[Chem. Pharm. Bull. 36(12)4963—4970(1988)]

Inactivation of Mutagenic Heterocyclic and Aryl Amines by Linoleic Acid 13-Monohydroperoxide and Methemoglobin

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(Received June 4, 1988)

The effect of the radical species generated by interaction of linoleic hydroperoxide (LOOH) with methemoglobin (MetHb) on the mutagenicity of 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1), 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2) and 2-aminofluorene to *Salmonella typhimurium* TA98 was investigated. Treatment of Trp-P-1 and Trp-P-2 with LOOH alone gave rise to mutagenicity in the absence of S-9 mix but the extent was very low. Mutagenicity of Trp-P-1 and Trp-P-2 in the presence of S-9 mix was decreased by LOOH. Mutagenicity of 2-aminofluorene in the presence of S-9 mix was increased by LOOH, probably owing to the conversion of its active metabolite into more active form(s). Treatment of Trp-P-1, Trp-P-2 and 2-aminofluorene with the radical species generated from LOOH/MetHb effectively decreased the level of the mutagens. The mutagenicity of the LOOH/MetHb-treated mutagens in the presence of S-9 mix was similarly retarded. The radical species effectively destroyed the mutagens by transforming them into non active form(s) that could not be activated by S-9 mix.

Keywords——3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1); 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2); 2-aminofluorene; linoleic acid 13-monohydroperoxide; methemoglobin; *Salmonella typhimurium* TA98

It has been demonstrated that the free radicals participate in the metabolic activation of mutagenic and/or carcinogenic aromatic hydrocarbons and arylamines.[1–7] Lipid hydroperoxides and the radical species generated during lipid peroxidation and suspected of participating in the metabolic conversion of the chemical mutagens or carcinogens.[3–6]

Mutagenic and carcinogenic heterocyclic amines are generated during pyrolysis of proteins and are present in processed foodstuffs.[8] Peroxidation of lipids takes place during processing of foodstuffs. It is thus important to investigate the effect of the free radical species generated during lipid peroxidation on the mutagenic heterocyclic and aryl amines. We have now investigated the effect of the radical species generated by interaction of linoleic acid 13-monohydroperoxide (LOOH) with methemoglobin (MetHb)[9–13] on the mutagenicity of 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1),[8] 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]-indole (Trp-P-2)[8] and 2-aminofluorene[14] to *Salmonella typhimurium* TA98. We report here that the radical species inactivated these heterocyclic and aryl amines rather than activated them.

Experimental

Materials——3-Amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1) acetate and 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2) acetate were obtained from Wako Pure Chemical Industries, Ltd., Osaka. 2-Aminofluorene was obtained from Aldrich Chemical Company, Inc., Milwaukee. Methemoglobin (MetHb) from human was the product of Sigma Chemical Company, St. Louis. Linoleic acid 13-monohydroperoxide (LOOH) was prepared by use of linoleic acid (Nippon Oil and Fats Company, Ltd., Tokyo) and lipoxidase type I, 130000 units/mg (Sigma Chemical Company) according to the method of Gardner.[15] Purity of the hydroperoxide estimated on the basis of the extinction coefficient at 234 nm (24500)[16] was 91—98%. It showed a peroxide value[17] of 5970—6350 meq/kg.

Analysis by High-Pressure Liquid Chromatography (HPLC)——HPLC was carried out on a Shimadzu LC-6A liquid chromatograph equipped with a column of YMC A-303 ODS (4.5 × 250 mm) (Yamamura Chemical Laboratories, Ltd., Kyoto). The chromatograph was operated with solvent systems of methanol/0.025 M phosphoric acid–disodium hydrogen phosphate (pH 3.0) (1 : 1, v/v) for Trp-P-1 and Trp-P-2, and acetonitrile/0.025 M phosphoric acid–disodium hydrogen phosphate (pH 3.0) for 2-aminofluorene at a flow rate of 0.5 ml/min. A 10 μl aliquot of the sample solution was injected for analysis. The peaks were detected by a Shimadzu SPD-6A UV spectrophotometric detector. The peaks due to Trp-P-1 and Trp-P-2 appeared at retention times of 17.5 and 12.3 min, respectively, when detected at 260 nm. The peak due to 2-aminofluorene appeared at a retention time of 16.0 min when detected at 280 nm. The peaks of these mutagens were not disturbed by those of LOOH and the other components formed. The amount of the mutagenic compounds was determined by comparing the peak height to that of the standard solution of the mutagenic compounds.

Bactericidal Effect of LOOH and LOOH/MetHb on *Salmonella typhimurium* TA98——A suspension (0.10 ml) of *Salmonella typhimurium* TA98[19] was incubated with a mixture of 0.10 ml of a solution of LOOH in dimethyl sulfoxide and 0.50 ml of 0.1 M phosphate buffer (pH 7.4) containing MetHb at 37 °C for 20 min. The S-9 mix preparation[19] was also used instead of the phosphate buffer. The bacterial suspension was diluted with 0.85% sodium chloride and plated on a nutrient agar plate in order to determine cell survival frequency.[20]

Mutagenicity of the Mutagens Treated with LOOH and LOOH/MetHb——Mutagenicity of the mutagens treated with LOOH and LOOH/MetHb was tested according to the method of Yahagi *et al.*[21] using *Salmonella typhimurium* TA98 with a slight modification. To 0.50 ml of 0.1 M phosphate buffer (pH 7.4) containing MetHb were added 0.05 ml of a solution of a mutagen in dimethyl sulfoxide and 0.05 ml of a solution of LOOH in dimethyl sulfoxide. The mixture was preincubated at 37 °C for 20 min, then a mixture of 0.10 ml of the *Salmonella typhimurium* TA98 srain suspension[19] and 0.50 ml of the phosphate buffer, or a mixture of 0.10 ml of the bacterial suspension and 0.50 ml of the S-9 mix preparation[19] was added. The reaction mixture was then incubated at 37 °C for 20 min. For mutagenicity assay, the whole incubation mixture was poured onto a minimal glucose agar plate after being mixed with top agar (2.0 ml). After a 2-d incubation at 37 °C the number of his^+ revertant colonies was counted. The microsomal system S-9 was prepared from the liver microsomes of rats which had been treated with polychlorinated biphenyls. The background numbers of his^+ revertant colonies/plate were 16—22 for TA98 without S-9 mix and 20—28 for TA98 with S-9 mix. The numbers of his^+ revertants of the positive controls in the present experiment were 196/0.1 μg of 4-nitroquinoline-1-oxide for TA98 without S-9 mix, 1030/0.1 μg of Trp-P-1, 2480/0.1 μg of Trp-P-2, and 1120/5 μg of 2-aminofluorene for TA98 with S-9 mix. Data are expressed after subtraction of the background number of his^+ revertant colonies. At least two experiments were done for each set of reactions and data are presented as mean values.

Results

Effect of LOOH and LOOH/MetHb on *Salmonella typhimurium* TA98

Prior to the examination of the effect of LOOH and LOOH/MetHb on the mutagenicity of the heterocyclic and aryl amines, their own bactericidal effect and mutagenicity to *Salmonella typhimurium* TA98 in the presence and absence of S-9 mix were investigated. In the absence of S-9 mix, LOOH revealed a bactericidal effect at doses higher than 100 μg but no significant effect at doses lower than 50 μg. In the presence of S-9 mix, no significant bactericidal effect was observed at LOOH doses up to 200 μg. A mixture of LOOH (20 μg) and various amounts of MetHb (< 1000 μg) did not show any significant bactericidal effect in the presence or absence of S-9 mix. It has been shown that linoleate hydroperoxides and related hydroperoxides are mutagenic to the TA98 strain in the presence of S-9 mix, but the number of his^+ revertant colonies does not exceed 300/100 μg of the hydroperoxide.[22-24] In the present investigations, the mutagenicity of LOOH (< 100 μg) and LOOH (20 μg)/MetHb (< 1000 μg) to the bacteria was not significant in the presence or absence of S-9 mix. Thus, LOOH and LOOH/MetHb at these doses could be used in the following experiments without taking their intrinsic bactericidal and mutagenic effects into consideration.

Effect of LOOH and LOOH/MetHb on the Mutagenicity of Trp-P-1 and Trp-P-2 to *Salmonella typhimurium* TA98

Trp-P-1 (2.5 μg) was preincubated with various doses of LOOH (< 100 μg) at 37 °C for 20 min. HPLC of the mixtures did not show any significant loss of the mutagen (Fig. 1A closed circles and solid line). The mixtures were then incubated with *Salmonella typhimurium*

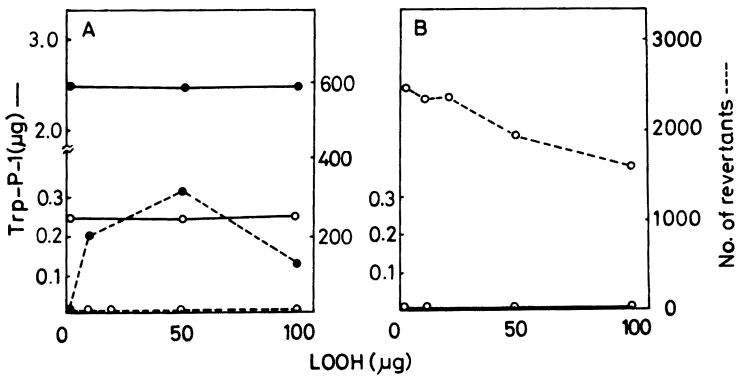

Fig. 1. Effect of LOOH on the Decrease and the Mutagenicity of Trp-P-1 in the Absence (A) and Presence (B) of S-9 Mix

Trp-P-1 (2.5 μg, ● or 0.25 μg, ○) was preincubated with the indicated amount of LOOH at 37 °C for 20 min. A: The amount of Trp-P-1 in the preincubation mixtures was determined by HPLC (——). Mutagenicity of the mixtures was measured on incubation with *Salmonella typhimurium* TA98 at 37 °C for 20 min (----). B: The preincubation mixture was incubated with S-9 mix at 37 °C for 20 min, and the amount of Trp-P-1 was determined by HPLC (——). The mutagenicity of the preincubation mixture was assessed by incubation with the bacteria and S-9 mix (----). All the data shown are the mean values of more than two experiments.

TA98 strain. Mutagenicity to the bacteria developed depending on the LOOH dose (Fig. 1A closed circles and dotted line), but the number of his^+ revertant colonies did not exceed 300 (12/0.1 μg Trp-P-1). When Trp-P-1 (0.25 μg) was treated similarly, neither loss of Trp-P-1 nor mutagenicity to the bacteria was observed (Fig. 1A open circles).

When the mutagenicity of untreated Trp-P-1 (0.25 μg) was tested in the presence of S-9 mix, it produced 2575 his^+ revertant colonies (1030/0.1 μg Trp-P-1). Metabolic conversion of Trp-P-1 into the active metabolite *N*-hydroxy-Trp-P-1[25] was complete, and this was little affected by LOOH (< 100 μg) (Fig. 1A solid line), indicating that the activity of S-9 was not destroyed by LOOH. Trp-P-1 treated with increasing doses of LOOH showed significantly decreased mutagenicity to the bacteria in the presence of S-9 mix; 40% of the mutagenicity was lost with 100 μg of LOOH (Fig. 1B dotted line). The active metabolite produced by S-9 mix might be partially converted into non active form(s) by LOOH. It seems likely that LOOH hardly reacted with Trp-P-1 but transformed its active metabolite into non active form(s).

A similar effect of LOOH was observed on the development of the mutagenicity of Trp-P-2. Trp-P-2 was not lost on incubation with LOOH alone (Fig. 2A), but it was inactivated by incubation with LOOH in the presence of S-9 mix (Fig. 2B). The active metabolite of Trp-P-2, *N*-hydroxy-Trp-P-2,[26] might be converted into non active form(s) by reaction with LOOH. This observation is consistent with that of Saito *et al.*[27] who demonstrated that the active metabolite was destroyed by peroxidized microsomal lipids.

Trp-P-1 (2.5 and 0.25 μg) was preincubated with mixtures of LOOH (10 μg) and various amounts of MetHb (< 1000 μg) at 37 °C for 20 min; the LOOH dose employed here hardly affected the mutagenicity of Trp-P-1 (Fig. 1B). HPLC of the mixtures revealed that the amount of the mutagen was dramatically decreased at the MetHb dose of 20 μg; 55% decrease for 2.5 μg of Trp-P-1 and more than 95% for 0.25 μg of Trp-P-1 (Fig. 3A solid line). This decrease was inhibited at higher doses of MetHb. The extensive loss of Trp-P-1 may be due to the conversion of the mutagen by the radical species (LOO· or LO·) generated by interaction of LOOH and MetHb.[9-13] The inhibition of the loss by a large amount of MetHb may be

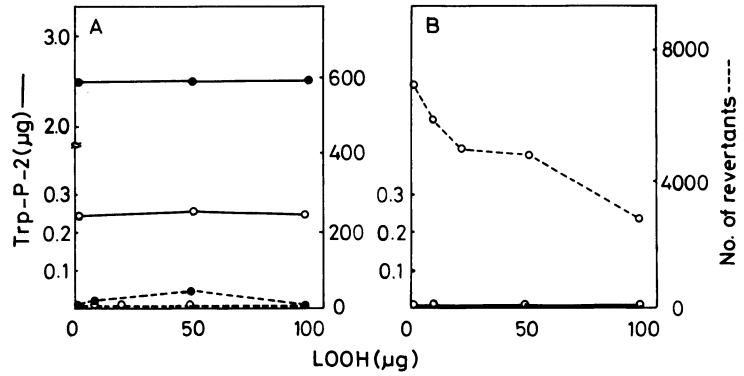

Fig. 2. Effect of LOOH on the Decrease and the Mutagenicity of Trp-P-2 in the Absence (A) and Presence (B) of S-9 Mix

Trp-P-2 (2.5 μg, ● or 0.25 μg, ○) was preincubated with LOOH. The amount of Trp-P-2 in the preincubation mixtures and the control incubation mixtures with S-9 mix was determined by HPLC (——), and the mutagenicity to *Salmonella typhimurium* TA98 (- - - -) was assayed as described in the legend to Fig. 1. All the data shown are the mean values of more than two experiments.

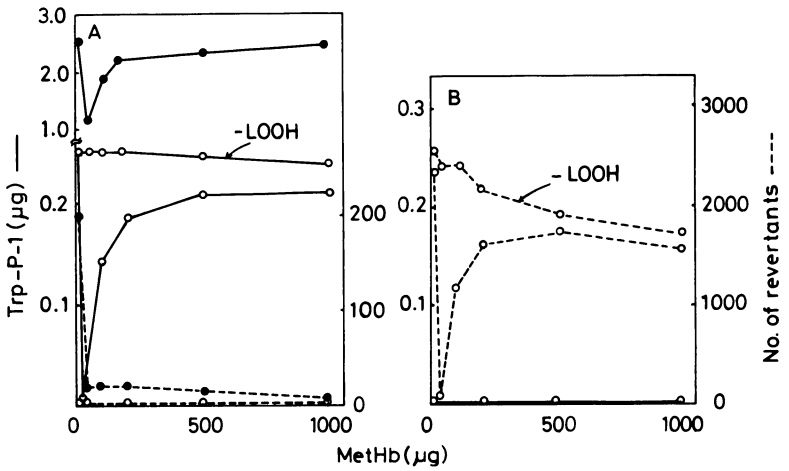

Fig. 3. Effect of LOOH/MetHb on the Decrease and the Mutagenicity of Trp-P-1 in the Absence (A) and Presence (B) of S-9 Mix

Trp-P-1 (2.5 μg, ● or 0.25 μg, ○) was preincubated with LOOH (10 μg) and the indicated amount of MetHb at 37 °C for 20 min. A: The amount of Trp-P-1 in the preincubation mixtures was determined by HPLC (——). Mutagenicity of the mixtures was measured on incubation with *Salmonella typhimurium* TA98 at 37 °C for 20 min (- - - -). B: The preincubation mixtures were incubated with S-9 mix at 37 °C for 20 min, and the amount of Trp-P-1 was determined (——). The mutagenicity of the preincubation mixtures was assayed by incubation with the bacteria and S-9 mix (- - - -). The effect of MetHb alone is also indicated. All the data shown are the mean values of more than two experiments.

due to scavenging of the radical species by MetHb.[9,28] Trp-P-1 treated with LOOH/MetHb at the MetHb dose of 20 μg showed no significant mutagenicity (Fig. 3A dotted line), indicating that the mutagen was converted into some non direct acting form(s) by the radical species.

When the mutagenicity of Trp-P-1 treated with LOOH/MetHb was tested in the presence of S-9 mix, the mutagenicity was completely lost at the MetHb dose of 20 μg, and this decrease was suppressed at higher doses of MetHb (Fig. 3B dotted line). This decrease of mutagenicity corresponded to the loss of the mutagens (Fig. 3A solid line). Thus, Trp-P-1 was effectively

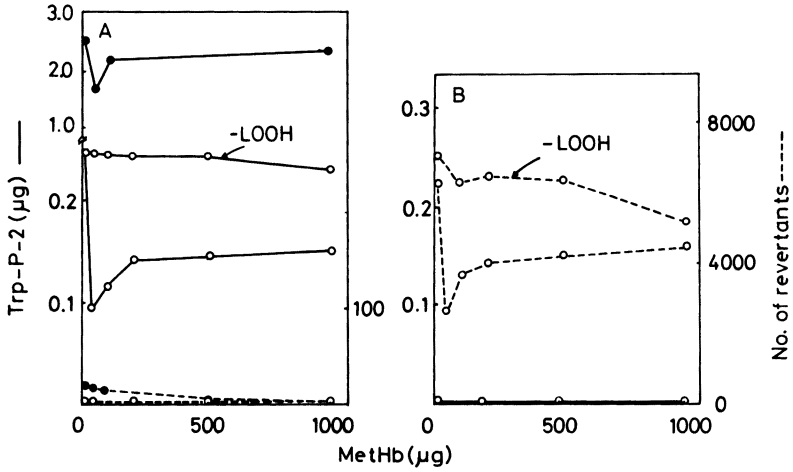

Fig. 4. Effect of LOOH/MetHb on the Decrease and the Mutagenicity of Trp-P-2 in the Absence (A) and Presence (B) of S-9 Mix

Trp-P-2 (2.5 μg, ● or 0.25 μg, ○) was preincubated with LOOH (10 μg) and MetHb. The amount of Trp-P-2 in the preincubation mixtures and the control incubation mixtures with S-9 mix was determined by HPLC (——), and the mutagenicity to *Salmonella typhimurium* TA98 (- - - -) was assayed as described in the legend to Fig. 3. The effect of MetHb alone is also indicated. All the data are the mean values of more than two experiments.

TABLE I. Summary of the Effect of LOOH and LOOH/MetHb on the Loss of the Mutagens and the Development of the Mutagenicity

Mutagen	LOOH				LOOH/MetHb			
	−S-9 mix		+S-9 mix		−S-9 mix		+S-9 mix	
	Loss	Mut.	Loss	Mut.	Loss	Mut.	Loss	Mut.
Trp-P-1	−	+	+++	++	+++	−	+++	−
Trp-P-2	−	+	+++	++	+++	−	+++	−
2-Aminofluorene	−	−	+++	+++++	++	+	+++	+
Control for each mutagen without LOOH and LOOH/MetHb	−	−	+++	+++	−	−	+++	+++

Mut. = mutagenicity.

degraded by the radical species into non mutagenic compound(s) that could not be activated in the presence of S-9 mix. MetHb alone slightly decreased the mutagenicity of Trp-P-1 in the presence of S-9 mix (Fig. 3B), which may be due to the interaction of MetHb with the active metabolite.[29] While each of LOOH (10 μg) (Fig. 1B) and MetHb (20 μg) (Fig. 3B) slightly decreased the mutagenicity of Trp-P-1, the combination of them much more effectively decreased the mutagenicity.

The effect of LOOH/MetHb on the loss and the development of the mutagenicity of Trp-P-2 (2.5 and 0.25 μg) was similar to that in the case of Trp-P-1 (Fig. 4). The radical species generated from LOOH/MetHb degraded the mutagen into non mutagenic compound(s) that could not be activated in the presence of S-9 mix.

The effects of LOOH and LOOH/MetHb on the loss of Trp-P-1 and Trp-P-2 and the development of the mutagenicity are summarized in Table I. LOOH might partially degrade

active metabolites into non active form(s), as has been shown by Saito *et al.*[27] The radical species (LOO· or LO·) generated from LOOH/MetHb effectively transformed Trp-P-1 and Trp-P-2 into non active form(s) that could not be activated by S-9 mix.

Effect of LOOH and LOOH/MetHb on the Mutagenicity of 2-Aminofluorene to *Salmonella typhimurium* TA98

2-Aminofluorene (5 μg) was incubated with various doses of LOOH (<100 μg). The incubation mixtures showed no decrease of the mutagen (Fig. 5A solid line) and no increase in the mutagenicity (Fig. 5A dotted line). Thus, 2-aminofluorene did not react with LOOH.

When the mutagenicity of untreated 2-aminofluorene (5 μg) was tested in the presence of S-9 mix, it produced 1120 his^+ revertant colonies. The conversion of the mutagen into its active metabolite *N*-hydroxy-2-aminoflorene by S-9 mix[30] was ascertained by complete loss of the mutagen. Treatment of 2-aminofluorene with increasing LOOH doses markedly increased the mutagenicity in the presence of S-9 mix (Fig. 5B dotted line). While treatment with LOOH (20 μg) gave only less than 20% increase, treatment with LOOH (100 μg) increased the mutagenicity by 2-fold. It seems likely that the active metabolite produced by S-9 mix was converted into more active form(s) by reaction with LOOH.

2-Aminofluorene (5 μg) was treated with a mixture of LOOH (10 μg) and various doses of MetHb (<1000 μg); the LOOH dose used did not affect the mutagenicity of the mutagen (Fig. 5B). The mutagen was decreased depending on the MetHb doses; maximal decrease (60%) occurred at the MetHb doses of 20—100 μg and slight decrease at the higher doses of MetHb (Fig. 6A solid line). While 2-aminofluorene treated with LOOH/MetHb showed a significant increase in the mutagenicity, the maximal number of his^+ revertant colonies was not more than 200/5 g of 2-aminofluorene at the MetHb doses of 20—100 μg (Fig. 6A dotted line). Certain active form(s) were produced during the loss of the mutagen.

When the mutagenicity of 2-aminofluorene treated with LOOH/MetHb was tested in the presence of S-9 mix, the mutagenicity was lowered to 60% at the MetHb dose of 100 μg (Fig. 6B dotted line). It is likely that LOOH/MetHb degraded the mutagen into non active form(s) rather than activated it. In contrast to the effective activation of 2-aminofluorene by LOOH (Fig. 5B), the active species generated from LOOH/MetHb must have destroyed the mutagen.

The effects of LOOH and LOOH/MetHb on the loss of 2-aminofluorene and the development of the mutagenicity are summarized in Table I. LOOH might convert the active

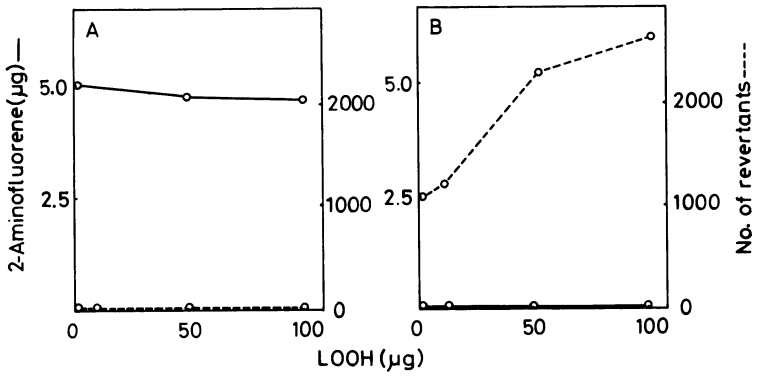

Fig. 5. Effect of LOOH on the Mutagenicity of 2-Aminofluorene in the Absence (A) and Presence (B) of S-9 Mix

2-Aminofluorene (5.0 μg) was preincubated with LOOH. The amount of 2-aminofluorene in the preincubation mixtures and the control incubation mixtures with S-9 mix was determined by HPLC (——), and the mutagenicity to *Salmonella typhimurium* TA98 (- - - -) was assessed as described in the legend to Fig. 1. All the data are the mean values of more than two experiments.

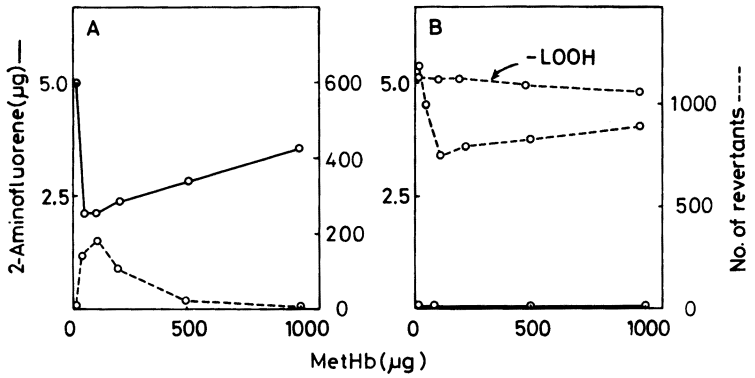

Fig. 6. Effect of LOOH/MetHb on the Decrease and the Mutagenicity of 2-Aminofluorene in the Absence (A) and Presence (B) of S-9 Mix

2-Aminofluorene (5.0 μg) was preincubated with LOOH (10 μg) and MetHb. The amount of 2-aminofluorene in the preincubation mixture and the control incubation mixtures with S-9 mix was determined by HPLC (——), and the mutagenicity to *Salmonella typhimurium* TA98 (– – – –) was assayed as described in the legend to Fig. 3. The effect of MetHb alone is also indicated. All the data are the mean values of more than two experiments.

metabolite of 2-aminofluorene into more active form(s). The radical species (LOO· or LO·) generated from LOOH/MetHb activated and destroyed the mutagen, and degradation was predominant.

Discussion

There are many reports demonstrating that free radical species from lipid oxidation participate in the metabolic conversion of heterocyclic and aryl amines. 2-Acetylaminofluorene is metabolized into *N*-hydroxy-2-acetylaminofluorene by microsomal enzymes and this is in turn converted into its nitroxyl radical by the radical from LOOH/MetHb or hematin as an intermediate of the more active carcinogenic species *N*-acetoxy-2-acetylaminofluorene and 2-nitrosofluorene.[31] 2-Aminofluorene is cooxidized into active free radical species during the conversion of prostaglandin G_2 into prostaglandin H_2 by the hydroperoxidase activity of prostaglandin endoperoxide synthetase.[32] Saito *et al.*[27] demonstrated that the active metabolite of Trp-P-2 is destroyed by peroxidized microsomal lipids.

In the present study, the direct effect of the radical species formed during lipid oxidation on the mutagenic heterocyclic and aryl amines was investigated. The radical species from LOOH/MetHb[9-13] effectively destroyed heterocyclic amine mutagens Trp-P-1 and Trp-P-2, and arylamine 2-aminofluorene. The radical species generated from LOOH/MetHb may be LOO· or LO· radical, which is the common species formed during lipid oxidation. The potency of the mixture of LOOH/MetHb depended on the amount of MetHb. In every experiment, a catalytic amount of MetHb (20—100 μg) was most effective to degrade the mutagens. It has been previously shown that the potency of the radical species (LOO· or LO·) was greatly lowered with increasing amount of MetHb.[28,33] The loss of potency caused by MetHb has been considered to be due to the radical scavenging property of the porphyrin ring of the MetHb.[9,28]

It may be postulated that treatment of heterocyclic and aryl amine mutagens with the radical species formed during lipid oxidation can effectively transform the mutagens into non active forms that cannot be activated by S-9 mix. Mutagenic and carcinogenic heterocyclic amines are generally produced and present in cooked foodstuffs.[8] The heterocyclic amines in

the foodstuffs can be partly destroyed by free radical species, when lipid oxidation simultaneously takes place during the cooking of the foods.

Acknowledgement We thank Mr. S. Horie and Mr. K. Mugiuda for their technical assistance. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

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