on the top agar layer. Each experiment was done in triplicate. The average and the standard deviation were calculated.

RESULTS

Analytical data of microsomal fraction

In this series of experiments, the S-9 prepared from the PCBs-treated rats were used. Analytical data of its microsomal fraction are shown in Table 1. By the pre-treatment of PCBs, marked increases in the content of cytochrome P-450 (3-fold) and cytochrome b₅ (2.5-fold), and in the activity of cytochrome c reductase (2-fold), aniline hydroxylase (3.0-fold), AHH (7-fold) and dimethylnitrosamine demethylase (1.5-fold) are noted. These data are comparable with those reported by other workers (Alvares and Kappas, 1977).

Variation of S-9 concentration in S-9 mix

The relationship between the amount of S-9 added and the number of revertant colonies induced (with a fixed amount of o-, m- and p-phenylenediamine) was studied (Fig. 1). o- and p-phenylenediamine induced more revertants when the concentration
Table 1. Effects of KC-400 on microsomal hemeproteins and drug metabolizing enzymes

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>KC-400</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein content, mg/g liver (S-9) (micro some)</td>
<td>72.0±4.9</td>
<td>77.1±1.9</td>
</tr>
<tr>
<td></td>
<td>17.5±0.8</td>
<td>28.2±0.6</td>
</tr>
<tr>
<td>Cytochrome P-450 content, n mol/mg protein</td>
<td>0.62±0.05</td>
<td>2.14±0.05</td>
</tr>
<tr>
<td>Cytochrome b, content, n mol/mg protein</td>
<td>0.20±0.02</td>
<td>0.49±0.01</td>
</tr>
<tr>
<td>NADPH-cytochrome c reductase, n mol cytochrome c reduced/mg protein/min</td>
<td>132.4±5.5</td>
<td>223.6±11.4</td>
</tr>
<tr>
<td>Aniline hydroxylase, n mol p-aminophenol/mg protein/hour</td>
<td>29.7±2.0</td>
<td>90.0±8.3</td>
</tr>
<tr>
<td>Aryl hydrocarbon hydroxylase, n mol 3-hydroxybenzo (a) pyrene/mg protein/hour</td>
<td>11.4±0.4</td>
<td>77.7±0.9</td>
</tr>
<tr>
<td>Dimethylnitrosamine demethylase, n mol HCHO/mg protein/hour</td>
<td>191.4±7.5</td>
<td>288.2±2.3</td>
</tr>
</tbody>
</table>

Rats were administered KC-400 (500 mg/kg) 5 days before sacrifice.
S-9 and microsomal fractions were prepared as described under Materials and Methods.
Analytical procedures were described in Materials and Methods.
Each value represents mean±S. D.

Fig. 1. Effects of protein concentration of the mutagenicity of phenylenediamines. The procedure was described in Materials and Methods except that the amount of S9 fraction in the mix was varied. (The protein concentration of 150 μl undiluted S9 fraction was 5.2 mg). Each plate contained 1 μmole (○) o-, (●) m- and (△) p-phenylenediamine. Values are means of triplicate estimations.
Mutagenicity of phenylenediamines of S-9 in the S-9 mix increased. For m-phenylenediamine, the number of revertants increased more drastically and then decreased with the concentration of S-9. The optimum was in the S-9 of 10-fold diluted (0.52 mg protein per plate). Even small amounts of S-9 (10-fold diluted) could activate m-phenylenediamine as well as the undiluted S-9 (5.2 mg per plate).

Subcellular localization of activating enzymes

The S-9, microsomal and soluble supernatant fractions were tested for their ability to activate three isomers (Table 2). For o- and m-phenylenediamine, a microsomal fraction increased the number of revertants. Microsomes gave a response similar to that observed with the S-9. A soluble supernatant fraction did not modify the mutagenic response of o- and p-phenylenediamine. None of fraction increased the number of revertants for p-phenylenediamine.

Effects of 7, 8-BF and SKF 525-A on the enzyme-mediated mutagenicity of phenylenediamines

Table 2. Mutagenicity of o-, m- and p- phenylenediamine with various subcellular fractions

<table>
<thead>
<tr>
<th>Subcellular fractions</th>
<th>Revertants TA98 per plate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>o-</td>
</tr>
<tr>
<td>9,000×g sup.*</td>
<td>827±72</td>
</tr>
<tr>
<td>105,000×g ppt **</td>
<td>901±35</td>
</tr>
<tr>
<td>105,000×g sup.***</td>
<td>30±1</td>
</tr>
</tbody>
</table>

The methods of the preparation of each fraction were described in Materials and Methods. The dose of three isomers was 1 μmole per plate for o- and m-phenylenediamine and 5 μmoles per plate for p-phenylenediamine, because of the lower mutagenic response of p-phenylenediamine. Microsomes** (2.1 mg protein per plate) or 105,000×g sup.*** (3.1 mg protein per plate) were used for activation instead of S-9 fractions* (2.2 mg protein per plate).

Each value represents mean±S.D.

As shown in Fig. 2, SKF 525-A reduced specifically the number of revertants induced by o-phenylenediamine after metabolic activation, but did not reduced the number of revertants induced by m- and p-phenylenediamine.

7, 8-BF lowered significantly the enzyme-mediated mutagenicity of three isomers of phenylenediamine. For o- and m-phenylenediamine the number of revertants increased with decrease in the concentration of 7, 8-BF, whereas these phenomena were not observed for p-phenylenediamine. 7, 8-BF was not bacteriostatic at these dose range. Similarly, SKF 525-A did not show any killing effects at any concentration below 50 nmoles per plate.

Effects of incubation time on the enzyme-mediated mutagenicity of m-phenylenediamine

The mutagenicity of m-phenylenediamine was almost inhibited by 7, 8-BF at a concentration of 500 nmoles per plate. In order to investigate the effects of incubation time on the activation of m-phenylenediamine, we have used this inhibitor.
Fig. 2. Effects of 7,8-BF (○) and SKF 525-A (●) on the mutagenicity of (A) o-, (B) m- and (C) p-phenylenediamine. The dose of three isomers was 1 μmole per plate for o- and m-phenylenediamine and 5 μmole per plate for p-phenylenediamine, because of lower mutagenic response of p-isomer. Each plate contained 150 μl S-9 fraction. The procedures of the inhibition study were described as under Materials and Methods. Values are means of triplicate estimations.

Fig. 3. Effects of the incubation time on the number of His° revertants induced by m-phenylenediamine after metabolic activation. The mean number of revertants in the control (○) was 970. The zero time value in the experiments after addition of the inhibitor (●) was 210. Each value represents the mean ± S. D..
Mutagenicity of phenylenediamines

As shown in Fig. 3, the revertants per plate increased linearly with time at least for 6 hours. However, in the control, the number of revertants was similar whenever the soft agar was added.

**DISCUSSION**

Phenylenediamines are components of hair dyes and reductive products of azo dyes. The mutagenicity of phenylenediamines was previously reported by Ames et al. (1975–1). These components were highly mutagenic only when S-9 was present. Only few reports, however, concerning the metabolism of phenylenediamines have been published.

Dybing et al. (1977–1) have used the bacterial mutagenesis test as an indicator for the metabolism of 2,4-diaminoanisol, an aryldiamine hair dye component. They suggested that 2,4-diaminoanisol could be activated via cytochrome P-448-dependent N-oxidation into reactive metabolites capable of inducing mutation (Dybing et al., 1977–2).

Our data also indicated that activation of phenylenediamines occurred via cytochrome P-448 metabolism. Among three types of inducers, 3-MC and PCBs were efficient (Yoshikawa et al., 1979). A microsomal fraction increased the mutagenicity of o- and m-phenylenediamine, whereas 105,000×g sup. did not (Table 2). The fact that the soluble supernatant fraction seemed to modify the mutagenic response of m-phenylenediamine may be due to the trace of microsomes contaminated in this fraction. NADPH was essential for the activation (data not shown). Addition of 7,8-BF, which is known to act as a specific inhibitor of cytochrome P-448, completely inhibited the enzyme-mediated mutagenicity of these three isomers, while SKF 525–A only slightly affected the enzyme-mediated mutagenicity of o-phenylenediamine (Fig. 2). Cytochrome P-448-dependent monooxygenase might mediate the first step of activation. For p-phenylenediamine, a much lower mutagenic response was observed when a microsomal fraction was used instead of S-9 (Table 2). A two-step activation mechanism, each being localized in a different cell component, could be involved. On the other hand, the protective effect of liver cytosol against lipid peroxidation may prolong the viability of the microsomal enzyme and thus increased the yield of mutagenic intermediates (Kamataki et al., 1977).

Activation procedures in mutation assay have been most commonly performed in a semisolid agar gel containing the chemical, bacteria and S-9 mix. In the case of simple enzyme assay in a buffer medium, reaction rates of metabolism remain maximal for only a few minutes (Wood et al., 1976). However, in the plate test, it is said that microsomes remain active for a relatively long period (Ames et al., 1975–2). Our data clearly indicated that monoxygenase involved in m-phenylenediamine biotransformation remained active at least 6 hours at 37°C in the agar layer. Similar results (9 or 7 hours at 37°C) have been reported by other workers when gaseous samples were used (Bartsch et al., 1975, Malaveille et al., 1977). The reason for the prolongation of the viability of microsomal enzymes is still uncertain. However, the stabilizing effect of agar and the delay of diffusion rates on the plate could contribute to this phenomenon.

The present paper demonstrated that cytochrome P-448 could play a main role in
the activation of phenylenediamines and these microsomal enzymes remain active for relatively long period as 6 hours in the agar overlayer.

ACKNOWLEDGEMENTS

This study was supported in part by the grants from the Ministry of Health and Welfare and the Technology Agency Japan.

REFERENCES


LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J., (1951): Protein
Mutagenicity of phenylenediamines

measurement with the folin phenol reagent, J. Biol. Chem., 193, 265-275.
Drug Metabolism and Drug Disposition. pp 546-582. The Williams & Wilkins Baltimore.
NEBERT, D. W. and GELBOIN, H. V., (1968): Substrate-inducible microsomal aryl hydro-
xylase in mammalian cell culture (1) Assay and properties of induced enzyme. J. Biol.
NOHMI, T., YOSHIKAWA, K., MIYATA, R., NAKAMURA, S. and ISHIDATE, M. Jr.,
OMURA, T. and SATO, R., (1964): The carbon monoxide-binding pigment of liver microsomes.
J. Biol. Chem., 239, 2370-2378.
RAZZOUK, C., AGAZZI-LEONARD, E., CUMPS, J., PONCELET, F., MERCIER, M. and
ROBERFROID, M., (1978): Induction, modification and inhibition of rat liver microsomal
benzo(a)pyrene hydroxylase; Correlation with the S9-mediated mutagenicity of benzo(a)pyrene.
cytochrome P-448 and P-450 from rat liver microsomes. Biochem. Biophys. Res. Com-
mun., 64, 1134-1141.
WOOD, A. W., LEVIN, W., LU, A. Y. H., YAGI, H., HERNANDEZ, O., JERINA, D. M. and
CONNEY, A. H., (1976): Metabolism of benzo(a)pyrene and benzo(a)pyrene derivatives to
mutagenic products by highly purified hepatic microsomal enzymes, J. Biol. Chem., 251,
4882-4890.
YOSHIKAWA, K., UCHINO, H. and KURATA, H., (1976): Studies on the mutagenicity of
YOSHIKAWA, K., NOHMI, T., HARADA, R., INOKAWA, Y. and ISHIDATE M. Jr., (1979):
Differential mutagenicities of triamino benzenes against Salmonella typhimurium TA98 in
the presence of S9 fractions from polychlorinated biphenyls, phenobarbital or 3-methyl-