Effect of 1,8-Pyrenequinone, a Mutagenicity Enhancing Agent, on Metabolisms of 2-Acetylaminofluorene in Liver Microsomal Fraction

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The addition of 1,8-pyrenequinone into the assay system containing rat liver homogenates (S-9) caused an approximately 10-fold increase in the mutagenicity of 2-acetylaminofluorene (AAF) in the current *Salmonella* reversion assay system. Since no chemical reaction between 1,8-pyrenequinone and AAF was observed, the *in vitro* effects of 1,8-pyrenequinone on the metabolisms of AAF with S-9 mix were studied. The enhancement of mutagenicity by 1,8-pyrenequinone was not dependent on the dose of NADPH under the present assay condition. The mutagenicity of AAF was increased approximately 4-fold by the addition of 1,8-pyrenequinone into microsomes, whereas it remained at the spontaneous level in the presence of cytosol. However, by reconstituting microsomes with cytosol, the mutagenicity enhancing activity was recovered to the original level. Since 1,8-pyrenequinone inhibited the AAF hydroxylase activity, chemical analysis of the incubation mixture of AAF was tried. This indicated that a higher amount of unmetabolized AAF remained and higher amounts of 2-aminofluorene and *N*-hydroxy-2-acetylaminofluorene were accumulated in the presence of 1,8-pyrenequinone compared with those in the absence of 1,8-pyrenequinone. From these results, it seems probable that 1,8-pyrenequinone inhibits C-hydroxylation (the detoxifying pathway) and promotes *N*-hydroxylation (the activating pathway) as well as deacetylation in the AAF metabolism.

We previously reported that certain pyrolysis products of carbohydrates enhanced the mutagenicities of aromatic amines such as 2-acetylaminofluorene (AAF), 2-aminofluorene (AF), 2-aminoanthracene (AA) and Trp-P,* a potent mutagen detected in the tryptophan pyrolysate¹⁾ and broiled fish cooked in the ordinary way.²⁾ We also reported that pyrene, 1-methylpyrene and 1-hydroxypyrene were isolated from cellulose pyrolysates and identified as the substances enhancing the mutagenicity of AAF.^{3~5)} The enhancement of mutagenicity by these pyrenes was revealed only in the presence of rat liver homogenates (S-9). When pyrene was metabolized in the presence of S-9 mix, 1-hydroxypyrene, 4,5-

dihydro-4,5-dihydroxypyrene, 1,6-dihydroxypyrene, 1,8-dihydroxypyrene, 1,6-pyrenequinone and 1,8-pyrenequinone were detected in the metabolites.^{6,7)} Among these metabolic products, 1,8-pyrenequinone showed the highest mutagenicity enhancing activity, and it was suggested that 1,8-pyrenequinone might play an important role in the exhibition of mutagenicity enhancing activity by pyrene.

We also reported that 1,8-pyrenequinone enhanced the mutagenicities of aromatic amines such as AAF, AF and AA, which usually require metabolic activation to reveal their mutagenicity.⁸⁾ However, the mutagenicities of the direct acting mutagens were not influenced by the addition of 1,8-pyrenequi-

* The mixture of Trp-P-1 (3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole) and Trp-P-2 (3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole).

none.⁸⁾

In this paper, the *in vitro* effects of 1,8-pyrenequinone as a modulator of the metabolism of AAF are studied and the mechanism for mutagenicity enhancing action of AAF by 1,8-pyrenequinone is discussed.

MATERIALS AND METHODS

Chemicals. AAF, AF, 1,4-naphthoquinone, 2-methylnaphthoquinone, 2-methylanthraquinone and Vitamin K₁ were of the purest grade available from commercial sources. 1,8-Pyrenequinone was synthesized by the method of Arnold *et al.*⁹⁾ N-Hydroxy-2-acetylaminofluorene (N-OH-AAF) was a generous gift from Dr. Y. Yamazoe, University of Keio, Tokyo. Ubiquinone 9 and ubiquinone 10 were generously supplied by Dr. T. Ikeda of this Institute. NADPH and glucose-6-phosphate (G-6-P) were purchased from Oriental Yeast Company Ltd., Tokyo.

Preparation of S-9, microsomes and cytosol. The S-9 fraction was prepared from the livers of 200 g male Sprague-Dawley rats induced 5 days prior to sacrifice by a single injection of polychlorinated biphenyl according to the method of Ames *et al.*¹⁰⁾ The microsomes and cytosol were prepared by centrifugation of the S-9 fraction at $105,000 \times g$ for 1 hr at 4°C. The supernatant and the pellet fraction were cytosol and microsomes respectively, and the latter was resuspended in cold 0.15 M KCl of the same volume as that of the original S-9. For the mutagenicity assay, cofactors such as NADPH and G-6-P were incorporated in the respective enzyme fractions.

Determination of AAF hydroxylase activity. The hydroxylase activity of AAF was determined according to the method of Cramer *et al.*¹¹⁾ The reaction mixture for assay contained 24 μmol of MgCl_2 , 100 μmol of KCl, 300 μmol of sodium phosphate buffer, pH 7.4, 10.44 μmol of NADPH, 13.44 μmol of G-6-P, 0.3 ml of S-9 and 0.23 μmol of AAF in 50 μl methanol with 0 to 0.86 μmol of 1,8-pyrenequinone in 0.2 ml methanol at the maximum. The total volume was between 3 and 3.25 ml. The mixtures were incubated in a shaker for various times at 37°C in a flask open to the air and the reaction was terminated by the addition of 1 ml of 38% KOH. Ten minutes later the neutral and basic compounds were extracted into 20 ml of a 1:1 mixture of diethyl ether and petroleum ether by vigorous shaking for 1 min, and the optical density of the organic phase was determined at 288 nm in a spectrophotometer.

Preparation of incubation mixture for chemical analysis. The incubation mixture contained 40 μmol MgCl_2 , 168 μmol KCl, 17.4 μmol NADPH, 22.4 μmol G-6-P, 0.5

mmol sodium phosphate buffer, pH 7.4, 0.5 ml microsomes and 0.45 μmol AAF in 0.1 ml methanol with (B) or without (A) 0.43 μmol 1,8-pyrenequinone in 0.2 ml methanol. The total volume was 5.1 ml or 5.3 ml and each reaction mixture was incubated for 30 min at 37°C in a flask open to the air.

Analysis of AAF and AF. The reaction mixture was extracted 3 times with approximately 40 ml of ether. The ether extracts were combined, evaporated to dryness and dissolved in 2 ml of methanol for analysis by high-pressure liquid chromatography (HPLC). HPLC was carried out using a JASCO Tri Rotar chromatograph connected with a JASCO UVIDEC-100-II spectrophotometer and a JASCO FP550 spectrofluorometer. A 25 cm Zorbax ODS column maintained at 50°C was eluted using 65% methanol in water at a flow rate of 1.5 ml/min. AAF was monitored by UV absorption at 280 nm. AF was detected by spectrofluorometer at an excitation wavelength (EX) of 290 nm and at an emission wavelength (EM) of 370 nm. The peak area of AAF was calculated by a Takeda Riken TR-2213 Integrator and for AF was calculated by a Shimadzu Chromatopac C-R1A. The AAF and AF contents of the sample solution were calculated from the calibration with authentic samples.

Analysis of N-OH-AAF. N-OH-AAF was analyzed according to the procedure described by Hinson *et al.*¹²⁾ The reaction was terminated by addition of 5 ml of acetone. The precipitated protein was separated by centrifugation, and the supernatant phase was decanted and extracted twice with approximately 40 ml of ether. The ether extracts were combined and evaporated to dryness. The residues were then dissolved in 2 ml of methanol and a sample of each was analyzed by HPLC. The HPLC assay was performed in a Shimadzu LC-3A chromatograph connected with a reverse-phase C₁₈ $\mu\text{Bondapak}$ column (3.9 $\times$ 300 mm) and a Shimadzu spectrophotometric detector SPD-1 at 535 nm. The carrier solvent consisted of 1 mM ferric chloride, 0.005 M heptane sulfonic acid and 1% acetic acid dissolved in 70% methanol in water. Peak area was calculated by a Takeda Riken TR-2213 Integrator. The N-OH-AAF content of the sample solution was calculated from the calibration with authentic sample.

Recovery. The methanol solutions of the authentic AAF, AF and N-OH-AAF were treated using the same procedure as that for the analytical sample. The percentages of AAF, AF and N-OH-AAF recovered by this method were 96.2 (± 1.2)%, 86.1 (± 1.1)% and 86.6 (± 3.6)%, respectively, from experiments repeated three times. The data indicated were corrected for the recovery.

Mutagenicity assay. Mutagenicity assays with *Salmonella typhimurium* TA98 were carried out using the standard plate test according to the method of Ames *et al.*¹⁰⁾ as previously described.³⁾ To a test tube containing

2 ml of molten top agar (0.6% agar, 0.6% NaCl, 0.1 μ mol histidine, 0.1 μ mol biotin) were added 0.1 ml of the tester strain, 3 μ g of AAF, the sample suitably diluted in dimethyl sulfoxide, and 0.5 ml of S-9 mix that contained 0.05 ml of S-9, 4 μ mol $MgCl_2$, 16.8 μ mol KCl, 1.74 μ mol NADPH, 2.24 μ mol G-6-P and 50 μ mol sodium phosphate buffer, pH 7.4. The tube contents were mixed and poured onto an agar over layer. The number of histidine revertant colonies per plate was counted after incubation for 2 days at 37°C and the results given are expressed as the average number of His⁺ colonies from duplicate plates.

RESULTS

Mutagenicity enhancing action of AAF by 1,8-pyrenequinone

The mutagenicity of AAF assayed with the addition of different amounts of 1,8-pyrene-

FIG. 1. Mutagenicity of the Mixture of 1,8-Pyrenequinone and AAF in the Presence or Absence of S-9 Mix. The mutagenicity of 1,8-pyrenequinone with (O) or without (●) AAF was assayed using *S. typhimurium* TA98 in the absence (----) or presence (—) of S-9 mix.

quinone is shown in Fig. 1. The number of His⁺ colonies in 3 μ g of AAF per plate was 170. The addition of 5 to 20 μ g of 1,8-pyrenequinone resulted in a 9- to 11-fold increase in the mutagenicity of AAF, but further increase in the amount of 1,8-pyrenequinone resulted in a decrease in the number of His⁺ colonies. 1,8-Pyrenequinone itself was not mutagenic toward *S. typhimurium* TA98.

Chemical reaction between AAF and 1,8-pyrenequinone

The mixed solution without S-9 mix, which contained AAF and 1,8-pyrenequinone, was shaken in a water bath maintained at 37°C. After 30 minutes an aliquot of the incubation mixture was subjected to thin-layer chromatography (TLC) and HPLC. Although the data are not shown, no incidence of any substances apart from AAF and 1,8-pyrenequinone was observed on analysis by TLC, and the amount of AAF eluted at the same retention time as the authentic AAF was unchanged on analysis by HPLC. This shows that a chemical reaction between AAF and 1,8-pyrenequinone did not occur in the solution and that 1,8-pyrenequinone would be comparatively stable in the assay medium.

Effect of 1,8-pyrenequinone on NADPH in S-9 mix

Quinones, particularly ubiquinones, play an important role as mediators of the electron transport from NADPH contained in S-9 mix.^{13,14} 1,8-Pyrenequinone may act as an electron acceptor from NADPH in S-9 mix.

TABLE I. EFFECT OF NADPH ON THE MUTAGENICITY ENHANCEMENT BY 1,8-PYRENEQUINONE

NADPH (μ mol/plate)	His ⁺ colonies per plate ^a			
	Spontaneous	AAF (3 μ g)	1,8-Pyrenequinone (10 μ g)	AAF + 1,8-Pyrenequinone
0	24	41	18	68
1.74	39	182	51	1098
3.48	47	179	50	1196
5.22	36	192	60	1215
8.70	44	197	49	1037

^a *S. typhimurium* TA98.

Consequently the mutagenicities of AAF with 1,8-pyrenequinone at various concentrations of NADPH and subsequently the effect of various quinones on the mutagenicity of AAF were firstly determined. As shown in Table I, the mutagenicity of the mixture of AAF and 1,8-pyrenequinone hardly changed with the addition of NADPH up to 5 times of the usual level (1.74 μ mol). In the absence of NADPH, the mutagenicity of AAF was not enhanced at all by the addition of 1,8-pyrenequinone. This suggests that NADPH is required for revealing the enhancement of mutagenicity, and that the mutagenicity enhancement by 1,8-pyrenequinone is not influenced by the amount of NADPH used in this assay condition.

Subsequent experiments examining the effect of various quinones on the mutagenicity of AAF were carried out. As shown in Table II, ubiquinone 9 and ubiquinone 10 did not enhance the mutagenicity of AAF. Naphthoquinone and its methyl derivative, and Vitamin K₁ also enhanced little the mutagenicity of AAF. 2-Methylanthraquinone resulted in a 2- to 3-fold increase in the number of His⁺ colonies of AAF, although the degree

of enhancement was much lower than that of 1,8-pyrenequinone. These results indicate that the enhancing action of mutagenicity may not be common for quinone compounds but it would be rather specific to 1,8-pyrenequinone.

Effect of 1,8-pyrenequinone on the mutagenicity of AAF in microsomes and cytosol

From the above results, it would seem that the mutagenicity enhancement of AAF is exhibited by the interaction between 1,8-pyrenequinone and S-9 mix. Therefore, the effects of 1,8-pyrenequinone on the mutagenicity of AAF were examined as to microsomes, cytosol, the reconstituted mixture of microsomes and cytosol, and S-9. As shown in Fig. 2, the mutagenicity of AAF was increased approximately 4-fold by the addition of 10 μ g of 1,8-pyrenequinone in the presence of microsomes, whereas it remained only at the spontaneous level in the presence of cytosol. The addition of microsomes in combination with cytosol to the assay medium markedly increased the mutagenicity of AAF with 1,8-pyrenequinone, which was approximately recovered at a level similar to the original S-9. These facts show that

TABLE II. EFFECT OF VARIOUS QUINONES ON MUTAGENICITY OF AAF

Experiment	Quinone	Dose (μ g/plate)	His ⁺ colonies per plate ^a	
			Without AAF	With AAF (3 μ g/plate)
1	None	—	41	158
	Ubiquinone 9	5	53	181
		10	48	162
	Ubiquinone 10	5	53	166
		10	37	191
2	None	—	30	173
	1,4-Naphthoquinone	5	34	209
		10	37	224
		50	31	221
	2-Methylnaphthoquinone	5	48	226
		10	34	210
	2-Methylanthraquinone	5	24	373
		10	29	496
	Vitamin K ₁	5	33	184
		10	35	171
		50	30	222

^a *S. typhimurium* TA98.

FIG. 2. Effect of 1,8-Pyrenequinone on the Mutagenicity of AAF in the Presence of Microsomes or Cytosol.

The mutagenicity of the mixture of AAF (3 μg) and 1,8-pyrenequinone was determined in the presence of microsomes (●—●), cytosol (○—○), S-9 (▲—▲) and both microsomes and cytosol (△—△).

cytosol plays a role together with microsomes in the mutagenicity enhancement of AAF by 1,8-pyrenequinone.

Effect of 1,8-pyrenequinone on hydroxylation of AAF

The activity of hydroxylation which is the first step of AAF metabolism was determined. The effect of incubation time on the hydroxylation of AAF by rat liver homogenates is shown in Fig. 3. Approximately 93% of AAF was converted to phenolic metabolites upon incubation for 30 min. The effect of 1,8-pyrenequinone on the hydroxylation of AAF is shown in Fig. 4. The incubation time with S-9 mix was 10 min. At equimolar levels, 1,8-pyrenequinone inhibited about 50% of the hydroxylation activity of AAF. The addition of 0.86 μmol of 1,8-pyrenequinone inhibited about 90% of the activity.

Determination of the metabolites of AAF with or without 1,8-pyrenequinone

The AAF, AF and N-OH-AAF contents contained in the incubation mixture of AAF with (sample B) or without (sample A) 1,8-pyrenequinone are shown in Table III. The amount of AAF detected in sample A corresponded to 1.6% of the original AAF, whereas the amount of AAF detected in sam-

FIG. 3. Effect of the Incubation Time on Hydroxylation of AAF by S-9 Mix.

Aliquots of individual mixtures containing 0.23 μmol of AAF were incubated at 37°C and extracted into 20 ml of a 1:1 mixture of petroleum ether and diethyl ether. The optical density of the organic phase was determined at 288 nm in a spectrophotometer.

FIG. 4. Effect of 1,8-Pyrenequinone on Hydroxylation of AAF by S-9 Mix.

The mixtures containing 0.23 μmol of AAF with or without 1,8-pyrenequinone were incubated at 37°C for 10 min. For details, see the legend of Fig. 3.

TABLE III. DETERMINATION OF THE METABOLITES OF AAF WITH OR WITHOUT 1,8-PYRENEQUINONE

Substrate	Metabolic product (nmol)		AAF (nmol)
	AF	N-OH-AAF	
AAF	2.9	5.8	7.1
AAF + 1,8-Pyrenequinone	43.4	42.3	179.6

Substrate: AAF, 450 nmol; 1,8-pyrenequinone, 430 nmol.

ple B corresponded to about 40% of the original AAF. This shows that the original AAF was mostly metabolized in the absence of 1,8-pyrenequinone while it remained relatively unmetabolized in the presence of 1,8-pyrenequinone.

The addition of 1,8-pyrenequinone caused about a 15-fold increase in the formation of AF from AAF. This shows that 1,8-pyrenequinone promoted the deacetylation of AAF.

The addition of 1,8-pyrenequinone caused about a 7-fold increase in the formation of N-OH-AAF from AAF. This shows that the addition of 1,8-pyrenequinone to rat liver microsomes stimulates the *N*-hydroxylation of AAF.

Effect of 1,8-pyrenequinone on the mutagenicity of N-OH-AAF in microsomes and cytosol

The effect of 1,8-pyrenequinone on the mu-

FIG. 5. Effect of 1,8-Pyrenequinone on Mutagenicity of N-OH-AAF in the Presence of Microsomes or Cytosol.

The mutagenicity of the mixture of N-OH-AAF (1 µg) and 1,8-pyrenequinone was determined in the presence of microsomes (●—●) and cytosol (○—○).

tagenicity of N-OH-AAF in microsomes or cytosol is shown in Fig. 5. N-OH-AAF showed higher mutagenic activity in the presence of cytosol than in the presence of microsomes. The mutagenicity of N-OH-AAF decreased almost linearly with the dose level of 1,8-pyrenequinone added in the presence of cytosol. However, approximately 40% of the mutagenic activity still remained with the addition of 20 µg of 1,8-pyrenequinone. On the other hand, there was little influence of 1,8-pyrenequinone on the mutagenicity of N-OH-AAF in the presence of microsomes.

DISCUSSION

A number of studies on the metabolism of AAF have provided important insights into the mechanism of chemical carcinogenesis. *N*-Hydroxylation is a rate-limiting step in the activation of AAF with subsequent deacetylation and this is the most important metabolic pathway in the activation of N-OH-AAF to form direct mutagens. Formation of a potent mutagen from N-OH-AAF by activation with a rat liver cytosolic fraction is shown in the *Salmonella* reversion system.¹⁵⁻¹⁸ This was also confirmed by our experiments (Fig. 5). Presumably the mutagenic species formed is *N*-hydroxy-2-aminofluorene (N-OH-AF), which is considered to be the ultimate mutagen.^{15,16,19} The main metabolic pathway of AAF and the working points of 1,8-pyrenequinone are summarized in Fig. 6.

About 40% of AAF remained unmetabolized in the incubation mixture of AAF by the

FIG. 6. Proposed Mechanism for the Mutagenic Activation of AAF with 1,8-Pyrenequinone.

The bold lines indicate the main pathway. NO-F, nitrosofluorene; PQ, 1,8-pyrenequinone; ×, inhibition by 1,8-pyrenequinone; ⊗, promotion by 1,8-pyrenequinone.

addition of 1,8-pyrenequinone (Table III). This was also supported by the fact that 1,8-pyrenequinone inhibited hydroxylation which is considered as the first step in the metabolism of AAF (Fig. 4). However, 1,8-pyrenequinone stimulated the metabolism of AAF to N-OH-AAF which is a weakly mutagenic intermediate of AAF (Table III). These facts demonstrate that 1,8-pyrenequinone do not inhibit the *N*-hydroxylation, *i.e.*, activating pathway, but inhibits the *C*-hydroxylation (ring-hydroxylation) of AAF, *i.e.*, detoxifying pathway.

1,8-Pyrenequinone inhibited the enzyme activity of cytosol which mediates the metabolism of N-OH-AAF to further mutagenic species (Fig. 5). However, cytosol maintained enough mutagenic potency to reveal the mutagenicity of N-OH-AAF (Fig. 5). As shown in Fig. 1, the addition up to 20 μ g of 1,8-pyrenequinone to the assay medium enhanced the mutagenicity of AAF, but the addition of 50 μ g of 1,8-pyrenequinone resulted in a decrease of mutagenicity. This may be due to an inhibition of cytosolic activity by the addition of 50 μ g 1,8-pyrenequinone.

When 1,8-pyrenequinone was added to the incubation mixture, a higher amount of AF was detected (Table III). Further metabolism of AF formed by microsomal enzymes may also produce N-OH-AF by the subsequent *N*-hydroxylation, whereas, as shown in Fig. 2, the mutagenicity of AAF showed only a few fold increase with the addition of 1,8-pyrenequinone in the presence of microsomes. Therefore, it seems likely that the metabolism of AAF to N-OH-AF through AF may not be the main pathway.

The mechanism of enhancing action on the mutagenicity of AAF by 1,8-pyrenequinone may be speculated as follows. In the metabolism of AAF, 1,8-pyrenequinone would inhibit the detoxification process (*C*-hydroxylation) and promote the activation process (*N*-hydroxylation). In consequence, N-OH-

AAF, a weak mutagenic intermediate, would be accumulated and then activated by the cytosolic fraction to form the ultimate mutagen, N-OH-AF.

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