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MAIN PATHWAY FOR MUTAGENIC ACTIVATION OF 2-ACETYLAMINOFLUORENE BY GUINEA PIG LIVER HOMOGENATES

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SUMMARY

The activation pathway of 2-acetylaminofluorene (AAF) to N-hydroxy-2-aminofluorene (N-OH-AF), a potent mutagen to Salmonella, by guinea pig liver post-mitochondrial supernatant fraction (S-9 fraction) was studied. 2-Aminofluorene (AF), as well as N-hydroxy-2-acetylaminofluorene (N-OH-AAF, Takeishi et al., Mutation Res. in press), was detected as a metabolite of AAF. The mutagenicities of AF and N-OH-AAF comparable to that of AAF were inhibited by antiserum against NADPH-cytochrome c reductase and by paraoxon, respectively. These data indicate that in the mutagenic activation of AAF, N-OH-AF can be produced by both N-hydroxylation of AF and deacetylation of N-OH-AAF. Furthermore, the data on the relative contribution of paraoxon-sensitive activation pathway to mutagenicities of AAF and N-OH-AAF led to a conclusion that deacetylation of AAF followed by N-hydroxylation to produce N-OH-AF is the main pathway for the mutagenic activation of AAF by guinea pig liver S-9 fraction.

INTRODUCTION

In the Salmonella assay system developed by Ames et al. (1,2), a carcinogen 2-acetylaminofluorene (AAF)* was shown to be highly mutagenic when activated by liver post-mitochondrial supernatant fraction (S-9 fraction) of guinea pigs (3, 4), in spite of the resistance of this species to AAF carcinogenesis. Our recent findings that both microsomal cytochrome P-450 mixed-function oxidases (4) and amidase(s) (5) were crucially involved in the mutagenic activation suggested that this highly mutagenic activity of AAF observed was due to the formation of N-OH-AF, a potent mutagen (6), because it can be produced from AAF by N-hydroxylation followed by deacetylation and/or vice versa (4).

In this study, both of the two pathways suggested above were shown to be involved in the formation of N-OH-AF from AAF, and furthermore their relative contribution to the mutagenic activation of AAF was determined. The results

*Abbreviations: AAF, 2-acetylaminofluorene; AF, 2-aminofluorene; N-OH-AAF, N-hydroxy-2-acetylaminofluorene; N-OH-AF, N-hydroxy-2-aminofluorene.

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revealed that deacetylation of AAF followed by N-hydroxylation to produce N-OH-AF was the main pathway for mutagenic activation of AAF by guinea pig liver S-9 fraction. This is in contrast with the widely accepted view in the case of rats or mice that N-hydroxylation is the first step for carcinogenic activation of AAF (7).

MATERIALS AND METHODS

Bacterial strain and chemicals *Salmonella typhimurium* TA98, and chromatographically pure AAF and N-OH-AAF were kindly provided by Dr. T. Matsushima of the University of Tokyo, Tokyo. 2-[9-^{14}C]AAF (46.16 mCi/mmole) and paraoxon (diethyl *p*-nitrophenyl phosphate) were purchased from New England Nuclear Corp., Boston, and Sigma Chemical Co., St. Louis, respectively. AF, a product of Aldrich Chemical Co., Milwaukee, was recrystallized from dilute ethanol prior to use.

Preparation of subcellular liver fractions Liver S-9 fraction from untreated guinea pigs was prepared as described previously (4). A microsomal fraction was obtained from the S-9 fraction by centrifugation at 105,000 x g for 60 min. The microsomal pellets were washed once with 150 mM KCl and resuspended in the same solution.

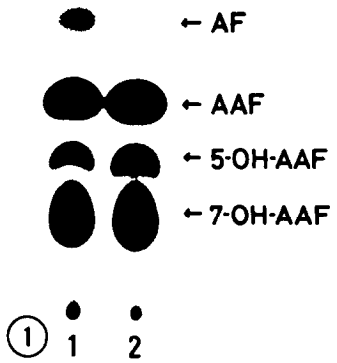
Detection of AF [^{14}C]AAF metabolites that were extractable with ether were chromatographed on a silica gel thin-layer sheet using a solvent system, chloroform/methanol (97:3, v/v) and the spots of the radioactive metabolites including [^{14}C]AF were made visible by autoradiography with Kodak XRP-1 X-ray film.

Others The *Salmonella* mutation assay was performed as described previously (5). Paraoxon was used in the mutation assay as well as in the experiment on the formation of AF from AAF at a concentration of 10^{-4} M, which completely inhibited the deacetylase activity (8) and the paraoxon-sensitive mutagenicity of AAF (5). Antiserum against NADPH-cytochrome *c* reductase was prepared as described by Kawajiri et al. (9). Protein concentration was determined by the methods of Lowry et al. (10) with bovine serum albumin as a standard.

RESULTS

Production of AF by the incubation of AAF with S-9 fraction To verify the ability of the guinea pig liver to activate AAF to a potent mutagen N-OH-AF *via* AF as an intermediate, metabolites of AAF were first analyzed. Fig.1 clearly shows that AF was produced when AAF was incubated with guinea pig liver S-9 fraction. AF accounted for about 5% of the total metabolites of AAF. The production of AF was completely inhibited in the presence of paraoxon.

Mutagenic activation of AF Fig.2 shows the mutagenicity of AF produced by incubation with guinea pig liver S-9 fraction. The number of His$^+$ revertant colonies obtained with AF at the concentration of 50 nmoles per plate was about double the number obtained with AAF at the same concentration (4). During in-



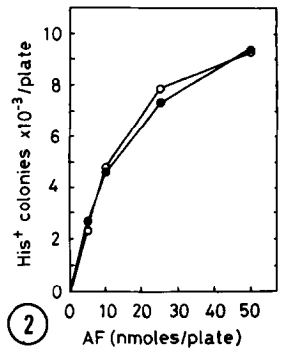
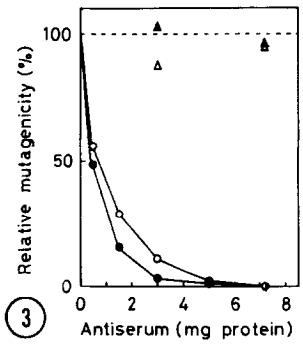


Fig. 1. Formation of AF by the incubation of AAF with liver S-9 fraction. [^{14}C]AAF (100 nmoles) was incubated for 3 h with 1 ml of S-9 mix in the presence (lane 2) or absence (lane 1) of paraoxon and ether extract of the reaction mixture was subjected to thin-layer chromatography. The amount of S-9 protein used per ml of S-9 mix was 6.2 mg.

Fig. 2. Mutagenic activation of AF by liver S-9 fraction in the presence (●) or absence (○) of paraoxon. The amount of S-9 protein per plate was 3.1 mg.

cubation of AF with guinea pig liver S-9 fraction at 37° for 3 h, about 5% of the original AF was found to be converted to AAF (data not shown). However, AF mutagenicity should not be due to such AAF produced by acetylation of AF, because the mutagenicity was not inhibited by paraoxon (Fig.2).

To determine whether or not a cytochrome P-450 mixed-function oxidase system is involved in the mutagenic activation of AF, we used antiserum against rat liver NADPH-cytochrome c reductase, which was shown to inhibit the reductase activity present in guinea pig liver S-9 fraction (4). Mutagenicity of AF produced by the S-9 fraction was clearly shown to be suppressed to zero by the antiserum (Fig.3, open circle), indicating that the mutagenicity completely depended on the cytochrome P-450 enzyme system. In order to confirm this result, AF was activated by a microsomal fraction instead of the S-9 fraction as shown in Fig.4. The level of mutagenicity of AF produced by the microsomal fraction (0.4 mg protein per plate) was comparable to that obtained by the S-9 fraction (see Fig.2). Also, the mutagenic activation of AF by the microsomal fraction was completely inhibited by the antiserum (Fig.3, closed circle). It should be noted that the mutagenic activation of AF was not caused by a 105,000 x g supernatant fraction (data not shown).



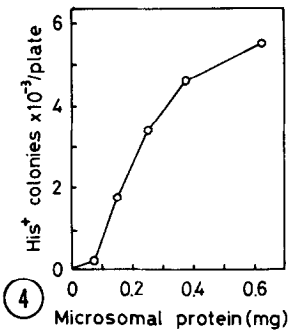


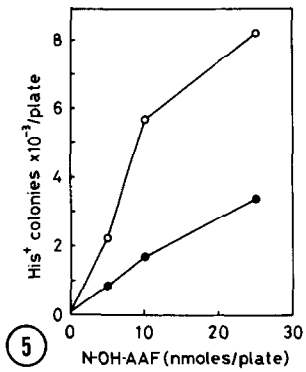
Fig. 3. Inhibition by antiserum against NADPH-cytochrome c reductase of mutagenic activation of AF by liver S-9 fraction (○ and △) and liver microsomal fraction (● and ▲). The amounts of S-9 protein and microsomal protein used per plate were 1.24 and 0.15 mg, respectively. 50 nmoles of AF were used per plate. ○ and ●, antiserum against NADPH-cytochrome c reductase; △ and ▲, control serum.

Fig. 4. Mutagenic activation of AF by liver microsomal fraction. 50 nmoles of AF were used per plate. 1 mg of microsomal protein was equivalent to 7.6 mg of S-9 protein.

Mutagenic activation of N-OH-AAF Our recent studies (4,5) suggested that a combination of N-hydroxylation and deacetylation was involved in the mutagenic activation of AAF by guinea pig liver S-9 fraction. The existence of the pathway starting with N-hydroxylation of AAF followed by deacetylation is supported by the previous finding that N-OH-AAF was produced by the incubation of AAF with liver S-9 fraction (4) or microsomes (11). To confirm the presence of this pathway, we examined the mutagenicity of N-OH-AAF. Fig.5 shows that N-OH-AAF was highly mutagenic when activated by liver S-9 fraction, and that 60% of the mutagenicity of N-OH-AAF was inhibited by paraoxon, indicating that about half of the mutagenicity of N-OH-AAF was produced by deacetylation.

DISCUSSION

Our previous studies (4,5) suggested that N-OH-AF was a major mutagen in AAF mutagenesis mediated by guinea pig liver S-9 fraction and was produced by N-hydroxylation of AAF followed by deacetylation or vice versa. The existence of the former pathway for activation of AAF was supported by the following facts: (i) N-OH-AAF was produced from AAF (4,11), (ii) guinea pig liver microsomes possessed a remarkably large capacity to deacetylate N-OH-AAF (8) and



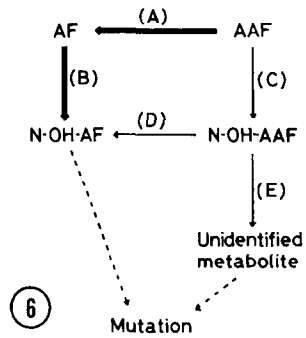


Fig. 5. Mutagenic activation of N-OH-AAF by liver S-9 fraction in the presence (●) or absence (○) of paraoxon. The amount of S-9 protein was the same as in Fig.2.

Fig. 6. Proposed pathways for the mutagenic activation of AAF by guinea pig liver S-9 fraction. The bold lines indicate the proposed main pathway.

(iii) the mutagenicity of N-OH-AAF produced was sensitive to paraoxon (Fig.5). Evidence for the existence of the latter pathway is as follows: (i) AF was produced from AAF by the action of paraoxon-sensitive deacetylase activity (Fig.1) and (ii) the mutagenicity of AF was completely dependent on microsomal cytochrome P-450 mixed-function oxidases (Fig.2-4). From these findings, it was concluded that a potent mutagen, N-OH-AF, was produced by the two pathways mentioned above in the AAF mutagenesis mediated by guinea pig liver S-9 fraction.

On the basis of the present and previous (4,5) data the pathways for the mutagenic activation of AAF by guinea pig liver S-9 fraction were proposed as illustrated in Fig.6. In Fig.6, pathway A and pathway D are paraoxon-sensitive and pathway E is paraoxon-resistant. Pathway B and pathway C are completely inhibited by antiserum against NADPH-cytochrome c reductase. We attempted to estimate the relative contribution of the individual activation pathway to the total AAF mutagenicity as follows. The previous finding that 90% of AAF mutagenicity was inhibited by paraoxon (5) represents the fact that 90% of the total AAF mutagenicity is due to the formation of N-OH-AF via both pathway A-B and pathway C-D, and 10% of the total is due to the formation of mutagens via pathway C-E. On the other hand, the ratio of N-OH-AAF mutagenicity via pathway D and pathway E is 3:2 (Fig.5). Therefore, the contribution of the acti-

vation via pathway C-D to the total AAF mutagenicity should be 15%. As a result, 75% of the total AAF mutagenicity is due to the activation via pathway A-B. In conclusion, AAF was activated to N-OH-AF by guinea pig liver S-9 fraction mainly via deacetylation followed by N-hydroxylation. This is in contrast with the widely accepted view that in the case of rats or mice AAF is activated to the ultimate carcinogen via N-hydroxylation of AAF as the first step (7). In parallel with the experiments with untreated guinea pig liver S-9 fraction as presented here, similar experiments were performed using liver S-9 fraction of guinea pigs treated with a combination of phenobarbital and 5,6-benzoflavone, cytochrome P-450 inducers (data not shown). Essentially the same results were obtained in both cases. Therefore, the conclusion obtained with untreated guinea pig liver S-9 fraction can be applied to the case of treated guinea pigs.

The question arises as to why about 75% of AAF mutagenicity was produced by the activation via AF, in spite of the fact that deacetylation of N-OH-AAF by guinea pig liver microsomes was 17 times faster than that of AAF (8). The most probable explanation for this paradox is as follows: In the present study a sufficient amount of AF was demonstrated to be produced during the incubation of AAF with guinea pig liver S-9 fraction. Therefore, if the molecular species of cytochrome P-450 involved in N-hydroxylation of AF is different from that involved in N-hydroxylation of AAF, and if the activity of the former species is much higher than that of the latter in guinea pig liver, it may easily be conceivable that N-OH-AF can be produced more efficiently via AF than via N-OH-AAF. In fact, there are observations which suggest that cytochrome P-450 induced by phenobarbital rather than 3-methylchoranthrene or 5,6-benzoflavone is involved in N-hydroxylation of AF by liver S-9 fraction of rats (12) or mice (unpublished). This may help to support the above-mentioned assumption.

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