THE SUPPRESSIVE EFFECT OF HEPATIC 9,000×G SUPERNATANT FRACTION FROM 3-METHYLCHOLANThRENE-TREATED RATS ON THE MUTAGENIC ACTIVITY OF 2-ACETYLAMINOFLUORENE

Akie KOJIMA, Sumie KAWANO and Kogo HIRAGA
Department of Toxicology, Tokyo Metropolitan Research Laboratory of Public Health, 24-1, Hyakunincho 3-chome, Shinjuku-ku, Tokyo, 160 Japan
Accepted January 21, 1980

Abstract—Hepatic 9,000×g supernatant (S9) fraction from 3-methylcholanthrene (MC)-treated rats showed a significantly low activity in the in vitro conversion of 2-acetylaminofluorene (AAF) to mutagen(s), while S9 fraction from untreated or phenobarbital (PB)-treated rats showed a remarkable activity. The S9 fraction from MC-treated rats actually suppressed the PB-S9 mediated AAF mutagenicity. Since the suppressive effect of MC-S9 was lost by the addition of 10⁻⁴ M α-naphthoflavone (α-NF), rat specific cytochrome P-448 may be responsible for this suppressive effect.

2-Acetylaminofluorene (AAF) is a widely studied carcinogen (1–3) and potent mutagen (4) in the Ames assay. The first step in the metabolic activation of AAF to mutagen(s) is considered to be N-hydroxylation via cytochrome P-450-dependent monooxygenase (5). Thorgeirsson et al. (6) reported that N-hydroxylation of AAF was closely associated with 3-methylcholanthrene (MC)-inducible arylhydrocarbon hydroxylase (AHH) activity and cytochrome P-448. In fact, in mice MC-treatment increased both of AHH activity and activation of AAF to mutagen(s) (6). In rats, several investigators (7–10) have shown that N-hydroxylation of AAF is markedly increased by the treatment of MC.

However, we have noticed that AAF which was used as a positive control in a routine assay shows only a slight mutagenic activity when MC-induced rat liver 9,000×g (S9) fraction was used, although non-induced or PB-induced S9 fractions remarkably activated AAF mutagenesis. This species difference between rats and mice prompted us to examine the activation of AAF to mutagen(s) in rat subcellular fractions.

MATERIALS AND METHODS

Chemicals: AAF and α-NF were purchased from Wako Pure Chemical Industries Co., MC and PB were from Fluka AG, Swiss and Yoshida Seiyaku Co., respectively.

Animals and treatments: Five-week-old male Wistar-JCL rats used for all experiments were kept in stainless steel wire cages, and were fed ad libitum a pelleted standard diet (CLEA Japan Inc., CE-2).

The animals were given the following treatments; MC, 20 mg/kg/day in corn oil i.p. for 4 days; PB, 0.1% in the drinking water for 1 week; a combined treatment of MC and PB.
Preparation of liver fractions: Rat liver was homogenized with three volumes of 0.15 M KCl. The homogenate was centrifuged for 10 min at 9,000 × g, and the resultant supernatant (the S9 fraction) was decanted and stored at −80°C. Microsomal fraction was prepared by centrifugation for 60 min at 105,000 × g of S9 fraction. The microsomes were suspended in 0.15 M KCl, and diluted to the same volume as that of S9 fraction.

Assay of mutagenic activity: The bacterial strain *Salmonella typhimurium* TA98 was provided by Dr. T. Kada, National Institute of Genetics, Japan. Mutagenicities were tested by modification (11) of Ames et al. (4, 12, 13).

0.1 ml of bacteria suspension and 0.1 ml of AAF (dissolved in dimethylsulfoxide) were added to tubes containing in a total volume of 0.5 ml; 4 μmoles of MgCl₂, 16.5 μmoles of KCl, 2.5 μmoles of glucose-6-phosphate, 0.25 units of glucose-6-phosphate dehydrogenase, 2 μmoles of nicotinamide dinucleotide phosphate, 50 μmoles of phosphate buffer (pH 7.4) and S9 fraction. The tubes were incubated for 20 min at 37°C with shaking, and 2 ml of soft agar (containing each 0.1 μmole of histidine and biotin) was added. The mixture was then overlayed on minimal agar plates. After incubation for 2 days at 37°C, revertant colonies were counted. Mean values ± SD of 3 experiments are listed. The average number of spontaneous revertants per plate in the presence of S9 fraction and cofactors was 35.

RESULTS

Comparison of different inducers given in vivo: Figure 1 shows mutagenic activation of AAF by S9 fraction from rats treated with well-known inducers of drug-metabolizing enzymes, i.e. MC, PB and MC plus PB. In the presence of 10 μg of AAF per plate, a
remarkable increase was observed in the revertant rate with PB-induced S9 fraction. However, with MC- and MC plus PB-induced S9 fractions a significantly low revertant rate was exhibited, compared with PB-induced or non-induced S9 fractions. In contrast, when 100 μg AAF per plate was used, no significant difference was observed in the revertant rate among these treatments.

Effect of in vitro addition of MC-induced S9 fraction on the PB-S9 mediated AAF mutagenicity: As above described, when S9 fraction from MC plus PB-treated rats was used, AAF mutagenicity was almost completely depressed, although S9 fraction from PB alone treated rats exhibited a remarkable mutagenic activation of AAF. These results prompted us to examine the combined effect of MC-S9 and PB-S9 in the in vitro assay system. Various amounts of S9 fraction (5 to 200 μl) from MC-treated or untreated rats were added to the AAF mutagenicity assay system containing a constant amount of S9 fraction (100 μl) from PB-treated rats. Figure 2 shows that the addition of 10 μl of MC-S9 per plate reduced the mutagenicity of AAF about three-fold, and the addition of more than 50 μl of MC-S9 per plate almost completely inhibited the mutagenicity. On the other hand, S9 fraction from untreated rats hardly reduced the mutagenicity.

In order to determine whether this suppressive effect of MC-S9 on the PB-S9 mediated AAF mutagenicity depended upon microsomes themselves or cytosol in the MC-S9, MC-S9 fraction was separated to microsomal and cytosol fractions by centrifugation, and these fractions were added to the AAF mutagenicity assay system. Figure 3 shows that micro-

![Fig. 2](image1.png)

**FIG. 2.** Effect of 3-methylcholanthrene (MC)-induced and non-induced S9 fractions on the phenobarbital (PB)-induced S9 mediated mutagenicity of 2-acetylaminofluorene (AAF). •, MC-induced S9 fraction; ○, non-induced S9 fraction. The designated amounts of MC-induced or non-induced S9 fractions were added to the incubation mixture containing 10 μg of AAF, 100 μl of PB-S9 and cofactors.

![Fig. 3](image2.png)

**FIG. 3.** Effect of 3-methylcholanthrene (MC)-induced microsomal and cytosol fractions on the 2-acetylaminofluorene (AAF) mutagenicity. •, MC-induced microsomal fraction; ○, MC-induced cytosol fraction; △, MC-induced S9 fraction. The assay was performed as described in Fig. 2.
somes are responsible for this suppressive effect.

Effect of α-NF on the AAF mutagenicity: α-NF inhibits the MC-inducible hydroxylase activity more than PB-inducible hydroxylase activity (14, 15). Since recent studies (16) have shown that α-NF also inhibits several other hydroxylases associated with cytochrome P-448 in MC-treated animals, α-NF is thought to be a cytochrome P-448 inhibitor, although the mechanism of inhibitions by α-NF is not clear. We examined the effect of α-NF on the AAF mutagenicity (Table 1). α-NF, when added at a concentration of $10^{-4}$ M, reduced PB-S9 mediated AAF mutagenicity only about 50%. The mutagenicity with MC-S9 was not affected by this level of α-NF. In contrast, addition of α-NF to the incubation mixture containing both MC-S9 and PB-S9 increased AAF mutagenicity more than ten-fold. This level was almost equal to the revertant rate observed in the presence of PB-S9 and α-NF.

| Subcellular fraction | No. of reversions/plate (mean±SD) |
|----------------------|-----------------------------------|
|                      | (-) α-NF                         | (+) α-NF                         |
| PB-S9                | 8322±83                          | 4072±333a                       |
| MC-S9                | 344±14                           | 338±18                           |
| MC-Microsomes        | 170±25                           | 52±18a                          |
| MC-Cytosol           | 258±20                           | 75±5a                           |
| PB-S9+MC-S9          | 306±22                           | 4237±479a,b                     |
| PB-S9+MC-Microsomes  | 268±35                           | 4047±1666a,b                     |
| PB-S9+MC-Cytosol     | 7676±27                          | 4135                             |

10$^{-4}$ M α-NF (in 50 μl of dimethylsulfoxide) was added to the incubation mixture. 100 μl of each subcellular fractions and 10 μg of AAF per plate were used for mutagenicity assay.

a These values are significantly different from each α-NF-non-added group (P<0.001)

b These values are not significantly different from PB-S9 group in the presence of α-NF.

Table 2. Effect of 3-methylcholanthrene (MC)-induced S9 fraction on the phenobarbital(PB)-S9 mediated 2-acetylaminofluorene (AAF) mutagenicity

| Incubation time with MC-S9 (min) | α-NF | No. of reversions/plate (mean±SD) |
|----------------------------------|------|-----------------------------------|
| A                                | -    | 384                               |
| 0                                | -    | 446                               |
| 20                               | -    | 446                               |
| B                                | -    | 5263±510                          |
| 0                                | +    | 4839±148                          |
| 2                                | +    | 4839±148                          |
| 5                                | +    | 4288±316                          |
| 10                               | +    | 5516±30                           |
| 20                               | +    | 5111±402                          |

After 10 μg of AAF, 100 μl of PB-S9 and cofactors were preincubated for 10 min at 37°C, 100 μl of MC-S9 was added, and incubation was continued for various periods. Bacterial suspension with or without 10$^{-4}$ M α-naphthoflavone (α-NF) was then added to this mixture.
Effect of incubation with MC-S9 after preincubation of AAF and PB-S9: In the following experiments, 10 \( \mu g \) of AAF, 100 \( \mu l \) of PB-S9 and cofactors were preincubated for 20 min at 37\(^\circ\)C, and thereafter 100 \( \mu l \) of MC-S9 was added. MC-S9 suppressed PB-S9 mediated AAF mutagenicity almost completely, and for this suppression incubation was not necessary (Table 2-A). After addition of MC-S9, incubation was continued for various periods, and thereafter 10\(^{-4}\) M \( \alpha\)-NF was added to the mixture. A high mutagenic rate was observed, in spite of incubation with MC-S9 (Table 2-B).

DISCUSSION

N-hydroxylation of AAF, which is an important step in the activation of AAF to mutagen(s), is closely associated with AHH (5). In fact, in mice, MC-treatment increased activities of AHH, AAF N-hydroxylation and the *in vitro* conversion of AAF to mutagen(s) (6).

This study has shown that liver S9 fraction (microsomes) from MC-treated rats exhibited a significantly low activity in conversion of AAF to mutagen(s) at the concentration of 10 \( \mu g \) AAF per plate, and addition of microsomes from MC-treated rats almost completely decreased the AAF mutagenicity seen in the presence of AAF and PB-S9. Since this suppressive effect was lost by the addition of \( \alpha\)-NF, a specific inhibitor of cytochrome P-448, MC-S9 suppressive effect might be attributed to the inhibition of degradation of AAF and/or its metabolites in the cytochrome P-448-dependent drug oxidation system. Recently, Thorgeirsson et al. (16) reported that hepatic cytochrome P-448 was composed of multiple cytochromes, which differ among animal species, each catalyzing different monooxygenase activities. Therefore, cytochrome P-448 specific for rats may be responsible for the suppressive effect of MC-S9.

In Table 2-A, the suppressive effect of MC-S9 was also observed, when MC-S9 was added after previous incubation of AAF and PB-S9, and for this suppressive effect incubation was not necessary. These results and the well-known inducing effect of MC on the AAF N-hydroxylation (7-10) indicate that the suppressive effect of MC-S9 on the AAF mutagenicity depends upon N-hydroxy AAF or subsequent metabolites rather than AAF itself. As shown in Table 2-B, AAF was incubated with PB-S9 and subsequently with MC-S9, and thereafter \( \alpha\)-NF was added to the incubation mixture. In this experiment, we had expected that mutagenic metabolites produced by incubation with PB-S9 might be destroyed during the subsequent incubation with MC-S9, and that the reversion rate might be decreased, irrespective of the addition of \( \alpha\)-NF. We found no such decrease. These unexpected results: a prompt inhibition in the absence of \( \alpha\)-NF and disappearance of inhibition in its presence, may be explained as follows: cytochrome P-448 does not destroy N-hydroxy AAF or subsequent metabolites, but rather inactivates by reversible binding, thus eliminating the suppression.

At the concentration of 100 \( \mu g \) AAF per plate, we found a high mutagenic rate, using the MC-S9 fraction (Fig. 1). It is not clear at present why the results differed with 10 \( \mu g \) and 100 \( \mu g \) doses of AAF. However, we tentatively postulate that at 10 \( \mu g \) per plate, AAF
or subsequent metabolites bind macromolecules of MC-S9, and only a low mutagenic rate is observed, while at 100 μg per plate, a portion of AAF or metabolites binds to macromolecules, the remainder escapes the binding and a high mutagenic rate is induced.

REFERENCES

1) HEIDELBERGER, C.: Chemical carcinogenesis. Ann. Biochem. 44, 79–121 (1975)
2) MILLER, J.A.: Carcinogenesis by chemicals: An overview—G.H.A. Clowes Memorial Lecture. Cancer Res. 30, 559–576 (1970)
3) WEISBURGER, J.H. AND WEISBURGER, E.K.: Biochemical formation and pharmacological, toxicological and pathological properties of hydroxylamines and hydroxamic acid. Pharmacol. Rev. 25, 1–66 (1973)
4) AMES, B.N., DURSTON, W.E., YAMASAKI, E. AND LEE, F.D.: Carcinogens are mutagens: a simple test system combining liver homogenates for activation and bacteria for detection. Proc. natn. Acad. Sci. U.S.A. 70, 2281–2285 (1973)
5) SCHUT, H.A.J. AND THORGEIRSSON, S.S.: Mutagenic activation of 2-acetylaminofluorene and N-hydroxy-2-acetylaminofluorene by mouse liver and kidney microsomes. Fedn. Proc. 36, 999 (1977)
6) THORGEIRSSON, S.S., FELTON, J.S. AND NEBERT, D.W.: Genetic differences in aromatic hydrocarbon-inducible N-hydroxylation of 2-acetylaminofluorene and acetaminophen-produced hepatotoxicity in mice. Mol. Pharmacol. 11, 159–165 (1975)
7) THORGEIRSSON, S.S., JOLLOW, D.J., SASAME, H.A., GREEN, I. AND MITCHELL, J.R.: The role of cytochrome P-450 in N-hydroxylation of 2-acetylaminofluorene. Mol. Pharmacol. 9, 398–404 (1973)
8) GUTMANN, H.R. AND BELL, P.: N-hydroxylation of arylamides by the rat and guinea pig. Evidence for substrate specificity and participation of cytochrome P-450. Biochem. Biophys. Acta. 498, 229–243 (1977)
9) MALEJKA-GIGANTI, D., McIVER, R.C., GLASEBROOK, A.L. AND GUTMANN, H.R.: Induction of microsomal N-hydroxylation of N-2-fluorenylacetamide in rat liver. Biochem. Pharmacol. 27, 61–69 (1978)
10) SCHUT, H.A. AND THORGEIRSSON, S.S.: In vitro metabolism and mutagenic activation of 2-acetylaminofluorene by subcellular liver fractions from cotton rais. Cancer Res. 38, 2501–2507 (1978)
11) YAHAGI, T., NAGAO, M., SEINO, Y., MATSUSHIMA, T., SUGIMURA, T. AND OKADA, M.: Mutagenicities of N-nitrosamines on salmonella. Mutation Res. 48, 121–130 (1977)
12) AMES, B.N., LEE, F.D. AND DURSTON, W.E.: An improved bacterial test system for the detection and classification of mutagens and carcinogens. Proc. natn. Acad. Sci. U.S.A. 70, 782–786 (1973)
13) AMES, B.N., McCANN, J. AND YAMASAKI, E.: Methods for detecting carcinogens and mutagens with the salmonella/mammalian-microsome mutagenicity test. Mutation Res. 31, 347–364 (1975)
14) WIEBEL, F.J., LEUTZ, J.C., DIAMOND, L. AND GELBOIN, H.V.: Aryl hydrocarbon (benzo[a]pyrene) hydroxylase in microsomes from rat tissues: differential inhibition and stimulation by benzo[b]furan and organic solvents. Arch. Biochem. Biophys. 144, 78–86 (1971)
15) GOUON, F.M., NEBERT, D.W. AND GIELEN, J.E.: Genetic expression of aryl hydrocarbon hydroxylase induction IV. Interaction of various compounds with different forms of cytochrome P-450 and the effect on benzo[a]pyrene metabolism in vitro. Mol. Pharmacol. 8, 667–680 (1972)
16) THORGEIRSSON, S.S., ATLAS, S.A., BOOBIS, A.R. AND FELTON, J.S.: Species differences in the substrate specificity of hepatic cytochrome P-448 from polycyclic hydrocarbon-treated animals. Biochem. Pharmacol. 28, 217–226 (1979)