Biochem. Biophys. **1950**, *25*, 231.
Nolte, A. J.; Pulley, G. N.; von Loesecke, H. W. *Food Res.* **1942**, *7*, 236.
O'Keefe, A. E.; Ortman, G. C. *Anal. Chem.* **1966**, *38*, 760.
Shaw, P. E. "Citrus Science and Technology"; Nagy, S., Shaw, P. E., Veldhuis, M. K., Eds.; Avi Publishing Co.: Westport, CT, 1977a; Vol. 1, p 427.
Shaw, P. E. "Citrus Science and Technology"; Nagy, S., Shaw, P. E., Veldhuis, M. K., Eds.; Avi Publishing Co.: Westport, CT, 1977b; Vol. 1, p 463.
Stahl, W. H., Ed., "Compilation of Odor and Taste Threshold Values Data"; American Society for Testing and Materials: Philadelphia, PA, 1973.
Veldhuis, M. K. "Fruit and Vegetable Juice Processing Technology", 2nd ed.; Tressler, D. K., Joslyn, M. A., Eds.; Avi Publishing Co.: Westport, CT, 1971; p 41.
Wolford, R. W.; Attaway, J. A. *J. Agric. Food Chem.* **1967**, *15*, 369.

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Mutagenicity of 2-Alkyl-*N*-nitrosothiazolidines

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N-Nitrosothiazolidine and its 2-alkyl derivatives, which formed in the browning reaction with nitrite, were tested for mutagenicity by using the Ames *Salmonella*/microsome mutagenicity assay. Some *N*-nitrosothiazolidines showed positive mutagenic responses toward *Salmonella typhimurium* TA 100, and the order of mutagenic potency relative to their 2-alkyl substituents was as follows: unsubstituted > isopropyl > propyl > ethyl > butyl > isobutyl > methyl. The metabolic activation system (S-9 mix) was not required to detect mutagenicity. In fact, the addition of S-9 mix strongly suppressed the mutagenic activity of *N*-nitrosothiazolidines. The factors which caused deactivation of the mutagens in S-9 mix were also investigated.

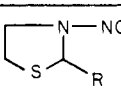
Tremendous numbers of chemicals are produced when foods are cooked. These chemicals include many nitrogen- and sulfur-containing compounds such as pyrazines, thiophenes, and thiazoles (dehydrogenated products of thiazolidines) (Wilson et al., 1973; Persson and von Sydow, 1973; Mussinan and Walradt, 1974). Extensive research in flavor chemistry has led to the isolation and identification of hundreds of these chemicals. Recently, increasing attention has been paid to the potential mutagenicity and carcinogenicity of these chemicals (Kosuge et al., 1978; Yamamoto et al., 1978; Yoshida, 1979).

Sakaguchi and Shibamoto (1978) observed the formation of thiazolidine and its alkyl derivatives by heating an aqueous cysteamine and acetaldehyde mixture in the course of their study of a nonenzymatic browning model system. Thiazolidines have a strong roasted flavor and can be readily nitrosated with sodium nitrite. The resultant nitroso derivatives showed some mutagenicity on *S. typhimurium* (Mihara and Shibamoto, 1979). In the present study, the effect of the alkyl side chain substituents of *N*-nitrosothiazolidines on their mutagenicities was investigated. The effect of the metabolic activation system (S-9 mix) and the mechanism of suppression of the mutagenicity of *N*-nitrosothiazolidines by S-9 mix were also discussed.

EXPERIMENTAL SECTION

Materials. Thiazolidine and its 2-alkyl derivatives were prepared from cysteamine and the corresponding fatty aldehydes (Nakarai Chemicals Ltd., Kyoto) as described by Ratner and Clarke (1973). Nitrosation of thiazolidines was performed as described by Ray (1978). The *N*-nitrosothiazolidines tested for mutagenicity are shown in Table I. Dimethyl sulfoxide (Me_2SO), polychlorinated biphenyl (PCB), and *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (NMNG) were purchased from Wako Pure Chemical (Osaka). 5,6-Benzoflavone (BF), benzo[*a*]pyrene (BP), and phenobarbital (PB) were obtained from Aldrich Chemical (Milwaukee, Wis.), Sigma Chemical (St. Louis, Mo.), and Yamazen Chemical (Osaka), respectively. 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide (AF-2) was donated by Dr. T. Matsushima (Tokyo University). All other chemicals were also obtained from reliable sources.

Table I. Chemicals Tested for Mutagenicity

	abbrev	R	mol wt
N-nitrosothiazolidine	NT		118
2-methyl-*N*-nitroso-thiazolidine	MNT	CH_3	132
2-ethyl-*N*-nitroso-thiazolidine	ENT	CH_3CH_2	146
2-propyl-*N*-nitroso-thiazolidine	PNT	$CH_3CH_2CH_2$	160
2-isopropyl-*N*-nitroso-thiazolidine	IPNT	$(CH_3)_2>CH$	160
2-butyl-*N*-nitroso-thiazolidine	BNT	$CH_3CH_2CH_2CH_2$	174
2-isobutyl-*N*-nitroso-thiazolidine	IBNT	$(CH_3)_2>CHCH_2$	174

Metabolic Activation System. Rat liver homogenate (S-9) was obtained from male Sprague-Dawley rats (200–210 g) which were injected either with PB and BF or with PCB, or from rats which were fed PB in their drinking water (Ames et al., 1975). Fifty microliters (per plate) of S-9 mix prepared from BP plus BF-induced rats was used in most cases. The amount of S-9 mix per plate (50 μL/plate or 100 μL/plate) did not lead to significant differences in the results.

Mutagenicity Test. Mutagenicity tests were conducted

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Table II. Suppression Effect of S-9 Obtained from Rats Treated with Various Inducers on the Mutagenicity of *N*-Nitrosothiazolidine and 2-Isopropyl-*N*-nitrosothiazolidine (The Number of Revertants without S-9 Was Taken as 100%[a])

chemicals tested	amount, μmol/plate[b]	PB + BF induced S-9, %	PCB-induced S-9, %	PB-induced S-9, %
N-nitrosothiazolidine (NT)	0.3	22	15	19
2-isopropyl-*N*-nitrosothiazolidine (IPNT)	1.0	40	37	42

[a] (Number of revertants with S-9)/(number of revertants without S-9) × 100. Number of revertants after substraction of blank values were used. [b] No killing of bacteria was observed at these concentrations.

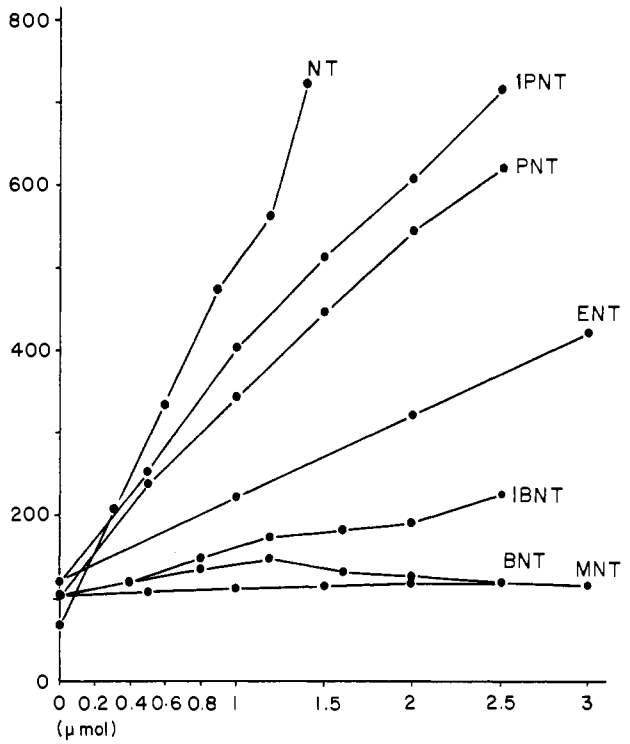

Figure 1. Mutagenicity of alkylnitrosothiazolidines on TA 100 without S-9. Number of revertants per plate is plotted against the amount of chemical per plate. Each point is the average of at least triplicate runs.

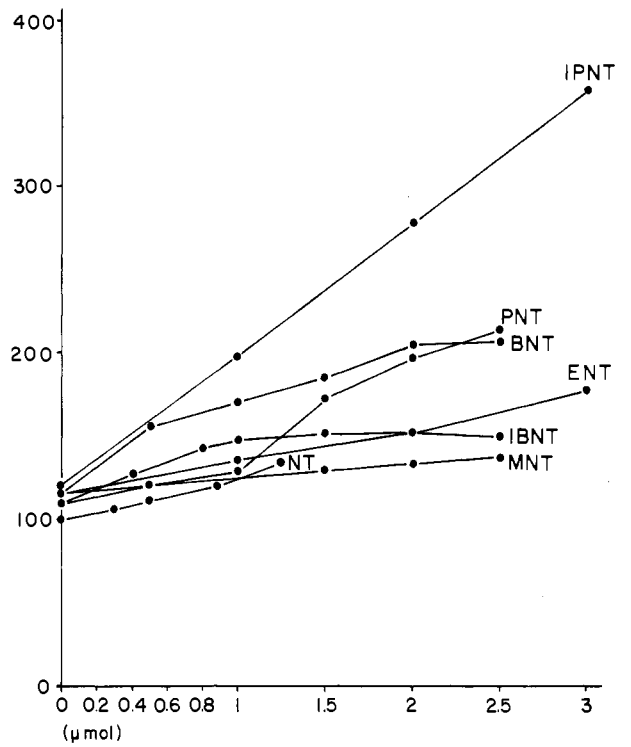

Figure 2. Mutagenicity of alkylnitrosothiazolidines on TA 100 with S-9 mix. Number of revertants per plate is plotted against the amount of chemical per plate. Each point is the average of at least triplicate runs.

as described by Umezawa et al. (1978), whose methods are a modification of those described by Ames et al. (1975). *Salmonella typhimurium* strains TA 100, TA 98, and TA 1535, which were developed by Dr. B. N. Ames, were used in preliminary tests in order to determine the strain most sensitive to the chemicals under study. Test chemicals were dissolved in 0.05 mL of Me_2SO immediately before the experiment and added to 0.5 mL of S-9 mix or 0.1 M sodium phosphate buffer (pH 7.4) with 0.1 mL of bacterial culture. The S-9 mix contained 50 μmol of sodium phosphate buffer (pH 7.4), 16.5 μmol of KCl, 4 μmol of $MgCl_2$, 2.5 μmol of glucose 6-phosphate, 2 μmol of NADH, 2 μmol of DADPH and 0.05 mL of S-9, which was replaced by boiled S-9 or by 0.15 M KCl in certain experiments. Preincubation was performed at 30 °C for 30 min before plating. AF-2 and BP were routinely used as positive controls to ensure the optimal activity of S-9 mix. Mutation frequency was determined from the slope value of a linear dose–response curve (an average of three–five replications). The number of spontaneous revertants per plate for each strains were as follows: 80-115 for TA 100, 26-33 for TA 98, and 17-18 for TA 1535.

RESULTS AND DISCUSSION

Strain TA 100 was used in all experiments since it exhibited the highest sensitivity toward *N*-nitrosothiazolidines. The preincubation method was adopted because it too gave higher sensitivity than the direct plate method. All *N*-nitrosothiazolidines showed various levels of mutagenic activity in the absence of S-9 mix (Figure 1). Unsubstituted nitrosothiazolidine showed the strongest mutagenicity and methylnitrosothiazolidine the weakest. Mutagenic responses increased, however, with increasing alkyl chain length in the order methyl < ethyl < propyl < isopropyl. Mutagenic responses were reduced by butyl or isobutyl substituents. Another distinctive feature in the

Table III. Suppression Effect of S-9 Mix of Various C Compositions on the Mutagenicity of Unsubstituted Nitrosothiazolidine and Several Well-Known Mutagens (Number of Revertants without S-9 Mix Were Taken as 100% for NT, AF-2, and NMNG[a] and with Intact S-9 Mix Was Taken as 100% for PB[b])

chemical	amount, μmol/plate	with intact S-9, %	with boiled S-9, %	with KCl, %
NT	0.9	24	21	60
BP	0.04	100	1	0.5
AF-2	0.0001	22	78	75
NMNG	0.01	0.5	74	130

[a] (Number of revertants with S-9 mix)/(numbers of revertants without S-9 mix) × 100. [b] (Number of revertants with S-9 mix)/(number of revertants with intact S-9) × 100. Number of revertants after subtraction of blank values were used.

mutagenic responses of these chemicals was that their mutagenic activities were suppressed by the addition of the S-9 metabolic activation system (Figure 2) Suppression of mutagenicity may have occurred due to the lower viability of the cells in the presence of S-9 mix. This possibility was ruled out, however, since not only was no growth inhibition detected under the microscope with respect to background colonies on the plate, but also the survival of the cells was found to be the same or even better in the presence of S-9 mix. PB plus BF-induced S-9, described as showing nearly the same levels of metabolic activity as PCB-induced S-9 for several nitroso compounds (Matsushima et al., 1976), was used. This S-9 may be able to specifically deactivate mutagenic metabolites of *N*-nitrosothiazolidines. Both PCB- and PB-induced S-9 showed suppression effects on the mutagenicity of NT or IPNT at the same level as that caused by PB plus BF-induced S-9 (Table II).

In order to examine the possibility that enzymes in the liver homogenate deactivate the mutagen, boiled S-9 was used in the S-9 mix. As shown in Table III, although boiling was sufficient to deactivate the liver enzymes, which were required for metabolic activation of BP, suppression of mutagenicity of NT was nonetheless observed with boiled S-9. Cofactors and ions in the S-9 mix were also examined to determine their roles in the suppression effect. The S-9 mix containing everything but S-9 was prepared for this purpose. Although S-9 mix without S-9 reduced the number of revertants slightly, it was not as effective as the complete S-9 mix (Table III). Well-known mutagens such as NMNG (a nitroso compound) and AF-2, which do not require liver enzymes for mutagenicity expression, were tested in parallel with nitrosothiazolidine. With S-9 mix addition, mutagenicity of NMNG was almost completely abolished and that of AF-2 was strongly suppressed (Table III). However, when boiled S-9 was used in the S-9 mix, the mutagenic activities of NMNG and AF-2 were reduced only slightly. This suggests that the active forms of these mutagens were deactivated by liver enzymes in these cases. Other factors may affect the results when S-9 mix without S-9 is used, but they are not clear at this stage.

Strain specificity in the mutagenicity of 2-isopropyl-*N*-nitrosothiazolidine and 2-ethyl-*N*-nitrosothiazolidine to revert strains TA 100 and TA 1535 (base-pair substitution strains) was reported by Rao et al. (1979). *N*-Nitrosothiomorpholine (NTM), *N*-nitroso-1,3-oxazolidine (NO), and *N*-nitrosopyrrolidine (NP), which have chemical structures similar to *N*-nitrosothiazolidine, have been reported as carcinogens (Garcia et al., 1970; Lijinsky and Taylor, 1976; Wiesler and Schmahl, 1976). NP requires S-9 mix for expression of mutagenicity, but its 3-hydroxy derivative is active without S-9 mix. Nitrosothiazolidines, which have sulfur in the β position to the nitrosated N in their rings, show mutagenic activity in the absence of S-9 mix. The mutagenicity of nitrosothiazolidines was partially suppressed by unknown factors in the S-9 mix. Apparently this suppression can proceed nonenzymatically since it was also observed when boiled S-9 was used. Ascorbic acid, creatine, and some amino acids such as serine and cysteine have been reported to inhibit the mutagenicity of NMNG (Gutterplan, 1977). Several antioxidants are also known to be effective in preventing mutagenicity and carcinogenicity of chemicals (Shamberger et al., 1979). There is a possibility that these compounds may also reduce the mutagenicity of nitrosothiazolidines. Further study will be needed to elucidate this point.

There have been several reports on the differences in mutagenicities of nitroso compounds with alkyl chains of various lengths. With dialkylnitrosamines, the longer the alkyl chain, the stronger the mutagenic activities up to butyl substitution (Yahagi et al., 1977). With alkylnitrosoguanidines, the bigger the alkyl group, the weaker the mutagenicity up to the hexyl derivative (Nagao et al., 1975). The reason for the differences in mutagenic responses with different alkyl substituents in the case of alkylnitrosothiazolidines is not yet clear. However, in a preliminary observation, NT was found to be metabolized faster than MNT or IPNT during preincubation.

Thiazolidines have not been reported in foods; thiazoles and thiazolines, however, which are dehydrogenated forms of thiazolidines, have been found in many foods (Maga, 1975). Further study is needed on the possible occurrence of preformed thiazolidines and *N*-nitrosothiazolidines in food systems and also on the potential toxicity and/or carcinogenicity of this class of nitrosamines.

LITERATURE CITED

Ames, B. N., MaCann, J., Yamasaki, E., *Mutat. Res.* **31**, 347 (1975).
Garcia, H., Keefer, L., Lijinsky, W., Wenyon, C. E. M., *Z. Krebsforsch.* **74**, 179 (1970).
Gutterplan, J. P., *Nature (London)* **268**, 368 (1977).
Kosuge, T., Tsuji, K., Wakabayashi, K., Okamoto, T., Sudo, K., Iitaka, Y., Itai, A., Sugimura, T., Kawachi, T., *Chem. Pharm. Bull.* **26**(2), 611 (1978).
Lijinsky, W., Taylor, H. W., *Cancer Res.* **36**, 1988 (1976).
Maga, J. A. *CRC Crit. Rev. Food Sci. Nutri.* **6**, 153 (1975).
Matsushima, T., Sawamura, M., Hara, K., Sugimura, T., "In Vitro Metabolic Activation in Mutagenesis Testing", Elsevier/North-Holland Biochemical Press, Amsterdam, 1976.
Mihara, S., Shibamoto, T., *J. Agric. Food Chem.* **28**, 63 (1980).
Mussinan, C. J., Walradt, J. P., *J. Agric. Food Chem.* **22**, 827 (1974).
Nagao, M., Yahagi, T., Nakadate, M., Kawachi, T., Sugimura, T., *Jpn. J. Genet.* **50**, 403 (1975).
Persson, T., Von Sydow, E., *J. Food Sci.* **38**, 377 (1973).
Rao, T. K., Ramey, D. W., Lijinsky, W., Epler, J. L., *Mutat. Res.* **67**, 21 (1979).
Ratner, S., Clarke, H. T., *J. Am. Chem. Soc.* **59**, 200 (1937).
Ray, S., *J. Chromatogr.* **153**, 173 (1978).
Sakaguchi, M., Shibamoto, T., *J. Agric. Food Chem.* **26**, 1179 (1978).
Shamberger, R. J., Corlett, C. L., Beaman, K. D., Kasten, B. L., *Mutat. Res.* **66**, 349 (1979).
Umezawa, K., Sawamura, M., Matsushima, T., Sugimura, T., *Cancer Res.* **38**, 1782 (1978).
Wiesler, M., Schmahl, D., *Z. Krebsforsch.* **88**, 25 (1976).
Wilson, R. A., Mussinan, C. J., Katz, I., Sanderson, A., *J. Agric. Food Chem.* **21**, 873 (1973).
Yahagi, T., Nagao, M., Seino, Y., Matsushima, T., Sugimura, T., Okada, M., *Mutat. Res.* **48**, 121 (1977).
Yamamoto, T., Tsuji, K., Kosuge, T., Okamato, T., Sudo, K., Takeda, K., Iitaka, Y., Yamaguchi, K., Seino, Y., *Proc. Jpn. Acad., Ser. B* **54**(5), 248 (1978).
Yoshida, D., *Kagaku to Seibutsu* **17**(1), 18 (1979) (Japanese), Abst. No. 90:180935y.

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