of oxidation. As expected, the rate of oxidation (as expressed by loss in reduced glutathione) by the hydroperoxide is somewhat slower than the rate observed using the more soluble oxidants and faster than the oxidation by the poorly soluble benzoyl peroxide.

In all of the reactions it appears that there is a rapid initial oxidation of reduced glutathione, followed by a slower oxidation and/or reduction of oxidized products by reduced glutathione. The reaction between the sulfhydryl group and sulfinic acid has recently been reported in detail by Finlayson et al. (1979). Snow et al. (1975) earlier reported the reaction between methionine sulfoxide and cysteine. We suggest that a similar interaction takes place with cystine monoxide and dioxide; i.e., cystine monoxide and dioxide can be reduced by cysteine to cystine.

Oxidations such as these, when taking place in food systems, may have great significance due to the reduction in the nutritional quality of the protein. Nutritional losses, however, may only be the tip of the iceberg. The oxidized products discussed here are quite reactive and can lead to further decomposition, losses in protein functionality, undesirable flavor, and potentially toxic compounds. Considerably more investigation in this area is needed, but this work has demonstrated that the oxidations do occur in model systems and that the products can be measured effectively by proton NMR.

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Chemical Aspects of Mutagen Formation by Sorbic Acid–Sodium Nitrite Reaction

Mitsuo Namiki,* Toshihiko Osawa, Harue Ishibashi, Kazuko Namiki, and Keiichi Tsuji

The effect of reaction conditions on the yield of individual products in the reacted mixture of sorbic acid and sodium nitrite was investigated by using TLC and high-performance LC methods. It became clear that mutagenicity of the reaction mixture that reached maximum by the reaction at pH 3.5–4.2 is due to the formation of the product Y (1,4-dinitro-2-methylpyrrole) and ethylnitrolic acid (ENA). The yields of Y and ENA reached maxima at 30 min (at 60 °C), but ENA decreased thereafter. Y and ENA gave maximal yield at 8-fold excess of nitrite to sorbic acid, but their formation was detected even by reaction at 1:0.5 molar ratio. These chemical results well explained the observed pronounced effects of reaction conditions on biological activities. Ascorbic acid and cysteine above certain levels inhibit effectively the mutagen formation in this reaction system.

Induction of mutagenic activity toward bacteria by heating sorbic acid with sodium nitrite in aqueous medium has been reported earlier (Kada, 1974). By subsequent studies we have isolated several products of this reaction and examined their biological activities (Namiki and Kada, 1975; Namiki et al., 1980; Kito et al., 1979). It was shown that the maximum mutagenicity was produced by the reaction at pH 3.5–4.2, by the rec assay (Kada et al., 1972) and by the standard Ames reversion assay without metabolic activation (Ames et al., 1975). The growth-inhibitory activity of the reaction mixture differed from the mutagenic activity in that it was intensified by lower reaction pH. Considering the widespread use of these chemicals as food additives, it seemed to warrant closer examination of the reaction conditions that produce mutagenic activity, as a preparatory study of the possibility of the occurrence of this type of reaction in actual food systems. The present work mainly deals with the reaction of the product dis-

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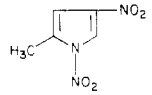
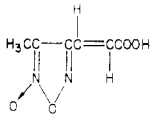
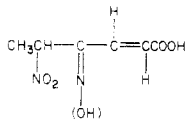
Table I. Biological Activities of Individual Isolated Products[a]

product	rec assay with *B. subtilis*	Ames assay with *S. typhimurium* TA 98	Ames assay with *S. typhimurium* TA 100	growth inhibition with *E. coli* B
ethylnitrolic acid (ENA) 	++	−	+	+
1,4-dinitro-2-methylpyrrole (product Y) 	+++	++	++	+
product B, $C_5H_6O_4N_2$ (−NO_2, −NO, −CHO)	±	−	−	++
product F 	−	−	−	(negative)
pre-F (syn + anti) 	−	−	−	(negative)

[a] Bioassay: see Namiki et al. (1980).

tribution of the biological activities of the mixture reacted under varying conditions. It is to be noted that the concentration of sorbic acid in the following experiments was in most cases 20 mM which is the officially permitted maximum level in foods, but that of the nitrite was usually very much higher than the permitted levels; therefore, the results do not directly apply to the actual situations in food processing. Effects of ascorbic acid and cysteine on the formation of mutagenic products were also briefly investigated.

MATERIALS AND METHODS

Sorbic acid and sodium nitrite were of guaranteed grade. TLC plates were prepared from Wakogel B5FM, a silica gel preparation containing several fluorescent materials that gave developed spots that appeared in characteristic and easily recognizable colors under mixed UV light. In the early stage of the study, the solvent was chloroform–methanol (95:5) and, later, *n*-hexane–acetic acid–ethyl acetate–chloroform (45:6:10:1). A Waters chromatograph was used for high-performance LC studies, with μBondapak C_{18} as the column and water–methanol (85:15) as the solvent. The products were detected by UV absorption at 254 nm, and a flow rate of 1.5 mL/min was employed throughout the work. Nitrite concentration in the solution was measured by a nitrogen oxide sensitive electrode and an ion meter (Orion 95-46-00 electrode and Ionalyser 407A).

The reaction of sorbic acid with sodium nitrite was carried out as described before (Namiki et al., 1980). In a typical example, 40 mL of 25 mM sorbic acid solution was stirred in 60 °C bath after rough adjustment of pH, and 10 mL of 0.8 M sodium nitrite was added to it at once. The pH of the solution was maintained constant by automatic addition of 1 N sulfuric acid by means of pH stat.

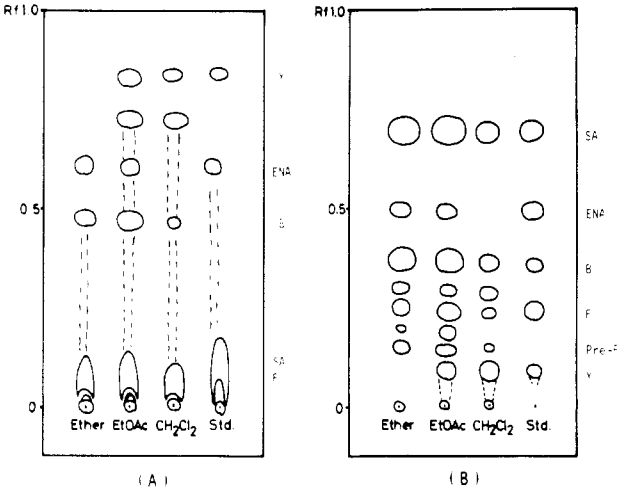


Figure 1. TLC of the reaction products of sorbic acid with sodium nitrite extracted with different solvents. Sorbic acid (10 mM) and sodium nitrite (80 mM) reacted at pH 3.5 and 60 °C for 30 min. TLC was developed by (A) chloroform–methanol (95:5) and (B) *n*-hexane–acetic acid–ethyl acetate–chloroform (45:6:10:1). Std: standard samples (isolated and purified substances from the reaction mixture). SA: sorbic acid. See Table I for identities of other products.

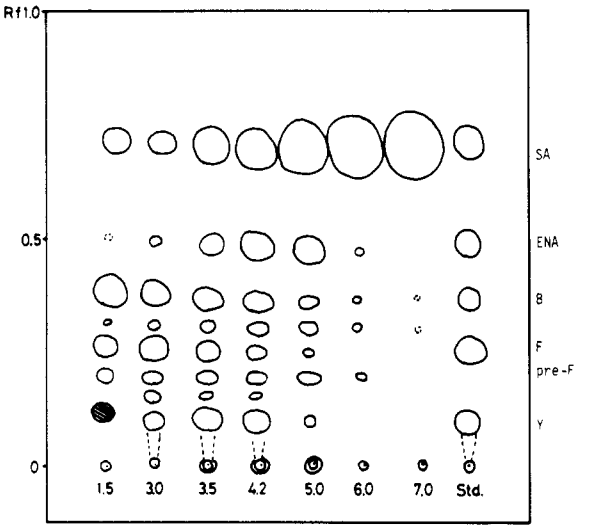


Figure 2. Effect of reaction pH on product formation. Sorbic acid (10 mM) with sodium nitrite (80 mM) reacted at 60 °C for 30 min. TLC was developed by *n*-hexane–acetic acid–ethyl acetate–chloroform (45:6:10:1). The shaded spot was found only at pH 1.5 and its color (pink) was apparently different from that of Y (yellowish green).

RESULTS AND DISCUSSION

The individual products that have been isolated from the reaction mixture are shown in Table I. Their structures have been elucidated except for the product B, and their biological activities have been assayed (Namiki et al., 1980).

Extraction of Products. In the previous report (Namiki et al., 1980), it was observed that the biological activities were exhaustively transferred to ethyl acetate by extraction, but only incompletely to dichloromethane or ethyl ether. Figure 1 shows that the results of a parallel TLC study of the extracted materials indicate that the product ethylnitrolic acid remains in the aqueous phase by the extraction with dichloromethane and that the product Y behaves similarly in the extraction with ethyl ether.

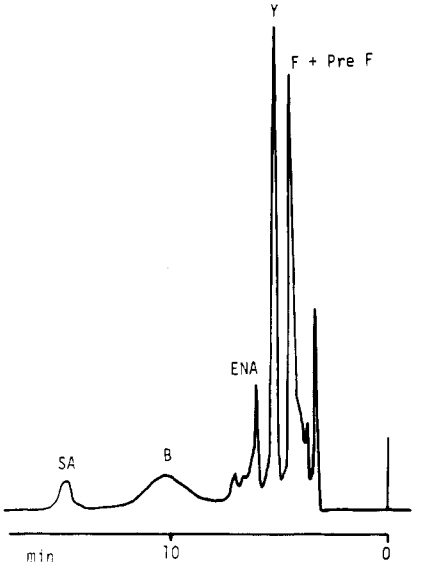


Figure 3. High-performance LC of the reaction mixture of sorbic acid with sodium nitrite. Sorbic acid (20 mM) and sodium nitrite (160 mM) reacted at pH 3.5 and 60 °C for 30 min. μBondapak C_{18}. Solvent: H_2O–methanol (85:15).

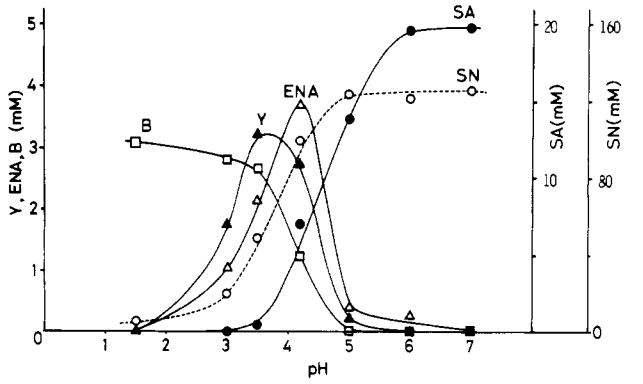


Figure 4. Effect of reaction pH on formation of the main products and decrease of the reactants. Sorbic acid (20 mM) with sodium nitrite (160 mM) reacted at 60 °C for 30 min.

As the solvent for separation of products by TLC, hexane–acetic acid–ethyl acetate–chloroform system was superior to the chloroform–methanol system, although the latter served well in the separation of ENA and Y (Namiki and Kada, 1975; Kito et al., 1978). By the separation of the products extracted with ethyl acetate with the former solvent system, nine discrete spots and at least one additional spot were observed. They are shown in Figure 2, in comparison with the spots of the purified products.

The choice of pH of the aqueous layer was sometimes found to be important in the extraction. When the mixture contained a significant amount of unreacted nitrite, the extraction at pH 1.5 caused the nitrogen oxide species to be extracted into the solvent, where they rapidly reacted with the dissolved sorbic acid and the products. This resulted in a different course taken by the reaction, as proved by the extraneous spots by TLC. That the extraction at pH 4.2 does not affect the composition was confirmed by polarographic analysis, the details of which will appear elsewhere.

Effect of Reaction pH on Product Distribution. The pronounced effect of the reaction pH on the biological activities (Namiki et al., 1980) can be quantitatively explained on the basis of the TLC results shown in Figure 2, which are for the ethyl acetate extracts of the mixtures reacted at pH ranging from 1.5 to 7.0. The product Y is

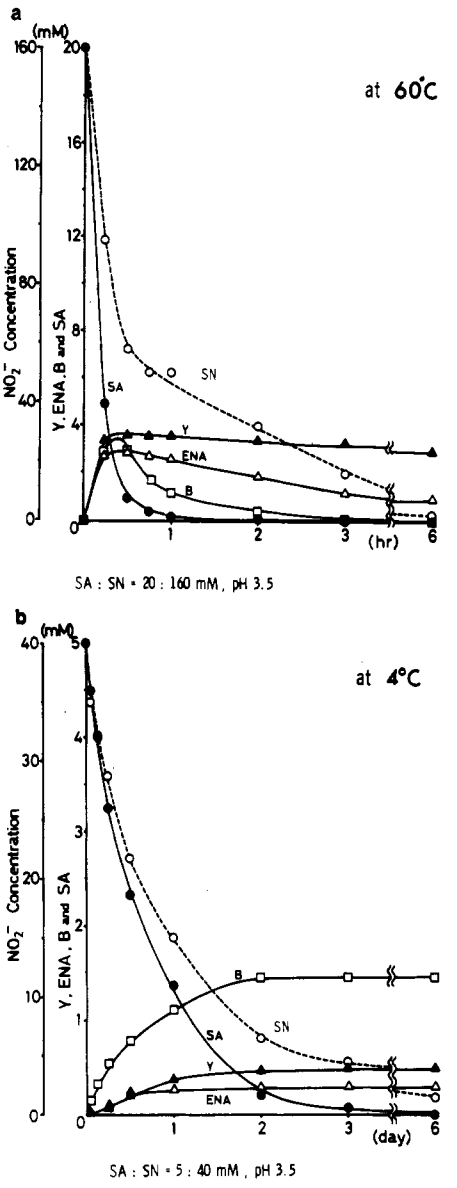


Figure 5. Time course of formation of the main products and decrease of the reactants. Sorbic acid (5 mM) with sodium nitrite (40 mM) reacted at pH 3.5. (a) At 60 °C. (b) At 4 °C. SA: sorbic acid. SN: sodium nitrite.

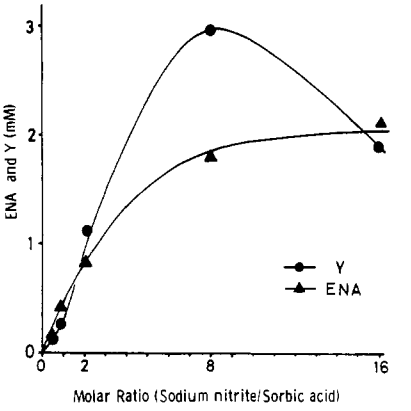


Figure 6. Effect of molar ratio of nitrite to sorbic acid on the formation of Y and ENA. Sorbic acid (20 mM) reacted with sodium nitrite at pH 3.5 and 60 °C for 30 min.

produced by the reaction at pH 3.0–5.0, with a maximum at pH 3.5, and ENA, between pH 3.5 and pH 5.0, with a maximum at pH 4.2. At pH 1.5, these mutagenic products are mainly replaced by the products B and F and other

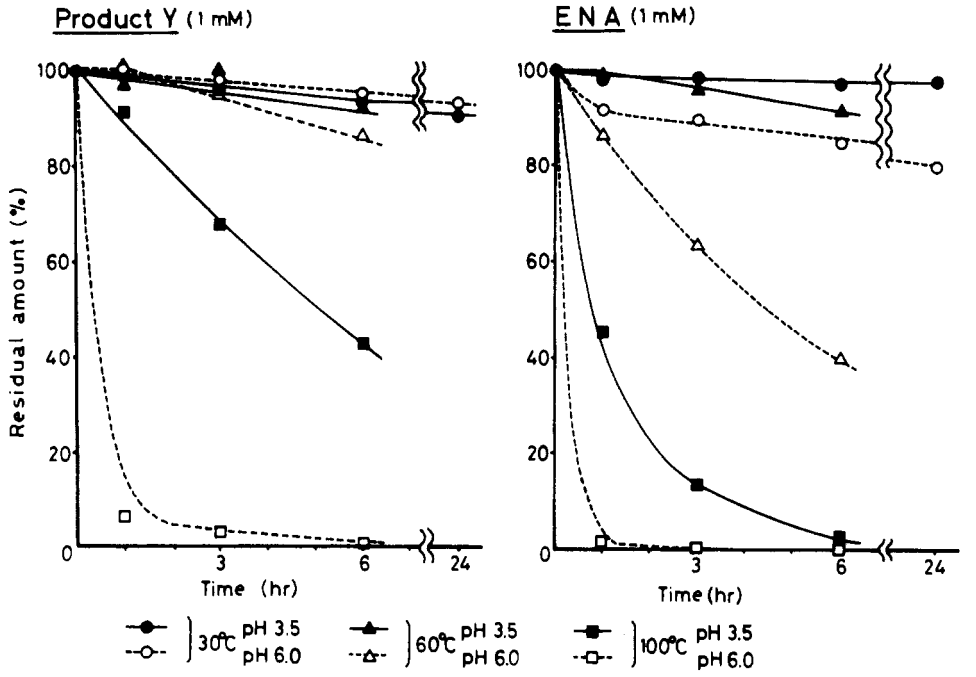


Figure 7. Stability of Y and ENA at different pH values and temperatures.

unknown minor products. Above pH 6.0, the chemical reaction hardly proceeds.

The mutagenic and growth inhibitory activities of the individual products have been studied [Namiki et al. (1980) and Table I]: Y is the strongest mutagen and also inhibitive, ENA is a mutagen to a lesser extent (negative to Ames test) but is inhibitive, B is not mutagenic but strongly inhibitive, and F and pre-F are not active in either way. It is then clearly understood that the mutagenic activity of the reaction mixture that becomes strongest by the reaction at pH 3.5–4.2 is mainly due to the formation of the product Y and, partly, of ENA and that the growth inhibition that is intensified by lower pH should be mainly due to that of the product B.

These results by TLC was also confirmed by a more quantitative study of the reaction mixture by high-performance LC, by which many of the main products were separated and estimated by comparison with standard specimen (Figures 3 and 4).

Course of Reaction. The TLC study of the product distribution of the mixture during the reaction (pH 3.5; 4 °C) showed formation of several products shortly after the start of the reaction. Among the predominant ones are the product pre-F and two unknown ones that appeared as the spots at R_f 0.2 and 0.3 on the TLC of the reaction mixture taken at 2 min after the start. Products B, ENA, and Y began to appear on TLC after 10 h of the reaction. Several unidentified products that are formed and disappeared during this time may be the precursors to the final products. Parts a and b of Figure 5 show the progress of reaction at 60 and 4 °C, respectively, at pH 3.5, as studied by high-performance LC. A similar reaction proceeded more slowly at 4 °C, where both Y and B reached constant levels after 2 days, which are not changed for at least 6 days. The product Y seems more stable than other products. These results are in good agreement with the previous biological results (Namiki et al., 1980). The yield of the active products, Y, ENA, and B, with respect to sorbic acid added are fairly high; the sum of these three products on a molar basis reached 40–50% of the sorbic acid after 2 days at 4 °C (Figure 5b).

Effect of Molar Ratio of Reactants. When the molar ratio of nitrite relative to sorbic acid (20 mM) is increased from 0.5 to 16, the yield of Y after 30-min reaction at 60

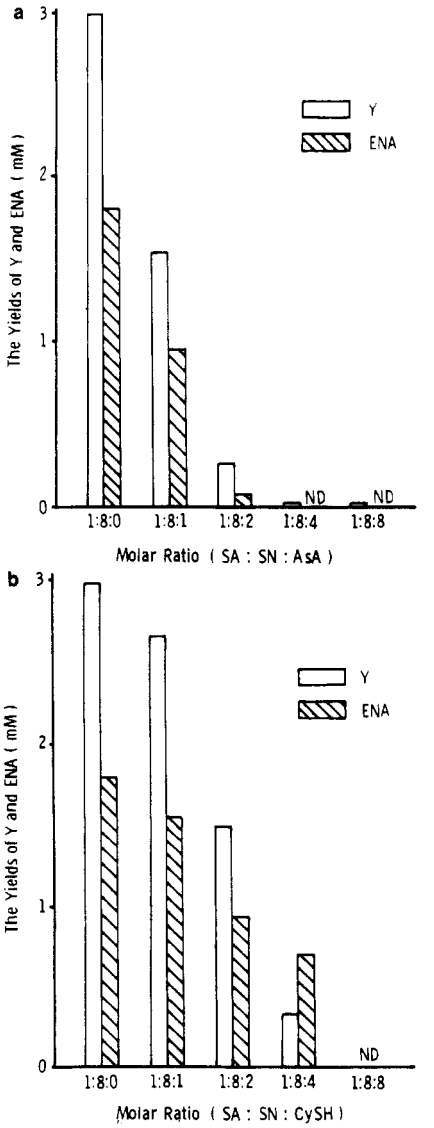


Figure 8. Effect of ascorbic acid and of cysteine on formation of Y and ENA. A mixture of sorbic acid (20 mM), sodium nitrite (160 mM), and ascorbic acid or cysteine reacted at pH 3.5 and 60 °C for 30 min. (a) Ascorbic acid. (b) Cysteine.

°C and pH 3.5 reached a maximum at 8-fold excess of nitrite and then decreased, while that of ENA gradually increased with an indication of leveling off, as shown in Figure 6. This is in accord with the previous biological results that the mutagenicity and the growth inhibition become stronger with increasing nitrite at least up to 8-fold (Namiki et al., 1980). It is to be noted, although such high levels of the nitrite as employed in this experiment are not very likely to occur in foodstuffs, that the mutagenic products are formed above the limit of detection even at a 1:0.5 molar ratio. This is also interesting in considering the mechanism of formation of these products, whose structures seem to require at least a 1:4 molar ratio for their formation.

Heat Stability of Active Products. Stabilities of the active products ENA and Y in pH 3.0 (citrate) or pH 6.0 (phosphate) buffer solutions were studied at 30, 60, and 100 °C. The determination was carried out by high-performance LC. The results (Figure 7) indicate labileness of both products at higher temperature and lower pH.

Under a different condition simulating that of cooking of bacon, i.e., 170 °C in corn oil, both ENA and Y decreased ~37% after 3 min.

Effect of Ascorbic Acid or Cysteine. It has been shown that presence of ascorbic acid and other food constituents effectively inhibit the formation of *N*-nitrosamine (Mirvish, 1975), mainly due to the faster rate of reaction of nitrite with ascorbic acid or others than that with secondary amino groups. To find out if ascorbic acid and also cysteine have similar inhibitory action on the formation of C-nitro mutagenic products, we carried out the following experiments.

High-performance LC analytical results for the products ENA and Y, of the mixtures of 20 mM sorbic acid and 160 mM sodium nitrite with and without addition of ascorbic acid, reacted for 30 min at 60 °C and pH 3.5, showed that addition of 80 mM ascorbic acid nearly eliminated the formation of these products (Figure 8a). Under more realistic condition, i.e., by the reaction of 20 mM sorbic acid and 20 mM sodium nitrite, the minimum concentration of ascorbic acid to inhibit the product formation was 10 mM for the product ENA and 5 mM for Y. Cysteine was also effective, but an equivalent molar concentration was required for nearly complete inhibition (Figure 8b).

It is not clear yet whether the above inhibition of mutagen formation depends entirely on the faster reaction rate of nitrite with ascorbic acid or cysteine than that with sorbic acid, since the mutagenic activity of Y has been found to be reduced effectively by the reaction with ascorbic acid or cysteine, at room temperature and at pH 6.8, though slightly at pH 3.5 (Osawa et al., 1980). The study of the process of the suppression of the formation of these mutagenic products is of importance from practical standpoint and is being pursued further.

Conclusion. Chromatographic studies of the reaction mixtures revealed the distribution of products variable with the condition of reaction, and it was correlated with the variation of biological, especially mutagenic, actions of the reaction mixture of sorbic acid with sodium nitrite. TLC study served to separate and identify most of the products, and high-performance LC was instrumental in partial separation and determination of the main products. The course of this reaction that readily gives variety of products by the action of nitrite on a conjugated unsaturated aliphatic carboxylic acid in aqueous system is of considerable interest, and further studies on the chemistry and kinetics of this reaction are desired. Ascorbic acid and cysteine are found to inhibit effectively the mutagen formation in this system. The effects of such food constituents on the course and on the produced biological activities are of importance from a practical viewpoint.

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