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INDUCTION OF MUTAGENIC ACTIVITY OF TETRACYCLINE BY SYNERGISTIC ACTION WITH NITRITE

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Abstract.....Synergistic mutagenicity of tetracycline(TC) and nitrite was investigated by the bacterial mutation test in the *Salmonella*/microsome system by using the reaction products obtained under neutral condition as well as under acidic condition (in simulated gastric juice).

Results from tests using *Salmonella typhimurium* TA 100 disclosed a significant increase in the appearance of histidine (+) (his^+) revertants by the reaction product between TC and nitrite in the presence of the rat liver-microsomal enzyme system(S9 mix), while the mutagenic potency of the reaction product of the two compounds in simulated gastric juice was extremely weak. In the process of the reaction of TC with and without nitrite in the presence of S9 mix, formaldehyde was detected, indicating the demethylation of TC by demethylase in S9.

To explain the induction mechanism of the synergistic mutagenicity of TC and nitrite, it was suggested that the alkylating reaction of nitroso compound formed by nitrosation of the 4-demethylated intermediate of TC by the aid of microsomal metabolism is more important than the well known nitrosation mechanism under acidic condition such as in gastric juice in rats and in simulated gastric juice.

Key words : Tetracycline, nitrosation, synergistic mutagenicity.

INTRODUCTION

In order to perform safety evaluations of the multiple chemicals contained in foods, studies on the synergistic effect following the interaction of multiple chemicals are of urgent importance since the daily human diet contains numerous kinds of chemicals.

Carcinogenic activity of *N*-nitroso compounds such as dimethylnitrosamine has been well established by administration of the compound (Clapp, et al., 1968 ; Clapp, 1973 ; Shimkin and Stoner, 1975) or by co-administration of nitrite and either secondary or tertiary amine in rodents (Shimkin and Stoner, 1975 ; Lijinsky and Greenblatt, 1972) and in other animals (Lijinsky and Epstein, 1970). Lijinsky has shown nitrosamine formation by the reaction of drugs and nitrite under acidic conditions in *in vitro* (Lijinsky et al., 1972 ; Lijinsky, 1974) and *in vivo* studies (Lijinsky and Greenblatt, 1972). The nitrite participating in nitrosation is considered to be readily formed by bacterial reduction of nitrate, which is frequently ingested as a common constituent of vegetables, a contaminant in drinking water or a preservative contained in meats and fish (Lijinsky and Epstein, 1970). TC is also widely used not only as an antibiotic for medical use but also as a food additive or a pesticide (Bacteriocide). The *N*-dimethylamine residue of TC as well as many other *N*-dimethyl compounds has been considered as an acceptor of the nitro-group to form dimethylnitrosamine (DMNA) under the acidic conditions mentioned above. Besides this, the mechanism of nitrosation of TC after single demethylation of the residue by demethylase in S9 may also be considered, because we observed synergistic mutagenicity of TC and nitrite at neutral pH in *S. typhimurium* TA 100 in the presence of S9 mix. In the present study, we pursued a possibility to induce synergistic mutagenicity by alkylation of bacterial DNA with methyl diazohydroxide (MDHO : known as a proximate carcinogen of DMNA) produced from the hypothetical nitroso-TC (III in Figure 1), which resulted from the reaction of TC with nitrite via the metabolic process as shown in Figure 1.

MATERIALS AND METHODS

Tetracycline, nicotineamide diphosphate (NADP) and glucose-6-phosphate (G6P) (each guaranteed grade) were purchased from Sigma Chemical Company. 2-Nitrofluorene and dimethylnitrosamine were from Wako Pure Chemical Industries, Ltd., aflatoxin B₁ from Makor Chemicals, Ltd. (Israel) and 4-dimethylaminoantipyrine (aminopyrine) from Aldrich Chemical Company, Inc.. Sodium nitrite, benzo(α)pyrene and the other chemicals (each guaranteed grade) were from Nakarai Chemicals, Ltd..

Rat liver microsome fraction (S9) was prepared from Sprague-Dawley rats treated with Aroclor 1254 by the method of Ames et al. (1975), and the fraction was purified further by differential centrifugation at 105,000 $\times g$ for 90 min to obtain partially

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Figure 1. Hypothetical metabolic pathway for nitrosation of tetracycline (TC) with nitrite (NO_2^-).

purified microsomes for some experiments.

S. typhimurium histidine-requiring (his^-) mutants TA 98 and 100 were kindly supplied by Dr. S. Iwahara of the Hatano Research Institute, Food and Drug Safety Center, Japan. The mutagenicity test was conducted in the *Salmonella*/microsome mutagenicity assay on plates according to the method of Ames et al. (1975) using TA 98 and 100 strains. The mutagenic potency was determined according to the protocol of Ames (MaCann et al., 1975 ; MaCann and Ames, 1976) ; results were regarded as positive when a reproducible, dose-related increase in the number of histidine-independent (his^+) revertant colonies per plate and a greater than two-fold increase in spontaneous mutation rate were obtained. The potency of chemicals was expressed by the number of his^+ revertants/nmol compound.

Formation of formaldehyde (CH_2O) resulted from *N*-demethylation of TC in the presence of S9 with cofactors (S9 mix) was measured by the Nash reaction described by Mazel (1972). Details of the experimental conditions are mentioned in the legend for Table 2. At the end of the reaction, zinc sulfate and saturated barium hydroxide were added in turn to stop the reaction, and precipitated protein was removed by centrifugation at 18,000 $\times g$ for 10 min. The supernatant fluid (5 ml) was mixed with 2 ml of Nash reagent, heated in a water bath for 30 min at 60°C and then cooled to room temperature. The absorbance (OD) was read at 415 nm. The amount of CH_2O formed was determined from a standard curve prepared using authentic CH_2O . Controls were also run without substrate or S9, and the average OD of the control flasks was subtracted from the average OD of the test flasks.

Nitrosation of TC with nitrite in simulated gastric juice (SGJ) was performed by the method of Lijinsky et al. (1972b, 1974). In brief, the SGJ was prepared with 2 g of NaCl and 24 ml of 10 % HCl/liter according to the recipe of Japan Pharmacopoeia VIII (Ishizawa et al., 1979). The reaction was carried out in the SGJ with an excess of nitrite at pH 3.5 for 4 hrs at 37°C, and was terminated by adding KOH up to pH 11. Nitrosated product was extracted twice either with ethyl ether, from which DMNA was extracted, or with *n*-butanol, from which TC and its metabolite were extracted. These extracts were back extracted with a small volume of 5 N hydrochloric acid and were evaporated to a smaller volume (1/10 volume of the original reaction mixture) at room temperature in a stream of nitrogen gas. For the Ames test, the evaporated material was adjusted to pH 7.2 with 0.1 M solution of sodium phosphate, dibasic.

For extraction of nitrosated TC in the reaction mixture treated under neutral condition in the presence of S9 mix, the reaction was stopped by adding 5,000 ppm ammonium sulfamate to decompose the residual nitrite. The mixture was extracted twice with butanol after the removal of lipid with *n*-hexane, and then evaporated to a small volume in a stream of nitrogen gas.

Amount of DMNA and nitrosated TC was determined using thin layer chromatography (TLC) on a silica gel plate (Linea-K plate, Whatman Inc.) with a solvent system of ether:*n*-hexane:ethanol (20:11:1) and butanol:*n*-hexane:ethanol (20:11:1), respectively, according to the modified methods by Weisburger, et al. (1980) and Ishizawa, et al. (1979). Five μl of the concentrated extracts from the reaction mixture under neutral and acidic conditions were spotted on the TLC plates and developed with the solvent described above. Authentic DMNA was dissolved in ether to 20 mg/ml and 5 μl was spotted on a TLC plate. DMNA was detected by spraying the plate with ninhydrin solution. The limit of DMNA detection by this TLC method was 0.2 μg . Authentic TC was dissolved in 5 mg/ml in butanol and 5 μl was spotted on a TLC plate. TC and its metabolites were detected by UV lamp. The limit of TC detection was 0.02 μg . Detection of nitroso compounds was made with combined eluate from the same spots on several TLC plates by Liebermann's nitroso reaction.

RESULTS

As a preliminary experiment to obtain synergistic mutagenicity of TC and nitrite, *S. typhimurium* TA 98 and 100 were incubated with various concentrations of TC and nitrite in the presence or absence of S9 mix. The reverse mutation of the his^- mutants was observed 48 hrs after incubation of each reaction mixture on histidine-deficient agar plates. As shown in Figure 2A and 2B, regardless of the presence or absence of S9 mix, neither nitrite nor TC alone showed distinct mutagenicity in either bacteria at concentrations of 25 to 200 $\mu\text{g}/\text{plate}$ and 0.2 to 1.0 $\mu\text{g}/\text{plate}$, respectively (only the result obtained with *S. typhimurium* TA 100 is shown). At higher concentrations, TC inhibited while nitrite stimulated slightly the appearance of spontaneous revertants. Based on these data, synergistic mutagenicity of TC and nitrite was examined at concentrations of 100 or 200 μg nitrite /plate and 0.1 to 1.0 μg TC/plate. As shown in Figure 2C and Table 1, a significant increase in his^+ revertants in TA 100 was observed only by co-existence of TC and nitrite in the presence of S9 mix. His^+ revertants in TA 98 also appeared under the same conditions ; however, in this case, the mutation rate was not significant when judged from the criteria for mutagenicity shown in the legend to Table 1. Under our experimental conditions, benzo(α)pyrene, aflatoxin B₁, 2-nitrofluorene and DMNA (all included as standard mutagens) induced a sufficient number of his^+ revertants (Table 1). These results suggested that TC, through its reaction with nitrite by the aid of metabolism of microsomal enzyme system, acquired a mutagenic potency detectable in the *S. typhimurium* TA 100 system.

As the mechanism(s) of induction of this synergistic action, it is possible to consider the alkylating reaction of MDHO via nitroso-TC, which was formed by nitrosation of 4-*N*-demethylated intermediate of TC by the microsomal metabolism as shown in Figure 1. To check this possibility, formaldehyde, which was expected to be released by oxidative *N*-demethylation of TC, was measured using Nash's method. As a preliminary experiment, *N*-demethylase activity of S9 was determined for various concentrations of S9 using aminopyrine as a standard substrate. The results showed that the maximum activity (Table 2) was obtained when 75 to 100 mg protein of S9 were incubated with 5 to 10 μmoles of aminopyrine in the presence of cofactors of the enzyme at 37°C for 30 to 60 min (only essentials of data are shown). When the partially purified microsome was used instead of S9, no significant release of CH_2O was detected. Based on these results, the experimental condition for the measurement of demethylation was arranged so that substrate was incubated with 100 mg protein of S9 for 40 min. Various concentrations of TC were incubated with the S9 and the cofactors in the absence or presence of nitrite. As shown in Table 2, significant CH_2O production was observed at 0.02 μmole or more of TC, and the maximum production was seen at more than 2 μmoles of TC. CH_2O production was slightly stimulated in the presence of nitrite at concentrations of more than 100 μmoles . Thus it was confirmed that TC was metabolized by *N*-demethylase to produce CH_2O , and

Figure 2. Synergistic mutagenicity of tetracycline and nitrite in *Salmonella typhimurium* TA 100.
(* : Spontaneous revertants were subtracted)

Table 1. Synergistic mutagenicity of tetracycline and nitrite by Ames test.

Chemicals	S9 mix	Revertants* per plate(μ g)		Revertants per nmole		Mutagenicity**
		TA 100	TA 98	TA 100	TA 98	
Tetracycline(TC)	- or +	0/0.5	0/0.5	0	0	—
Nitrite(NaNO_2)	- or +	<65/800	<18/800	<0.005	<0.002	—
TC(0.5 μ g) +	+	128/(0.5) +		(113) +		+
NaNO_2 (200 μ g)	—	0/(0.5) +		0		—
	+		23/(0.5)		46	— ?
	—		<18/(0.5)		<36	—
Dimethylnitrosamine	+	636/1,000		0.05		+
	—	<65/10,000		<0.001		—
	- or +		<18/10,000		<0.001	—
Benzo(α)pyrene	+	944/5.0	260/1.0	48	66	+
Aflatoxin B ₁	+	380/0.025	590/0.1	4750	1844	+
2-Nitrofluorene	—	1887/1.0	870/5.0	402	37	+

* : Histidine revertants on a petri plate per μ g chemicals tested (spontaneous revertants have been subtracted). For non-mutagenic compounds, a less-than figure is used to indicate the results; the numerator is 1/2 the spontaneous revertant colonies of the strain used (usually 18 for TA 98 and 65 for TA 100), and the denominator is the maximum allowable dose which is not inhibitory to the test system. For mutagenic compound, a positive results is defined as a reproducible, dose-related increase in the number of revertant colonies per plate and a greater than twofold increase in spontaneous mutation rate were obtained; the figures indicate number of revertants per amount of chemical(μ g) at the middle point on linear region of the dose-response curve.

** : —, for non-mutagenic (<0.01 revertant/nmol), +, for mutagenic.

() + : indicated as amount of TC.

that the metabolic rate was slightly stimulated by the addition of nitrite, probably by consequent progression of the reaction from *N*-monomethylated TC to nitroso-intermediates, as shown in the scheme of Figure 1. A large volume of reaction mixture of TC and nitrite was prepared under the same conditions as those for the maximum demethylating reaction in Table 2 (at least 5 times larger volume and the same concentrations), and the formation of metabolites of TC was examined by the TLC method after butanol extraction of the reaction mixture. A new spot was obtained in addition to TC, and the eluate from this spot showed Liebermann's nitroso reaction-positive (blue color).

Lijinsky et al. reported that TC was nitrosated with nitrite to produce DMNA in gastric juice in *in vivo* (1972a) or *in vitro* systems (1972b, 1974). To compare the result obtained under the metabolic nitrosation condition mentioned above, the mutagenic potency of the product derived from TC and nitrite under acidic condition was then investigated. Various amounts of TC and nitrite were incubated in SGJ according to the method described in METHODS, and the resultant extracts from the

Table 2. Determination of microsomal tetracycline demethylation by rat liver S9 fraction.

Chemicals (μmol)		NaNO_2 (μmol)	CH_2O released [#] (nmol)
AP	0.0	0.0	0.0
	5.0	0.0	$328.4 \pm 9.8(4.6)^*$
	10.0	0.0	$340.2 \pm 9.6(3.8)^*$
TC	0.002	0.0	0.0
		0.2	2.5
		2.0	1.7
	0.02	0.0	9.0
		0.2	25.0
		2.0	18.5
	0.2	0.0	46.0 ± 3.1
		2.0	56.3 ± 4.0
		20.0	54.8 ± 5.1
		100.0	49.3 ± 5.8
	2.0	0.0	102.4 ± 6.1
		20.0	110.9 ± 6.3
		100.0	127.6 ± 6.8
		200.0	103.3 ± 5.2
	5.0	0.0	100.0 ± 4.8
		20.0	108.1 ± 5.5
		100.0	133.9 ± 7.0
		200.0	133.6 ± 6.7
	10.0	0.0	103.2 ± 5.3
		20.0	111.7 ± 5.7
		100.0	127.4 ± 6.4
		200.0	136.6 ± 6.9

A various amount of TC or aminopyrine (AP) was incubated with 100 mg protein of S9 fraction in the presence of NADP (0.65 μmol), G6P (10.0 μmol), nicotineamide (50 μmol), and MgCl_2 (25 μmol) in 0.5 M Na^+/K^+ phosphate buffer (pH 7.4) (total volume 6.0 ml) at 37°C for 40 min. After the incubation, formaldehyde (CH_2O) production was determined by the Nash reaction.

: CH_2O nmol/100 mg of S9 protein/40 min.

()* : AP was incubated with 100 mg protein of the partially purified microsome instead of S9 fraction.

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Table 3. Mutagenic activity of the reaction product of TC and nitrite in simulated gastric juice.

Expt. No.	Chemicals (μ mol) in SGJ reaction		Solvent for extraction	Dilution of sample	S9 mix in the test system	Number of revertants* in	
						TA 100	TA 98
	TC	NaNO ₂					
I	0.1 ^a	0	Ether	no	+or—	6	0
		100				0	1
		200				8	3
		300 ^a				5	0
	0.5	0	"	"	"	3	5
		100				4	0
		300				0	3
	2.0	0	"	"	"	0	0
		100				7	3
		300				3	2
II	0.1 ^a	0	Butanol	1/100	+	0	0
		100				68	10
		300 ^a				70	13
	0.5	0	"	1/500	+ ^b	0	0
		100				72	11
		300				64	9
III	0.5	AP	Butanol	1/50	+	0	
		NaNO ₂				138**	0
		0				230**	3
		100					5
		300					

TC with or without nitrite was incubated in SGJ (total volume 2.5 ml) at 37°C for 4 hrs. Extraction from the reaction mixture was made with ether or butanol. The extracts obtained were then back extracted with HCl, and concentrated as described in "METHODS". The extract with ether was subjected to the Ames test without any dilution. The extract by butanol was diluted 100 or 500 times, because of lower dilution inhibited the appearance of spontaneous revertants, possibly due to residual TC in the reaction mixture.

* : Subtracted with spontaneous revertants.

** : Significant (positive) mutagenicity.

a : The same ratio but 100 times greater amounts of TC and nitrite than those for the synergistic mutagenicity in TA 100 under neutral condition (Table 1).

b : Purified microsomal fraction was used instead of S9.

reaction mixture were subjected to the Ames test. As shown in Table 3, the ether extracts showed no mutagenic potency in either bacteria, neither in the presence or absence of S9 mix (Expt. 1 in Table 3), while the butanol extract showed a certain (though not positive) mutagenic potency in TA 100 in the presence of S9 mix. In this mutagenicity test, it was found that the purified microsomal fraction (lacking demethylase) was equally as effective as S9, providing evidence against the possibility of enzymatic nitrosation of TC with nitrite being carried over into the test system by the extraction. The extracts from reaction mixtures of aminopyrine and nitrite induced considerable increase of the his⁺ revertants in TA 100 in the presence of S9 mix (Table 3-III). DMNA was not detected in the ether extract from the reaction mixture of TC and nitrite; however, when the butanol extract was examined, a new spot, which was the same as that seen in the extract from the reaction mixture for enzymatic nitrosation, was detected by the TLC method.

DISCUSSION

While safety evaluation of food chemicals is progressing, the synergistic effect of multiple chemicals remains to be evaluated. We have studied a particular mutagenesis by using a combination of multiple food chemicals and have found synergistic mutagenicity in the bacterial mutation of TC and nitrite in the presence of S9 mix. The formation *in vitro* or *in vivo* of DMNA from nitrite and either TC, other amines or amides under acidic conditions has been reported by Lijinsky et al. as described above. In our present study, significant synergistic mutagenicity of TC and nitrite was observed at neutral pH in *S. typhimurium* TA 100 only in the presence of S9 mix. There are no reports on the formation of nitrosamines under neutral conditions except that of microbial metabolism in saliva (Tannenbaum et al., 1978) or the *in vitro* study with *E. coli* strains (Calmels et al., 1985). To explain the results of the present study, we proposed a hypothetical scheme for the formation of nitrosamine (nitroso-TC) from TC and nitrite by metabolism in the microsomal enzyme system as shown in Figure 1. One of the key points in this hypothetical metabolic pathway is the *N*-demethylation process of TC by S9 with the concomitant release of formaldehyde, indicating the presence of active *N*-demethylase in S9 fraction. In fact, a significant release of formaldehyde was proven in our reaction system. Further, the release was slightly stimulated by addition of nitrite, suggesting an acceleration of the reaction by exhausting the demethylated intermediates through concomitant nitrosation. The analysis of the metabolite in the reaction mixture by the TLC method suggested the formation of nitroso-TC, as indicated by the positive Liebermann's nitroso reaction of the eluate from the metabolite spot.

When the ether extract from the reaction mixture of TC and nitrite in SGJ was tested, synergistic mutagenicity was not observed regardless of the presence or absence of S9 mix, showing no production of DMNA. In fact, no DMNA by TLC was detected in the ether extract from the reaction mixture of various concentrations of TC

and nitrite. Kawanishi et al. (1981) also reported a negative result in the formation of DMNA by gastric incubation of oxytetracycline and nitrite in rats and guinea pigs. When butanol extract was used as the reaction product, however, a weak (though not significant) mutagenicity was observed in TA 100 in the presence of either S9 mix or the partially purified microsomal enzyme system. In this case, in addition to TC, a faint new spot, which was the same as that produced by the microsomal reaction system, was observed on the TLC plate, suggesting the formation of a nitroso-TC (Figure 1-III). These results seem to suggest that TC is nitrosated to produce nitroso-TC but not DMNA in SGJ, and that the nitroso-TC extracted with butanol induces reverse mutation in TA 100 through further metabolism to form MDHO by the microsomal enzyme system as shown in Figure 1. This assumption is based on the fact that 1) it is not possible to consider the synergistic mutagenicity of residual TC and nitrite in this test system because the butanol extract may have contained unreacted TC but not nitrite, and 2) the purified microsomal fraction (lacking demethylase) was also effective as well as S9.

In general, the major organ in the mammalian body where nitrosation of amines and amides takes place is considered to be the stomach, and nitrosamine formation in the stomach is considered to be intimately related to the genesis of gastric cancer. However, the results of the present experiments revealed that the mutagenic activity of the product derived from the reaction between TC and nitrite in SGJ was considerably weak as compared with that of the product synthesized by the microsomal enzyme system. This finding seems to indicate that if the liver is an additional site for nitrosamine formation by some amines (or amides) and nitrite, the risk of liver cancer by the same proximate carcinogen (MDHO) as DMNA might increase, since the liver of mammals including man is known to be susceptible to DMNA (Lijinsky and Epstein, 1970 ; Clapp et al., 1968), and since methylated purines were detected in human or rat liver DNA after exposure to DMNA (Herron and Shank, 1980 ; Cooper et al., 1985). Recently a significant increase of bladder cancer mortality among workers occupationally exposed to aromatic amines was reported by Decarli et al. (1985). Although the induction mechanisms of the cancer were not studied in this case, if some of the cancers were due to nitrosamines from the aromatic amines, nitrosamine formation under non-acidic condition(s) might be responsible for the mechanism, considering the non-oral exposure of the workers to the chemicals. These results indicate the need for further studies on the nitrosation of other amines or amides by the enzyme system in liver as well as other organs.

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