Rapid Paper

Reduction of Induced Mutability with Xanthine- and Imidazole-derivatives through Inhibition of Metabolic Activation[†]

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The xanthine-derivatives; theophylline, caffeine, and 3-isobutyl-1-methylxanthine showed a tendency to reduce the induced mutagenicities of Trp-p-2, AAF and B(a)P. This effect was seen only on the mutagens which show their mutagenicities by metabolic activation. As for the relationship between this effect and the structural specificity among these derivatives, the imidazole rather than the uracil moiety in xanthine was found to be more effective. Judging from the compounds tested, 1(N)-methyl-derivatives were more effective than their original compounds. It would be probable that the effect of these compounds may be due to their interference with the metabolism of these mutagens by S-9.

It has been found that the pyrolysis products of some amino acids and proteins are strongly mutagenic in *Salmonella typhimurium*^{1,2)} and some of them were confirmed to be carcinogenic in rats. With a view to securing our food supplies and environment, studies on the screening of factors suppressing such environmental mutagens have been made by many workers.^{3~5)}

In our previous paper,⁶⁾ we reported that some biologically active quinones, such as menadione, coenzyme Q, and adrenochrome, reduced the mutagenicity of the pyrolysis products of tryptophan when these quinones were added in the process of the activation of this mutagen. In the course of these studies, theophylline and caffeine were also found to reduce the mutagenicity of this mutagen. Therefore, we made further studies about the

effects of various xanthine-derivatives on the mutagenicities of various mutagens.

This paper deals with the connection between the reductability of mutagenicities and the structural specificity of these compounds.

MATERIALS AND METHODS

Assay method of mutagenicity. A mutation assay was carried out according to Ames *et al.*⁷⁾ with some modification.⁸⁾ The histidine-requiring strains of *Salmonella typhimurium* TA 98 and TA 100 were kindly supplied by Dr. B. N. Ames. Liver microsomal fractions (S-9) were prepared from rats previously treated with PCB as described by Ames *et al.*⁹⁾ and S-9 mix was prepared by the addition of NADPH, G-6-P and the other cofactors to the S-9 fraction. The test samples were dissolved in distilled water.

The mixture of the test strain, a properly diluted mutagen and S-9 mix or phosphate buffer was incubated at 37°C for 20 min and then poured onto an agar layer. After incubation at 37°C for 48 hr, His⁺ revertant colonies were counted. The mutagenicity test was at five concentration levels for each sample, but only the results giving the highest potency are shown in this paper.

Materials. Benzo(a)pyrene (B(a)P), 2-acetyl amino-fluorene (AAF), and 4-nitroquinoline-1-oxide (4NQO) were purchased from Sigma Chemicals Co. 3-Amino-1-methyl-5H-pyrido[2,3-b]indol (Trp-p-2) was kindly supplied by Dr. T. Kada, National Institute of Genetics, Japan. Xanthine, imidazole and uracil derivatives were obtained from Nakarai Chemicals Co. All other reagents used were of guaranteed grade.

Inhibitory test on mutagenicity. Among the various mutagens, we selected some varieties from the following two groups; Chemicals of the first group show their mutagenicities by metabolic activation with S-9 mix, such as Trp-p-2, AAF or B(a)P, and the other chemicals are those which show their mutagenicities without metabolic activation, such as 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (AF-2) and 4NQO.

Cells of *Salmonella typhimurium* TA 98 or TA 100 were treated at 37°C for 20 min with 0.1 ml of mutagen, with or without 0.25 ml of S-9 mix and 0.1 ml of a sample solution and the mutagenicity was tested by the standard method.⁸⁾ The result was compared with a control in which distilled water was used instead of the sample solution.

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RESULTS AND DISCUSSION

Effect of various xanthine-derivatives on mutagenicity

Table I(a) shows the effects of various xanthine-derivatives on the mutagenicities of Trp-p-2, AAF and B(a)P, all of which show their mutagenicities by metabolic activation with S-9 mix.

As shown in this table, the mutagenicities of the three markedly decreased when they were

incubated with S-9 mix in the presence of caffeine or theophylline. But in the cases of xanthine, theobromine, and adenine, the mutagenicities of the three decreased only moderately. On the other hand, the mutagenicities of AF-2 and 4NQO, which show their mutagenicities without metabolic activation, were scarcely affected by the addition of these compounds (data not shown). Reduction in survival by the addition of these compounds was not significant in the range of concentra-

TABLE I. EFFECTS OF VARIOUS XANTHINE, URACIL AND IMIDAZOLE DERIVATIVES ON THE MUTAGENICITIES OF SOME MUTAGENS

Cells of *Salmonella typhimurium* TA 98 or TA 100 were treated at 37°C for 20 min with 0.1 ml of mutagen, 0.25 ml of S-9 (5 µg/plate) containing cofactors with or without 0.1 ml of a sample solution, then poured onto a histidine-deficient agar medium. The plates were incubated at 37°C for 48 hr and the His⁺ induced revertant colonies were counted. The mean number of spontaneous revertants (40 for TA 98 and 120 for TA 100) was subtracted. The concentrations of all these samples were 200 µg per plate. The test strains used were *Salmonella typhimurium* TA 98 for the assay of Trp-p-2 and AAF, and TA 100 for that of B(a)P. The concentrations of the mutagens per plate were: for Trp-p-2, 0.1 µg; AAF, 10 µg; B(a)P, 5 µg.

(a) Xanthine derivatives

Sample	Trp-p-2	AAF	B(a)P	Surviving cells (× 10 ⁸ /0.1 ml)
Control	4,785	3,523	363	1.52
Xanthine (X)	1,374	866	295	1.53
Hypoxanthine	2,215	1,632	326	1.50
1,3-Dimethyl X (Theophylline)	257	232	183	1.52
3,7-Dimethyl X (Theobromine)	2,132	1,248	335	1.53
1,3,7-Trimethyl X (Caffeine)	112	123	162	1.51
3-Isobutyl-1-methyl X	43	92	125	1.50
Adenine	1,014	574	256	1.52

(b) Uracil derivatives

Uracil (U)	3,366	3,352	368	1.52
5-Methyl U (Thymine)	3,284	3,124	355	1.53
1,3-Dimethyl U	2,512	2,215	214	1.51

(c) Imidazole derivatives

Imidazole (I)	75	116	152	1.51
2-Methyl I	4,543	3,234	356	1.52
1-Methyl I (NMI)	32	73	112	1.51
2-Ethyl-4-methyl I	4,575	3,452	346	1.50

tions tested. Theophylline and caffeine were known to have an inhibitory activity on cyclic AMP phosphodiesterase [3.1.4.17]. From these results, we became interested in whether another inhibitor of this enzyme, 3-isobutyl-1-methylxanthine (IBMX), would have the same effect or not.

As shown in this table, among the xanthine derivatives tested IBMX was the most effective in decreasing the mutagenicities of these mutagens.

Structural specificity of the reduction of mutagenicity

Then we tried to confirm which moiety in the xanthine structure, uracil or imidazole, is responsible for the decrease of the mutagenicities of these mutagens.

Table I(b) shows the effect of uracil derivatives on the mutagenicities of these mutagens.

In this table, only 1,3-dimethyluracil among the uracil derivatives tested showed a moderate decrease of the mutagenicities of these

FIG. 1. Changes of the Reduction of Mutagenicities by the Concentrations of S-9.

A reaction mixture containing a test mutagen, cells of the test strain, IBMX and various concentrations of S-9 containing co-factors was incubated at 37°C for 20 min. After these treatments, the mutagenicities were assayed by the standard method. The test strains used were *Salmonella typhimurium* TA 98 for assay of Trp-p-2 and AAF, and TA 100 for that of B(a)P. Concentrations of the mutagens per plate were: for Trp-p-2, 0.1 μg; AAF, 10 μg; B(a)P, 5 μg. Concentration of IBMX was 200 μg per plate. Other experimental conditions are described in the text and in Table I. ●—●, Trp-p-2; ○—○, AAF; □—□, B(a)P.

mutagens.

As shown in Table I(c), however, imidazole itself and 1-methylimidazole (NMI) markedly decreased these mutagenicities. NMI especially was the most effective among all these compounds in Table I.

It is suggested through all these experiments than *N*-methyl derivatives are more effective in decreasing these mutagenicities than the original compounds.

Imidazole is known to have an inhibitory activity on cyclic AMP phosphodiesterase like xanthine derivatives. From these facts, inhibitory activity on this enzyme seem to have something to do with the decrease of mutagenicities. And it may be interesting to do further studies on this relationship.

Effect of S-9 concentrations on the reduction of mutagenicity

Figure 1 shows the effect of IBMX on the

FIG. 2. Dose-response Curves of IBMX on the Mutagenicities of Various Mutagens.

A reaction mixture containing a test mutagen with or without S-9 mix, cells of the test strain, and various concentrations of IBMX was incubated at 37°C for 20 min. After these treatments, the mutagenicities were assayed by the standard method. Concentrations of the mutagens per plate were: for Trp-p-2, 0.1 μg; AAF, 10 μg; B(a)P, 5 μg; AF-2, 0.047 μg; 4NQO, 5 μg. The mutagenicities of Trp-p-2, AAF and B(a)P were assayed with S-9 mix. The test strains used were *Salmonella typhimurium* TA 98 for assays of Trp-p-2 and AAF, and TA 100 for those of B(a)P, 4NQO and AF-2. Other experimental conditions are described in the text and in Table I. The numbers of revertant colonies of controls without IBMX were: for Trp-p-2, 5351; AAF, 3854; B(a)P, 365; AF-2, 932; 4NQO, 2216. ●—●, Trp-p-2; ○—○, AAF; □—□, B(a)P; ▲—▲, AF-2; △—△, 4NQO.

reduction of mutagenicities of Trp-p-2, AAF and B(a)P when they were activated in various concentrations of S-9.

The optimal dose of S-9 for revelation of the mutagenicities of these mutagens was 10 to 15 μg per plate (data not shown). As for the reduction of their mutagenicities, IBMX was the most effective at lower doses of S-9 than the optimum for the revelation. And as higher levels of S-9 were added, less effect of IBMX were observed. It would be probable that the effect of xanthine-derivatives may be due to their interference with the metabolism of these mutagens by S-9.

Dose-response curves of xanthine-derivatives

Figure 2 shows the dose-response curves of IBMX on the mutagenicities of the various mutagens assayed.

The mutagenicities of Trp-p-2 and AAF were markedly decreased by increasing the concentration of IBMX. The mutagenicity of B(a)P was also decreased moderately in the presence of IBMX. On the other hand, the mutagenicities of AF-2 and 4NQO were scarcely affected in the range of the concentrations of IBMX tested.

Effect of xanthine-derivatives on activated mutagens

It would be probable that xanthine-derivatives might inhibit the function of certain enzymes in S-9 by which Trp-p-2, AAF and B(a)P are activated. To ascertain this assumption, experiments were carried out where a sample was added before or after metabolic activation of the mutagens by S-9 mix, followed by an assay of their mutagenicities.

Figure 3 shows the reductive effect of NMI and IBMX added at the indicated periods during the process of activation of Trp-p-2 and AAF.

In this figure, maximum effectiveness of both of them were observed when they were added prior to the process of activation. The later the period of the process in which they were added, the less effects were observed. And

FIG. 3. Changes of the Reduction of Mutagenicities by Addition Times of Xanthine-derivatives.

A reaction mixture containing a test mutagen and S-9 mix was incubated at 37°C. At the regular intervals indicated, cells of the test strain and each sample were added to the mixture and the incubation was continued at 37°C for 20 min. After these treatments, the mutagenicities were assayed by the standard method. The test strain used was *Salmonella typhimurium* TA 98 for the assay of both mutagens. The concentrations of the mutagens per plate were: for Trp-p-2, 0.1 μg ; AAF, 10 μg . The concentrations of both NMI and IBMX were 200 μg per plate. Other experimental conditions are described in the text and in Table I. ●—●, Trp-p-2; ○—○, AAF.

if these compounds were added after 60 min, no effects on a decrease of mutagenicities were observed at all. From these results, the assumption was confirmed that the main effect of these compounds may be their inhibitory action on S-9 activating reactions on the mutagens.

Nagao *et al.*^{10,11)} reported that norharman may act as a co-mutagen with AAF with its interference of the balance between the activation and inactivation pathways of the mutagen. Kushi *et al.*¹²⁾ and we⁶⁾ reported that the effect of emodin or many biologically active quinones on the mutagenicities of Trp-p-2 or AAF may also be of the same mechanism as that for norharman. In this study, the xanthine and imidazole derivatives may also affect the mutagenicities of certain mutagens by their interference with the S-9 metabolic activation.

All of the mutagens used in this study are known to exist in food or in the environment, and their carcinogenicities have been proved on experimental animals. It is worth notice

that some foods, such as tea or coffee, contain these xanthine and imidazole derivatives, which may play an important part in preventing the toxicities of various carcinogens derived from food and the environment. And it might be reasonable to take tea or coffee during or after meals as a kind of counterpoisons for mutagens in foodstuffs.

Further studies might clarify how these xanthine and imidazole derivatives act *in vivo* against these mutagenicities.

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REFERENCES

- 1) T. Sugimura, T. Kawachi, M. Nagao, T. Yahagi, Y. Seino, T. Okamoto, K. Sudo, T. Kosuge, K. Tsuji, K. Wakabayashi, Y. Iitaka and A. Itai, *Proc. Japan Acad.*, **53**, 58 (1977).
- 2) T. Matsumoto, D. Yoshida, S. Mizusaki and H. Okamoto, *Mutation Res.*, **48**, 279 (1977).
- 3) T. Kada, K. Morita and T. Inoue, *Mutation Res.*, **53**, 351 (1978).
- 4) T. Inoue, K. Morita and T. Kada, *Agric. Biol. Chem.*, **45**, 345 (1981).
- 5) T. Yamaguchi, Y. Yamashita and T. Abe, *Agric. Biol. Chem.*, **44**, 959 (1980).
- 6) T. Yamaguchi, *Agric. Biol. Chem.*, **46**, 2373 (1982).
- 7) B. N. Ames, J. McCann and E. Yamasaki, *Mutation Res.*, **31**, 374 (1975).
- 8) T. Yahagi, *Tampakushitsu Kakusan Koso*, **20**, 1178 (1975).
- 9) B. N. Ames, W. E. Durston, E. Yamasaki and F. D. Lee, *Proc. Natl. Acad. Sci., U.S.A.*, **72**, 1132 (1975).
- 10) M. Nagao, T. Yahagi and T. Sugimura, *Biochem. Biophys. Res. Commun.*, **83**, 373 (1978).
- 11) M. Nagao, T. Yahagi, M. Honda, Y. Seino, T. Matsushima and T. Sugimura, *Proc. Japan Acad.*, **53**, 34 (1977).
- 12) A. Kushi, A. Koiwai, D. Yoshida and F. Goto, *Agric. Biol. Chem.*, **44**, 2513 (1980).