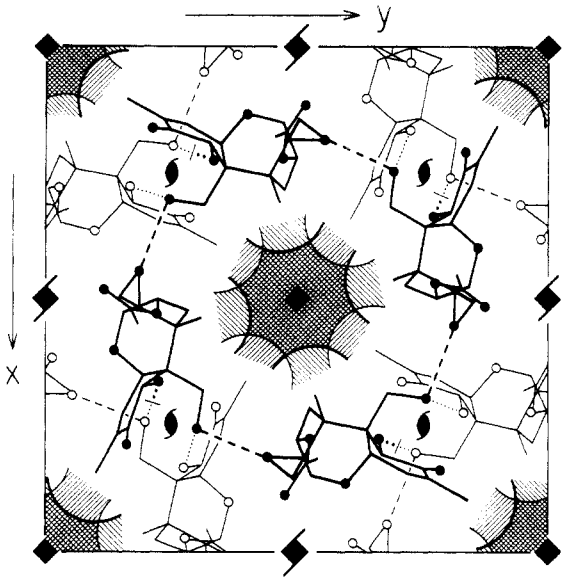

Figure 2. The unit cell contents projected along *c*. Of the two layers of molecules shown, the upper is depicted in bold outline. Oxygen atoms are represented as circles (solid or open). Intermolecular hydrogen bridges are shown as dashed lines if they join molecules in the same layer and as dotted lines otherwise. Interrupted H bridges join the upper layer to the one above it or the lower layer to the one below it. The holes in the layers (see the text) are bounded by arcs of (arbitrarily selected) radius 1.7 Å. "ring" holes are hatched one way, "interstitial" holes are hatched another, and the overlapped region (indicating the minimum channel dimensions) is crosshatched.

symmetry, an effective local radius can be recognized and could reasonably be defined as the distance from 0, 0, *z* to the nearest non-hydrogen atom. This radius ranges from the local minima (noted above) of 3.49 and 3.35 Å to local maxima of 3.83 and 4.25 Å. The greater of these maxima occurs at $z = 0.55$, and it is just here that the large concentration of residual electron density (described above) is centered. It is concluded that the concentration is a small molecule (perhaps H_2O), disordered, and possibly of partial occupancy. The mobility of the molecule along the channel is presumably restricted by the narrow parts.

If these channels accommodate small molecules (e.g., water and methyl alcohol), their occupancy by such solvents may explain the occurrence of different crystal habits of different melting point such as has been observed in this and closely related molecules (Blight and Grove, 1974). Presumably such occluded solvents are lost in the preparation of samples for elemental analysis. It is also clear that care should be exercised in preparation of samples in isotope dilution studies.

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Registry No. I (R = Ac), 50722-38-8; I (R = H), 51481-10-8.

Supplementary Material Available: Coordinates of Hydrogen atoms (Table III), thermal parameters of all atoms (Table IV), and observed and calculated structure factors (Table V) (13 pages). Ordering information is given on any current masthead page.

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Mutagen Formation by Nitrite–Spice Reactions

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Among several spices treated with sodium nitrite, pepper exhibited the strongest mutagenic activity by Ames method and nutmeg, chili pepper, and laurel the strong activity. No mutagenicity was observed for spices alone. The mutagen production was observed between pH 2 and pH 6, with the maximum between pH 3 and pH 3.5, and the reaction was very fast at 40 °C or above, even at the low levels of nitrite permitted by legal regulations. The mutagenicities of spice–nitrite reaction products were completely inactivated by S9 mix, but the activity of pepper–nitrite products toward TA 100 remained unchanged. By preparative TLC of spice–nitrite reaction mixtures, pepper provided two very active fractions, while nutmeg and chili pepper showed a different active fraction.

Studies on the possible formation of various mutagens by the reaction of food additives with food components or by that between coexistent food additives are no less important than those of the mutagens primarily contained in foods. An outstanding example has been the formation of *N*-nitrosamines by the reaction of secondary amines with added nitrites. Our group has also clarified the formation of a group of mutagenic *C*-nitro or *C*-nitroso products by the reaction between two commonly used food additives, sorbic acid and nitrite (Kada, 1974; Namiki and

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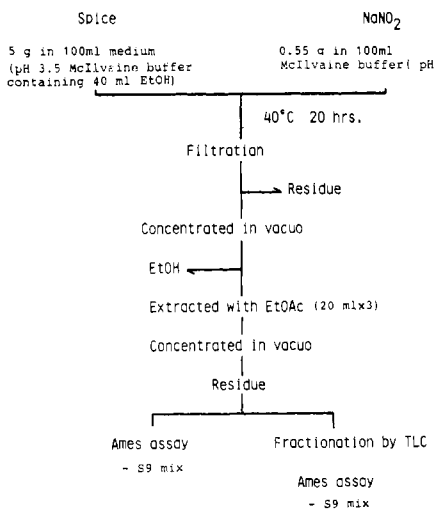

Figure 1. Experimental procedure.

Kada, 1975; Namiki et al., 1980, 1981; Osawa et al., 1979). Continued study has shown that some other food constituents with conjugated dienoic partial structures identical with sorbic acid produce similar mutagenic products (Osawa et al., 1982), as exemplified by that of piperine, the main pungent principle in pepper. Pepper powder that contains about 5% piperine also gave mutagenic products upon reaction with nitrite solution (Osawa et al., 1981). The study was extended further to include nitrite-treated other spices such as nutmeg, laurel, chili pepper, and others. The present paper deals with the mutagen production by these common spices and with the effects of reaction conditions and metabolic activation by S9 mix.

MATERIALS AND METHODS

Spices and Reagents. Fourteen kinds of common spices consumed in quantities, viz., pepper, garlic, nutmeg, laurel, paprica, allspice, thyme, clove, chili pepper, sage, cardamon, fennel, savory, and wuxiang fen were used in commercially available powder form. Chemical reagents were of guaranteed grade.

Reaction of Spices with Nitrite. Unless otherwise stated the reaction was carried out as follows (Figure 1). A suspension of 5 g of pepper in a mixture of 40 mL of EtOH and 60 mL of 0.2 M McIlvaine buffer (pH 3.9) (Perrin and Dempsey, 1974) was mixed with a solution of 0.55 g of $NaNO_2$ in 100 mL of the same buffer. The final apparent pH was 3.5. The mixture was shaken for 20 h at 40 °C, unless otherwise stated. Then the filtrate of the mixture freed from ethanol in vacuo was extracted with 20 mL of ethyl acetate 3 times. The extract was dehydrated with anhydrous sodium sulfate and concentrated in vacuo. A part of the concentrate equivalent to 20–50 mg of the material spice was dissolved in a small amount of ethanol and mutagenicity was assayed by the Ames test using *Salmonella typhimurium* TA 98 and TA 100 strains. (Ames et al., 1975) A control, the ethyl acetate extract from the identically incubated solution of sodium nitrite alone, of the same concentration used in this experiment showed 35 revertants for TA 98 and 135 revertants for TA 100 per plate. A positive control that used 0.1 μg of AF-2 [2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide] per plate produced 1400 revertants for TA 100 and 522 for TA 98, per plate. Spontaneous reversion counted was 33 for TA 98 and 122 for TA 100 per plate, under identical conditions.

TLC Fractionation of the Products. The above extract was dissolved in a fixed volume of ethanol and was developed on a silica gel plate (Wakogel FM) containing fluorescent agents. The solvent was hexane–acetic acid–ethyl acetate–chloroform (90:9:12:2). The chromatogram was divided into 10 equal fractions, which were separately extracted with ethyl acetate and were tested for their mutagenic activities.

Heat Stability Test. The stability of the reaction products obtained from nitrite-treated pepper and nutmeg toward heat was examined, roughly simulating the conditions of actual food cooking. The extracted reaction products were tested either by (a) heating the product as they were for 2 and 5 min at 180 °C in an oil bath or by (b) heating 10 mg suspensions of the products in 2 mL of 0.2 M McIlvaine buffer (pH 3.5 and 6.5) for 1 and 2 h at 90 °C in a boiling water bath. The heat-treated samples were extracted with ethyl acetate and were microbioassayed.

Mutagenicity Test. Ames assay using *S. typhilimurium* TA 98 and TA 100 was employed for the purpose. Metabolic activation was carried out by adding 0.3 mL of S9 mix from rat liver activated by phenobarbital, per plate. The fractionated extract was dissolved and diluted with ethanol according to its activity for application to the plate. Relative activities were shown as the number of revertant colonies per plate.

RESULTS AND DISCUSSION

Mutagenicity of the Spice–Nitrite Reaction Mixture. Five grams of spice powder was reacted with 0.55 g of sodium nitrite, in 200 mL of medium at 40° C as was described earlier. The portions of the concentrated extract corresponding to 10, 30, or 50 mg of the spice were dissolved in ethanol and used for the Ames test, without activation by S9 mix. As shown in Figure 2, reaction mixtures of pepper, nutmeg, laurel, chili pepper, and wuxiang fen were found to produce strong mutagenic activity. The nitrite-treated wuxiang fen showed a significant activity roughly equal to that of nutmeg. (This may not be true for other preparations because the composition of this mixed spice is not definite.) Since it has been reported that certain spices are mutagenic themselves, probably because of the mutagenic constituents such as flavonoids (Seino et al., 1978), the Ames test was carried out on the extracts of the unreacted spices. The results in Figure 2 indicate no appreciable mutagenicity in the unreacted spices, at least under the present experimental conditions.

Effect of Reaction Temperature. Similar tests were carried out on the spices reacted at 5, 20, 40, and 60 °C at pH 3.5. It is evident from Figure 3 that higher temperature promotes the formation of the mutagenic products from the nitrite-treated spices, while virtually no mutagenicity was observed at 5 °C.

Effect of Reaction pH. The effect of pH on mutagen formation was examined with the suspensions of pepper or nutmeg powder in nitrite solutions buffered at different pH. the reaction temperature was 40 °C. As shown in Figure 4, the activity reaches maximum between pH 3 and pH 3.5, in the examined pH range of 2–6.

Effect of Nitrite Concentration. As is well-known, nitrites have been extensively used as coloring agents and preservatives for meat, and the levels legally permitted by most governments range from 50 to 100 mg/kg of meat (as NO_2^-). Nitrites may also be derived from nitrates, which are abundant in vegetables. Thus, the total amount of nitrites contained in ingested food can be sometimes considerably larger than the above levels. On the other hand, spices, especially pepper and nutmeg, are commonly used in meat dishes. The average amount of pepper for a dish of cooked meat would be, on a rough estimation, around 0.1–0.3 g. The following experiment was intended

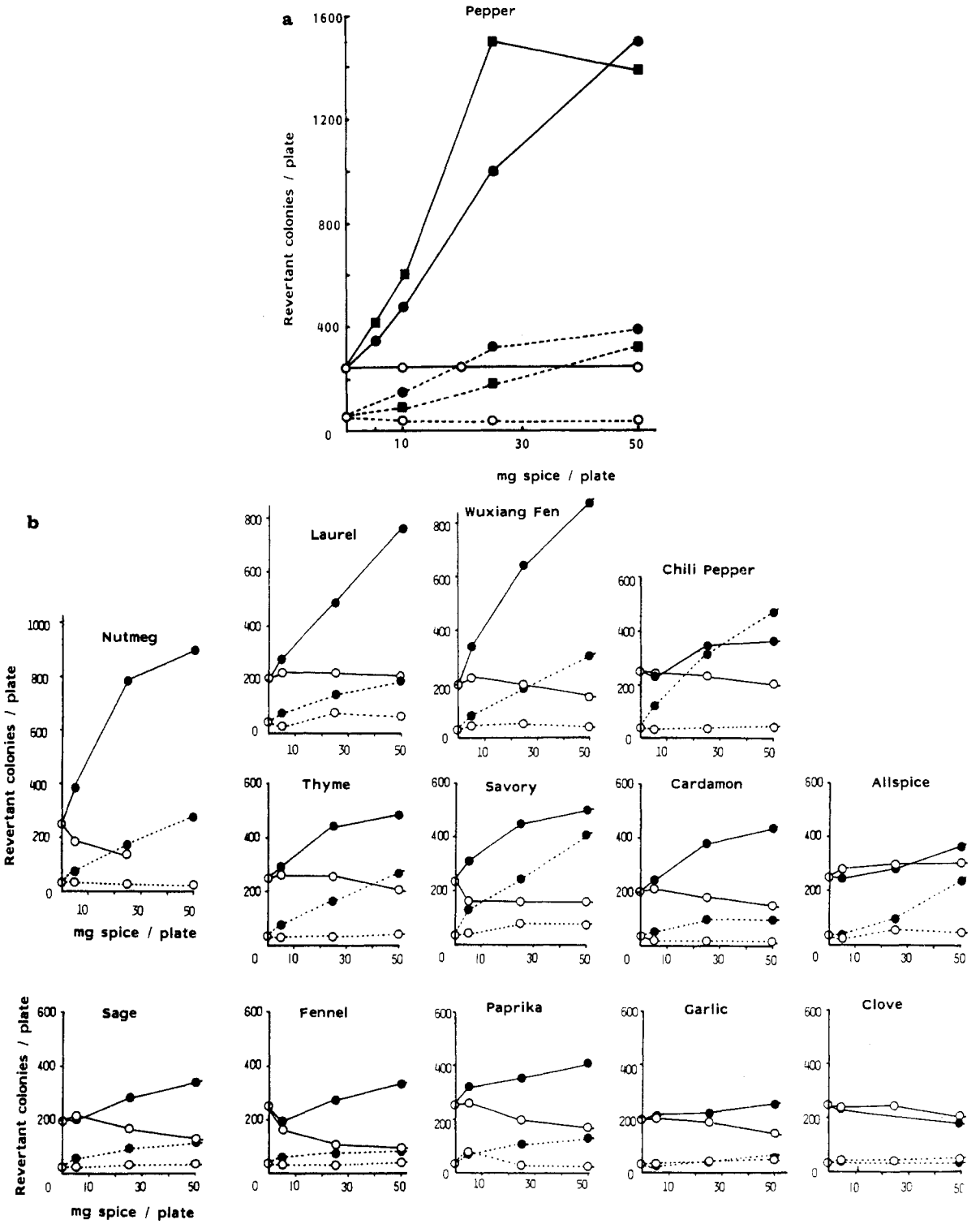

Figure 2. Mutagenicity of the spice–nitrite reaction mixture. Reaction procedure as shown in Figure 1. Ames test (-S9 mix): (---) TA 98; (—) TA 100. (a) (○) Pepper only; (●) black pepper–nitrite; (■) white pepper–nitrite. (b) (○) spice only; (●) spice–nitrite.

to simulate partly the actual conditions at the normal food consumption.

One gram of pepper or nutmeg was reacted with 27.6–138 mg of sodium nitrite (concentration, 0.4–2 mM) in 1 L of aqueous medium (pH 3.5, McIlvaine buffer) at 40 °C for 20 h. The mutagenicity of the extracted product is illustrated in Figure 5, which indicates that the mutagenicity is low at 0.4 mM nitrite but increases significantly with increased nitrite concentration. This effect was more marked for pepper than for nutmeg.

Fractionation of the Products of Spice–Nitrite Reaction. The results of mutagenicity tests on the 10 TLC fractions are shown in Figure 6. The strongest activity was found in the fractions 2 and 7 for pepper, while it was found in the fraction 1 for nutmeg, suggesting different constituents are responsible for mutagenicity.

Effects of S9 Mix on Mutagenicity. Metabolic activation of phenobarbital-induced S9-mix on mutagenic products showed different effects on the mutagenicity as was tested by TA 98 or TA 100 strains. As shown in Figure 7, the mutagenic acitivity of pepper–nitrite reaction products toward TA 98 was decreased considerably by addition of S9 mix but had little or no effect on the activity to TA 100.

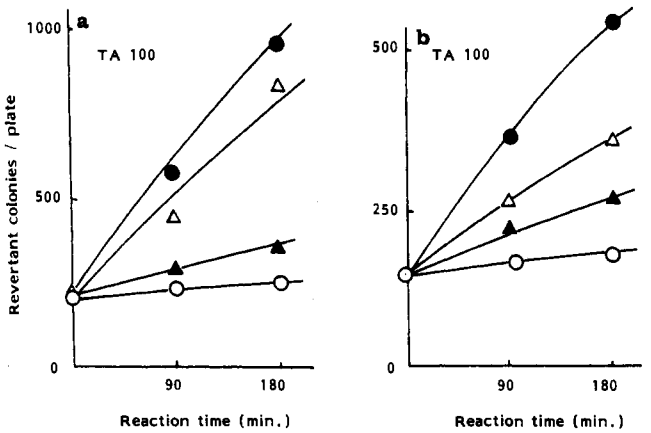

Figure 3. Effect of reaction temperature on generation of mutagenicity. Reaction procedure as shown in Figure 1 except for the temperature. (a) Pepper–nitrite; (b) nutmeg–nitrite. Equivalent milligrams of the spices is shown. TA 100 without metabolic activation. Ames test (–S9 mix): (●) 60 °C; (△) 40 °C; (▲) 20 °C; (○) 5 °C.

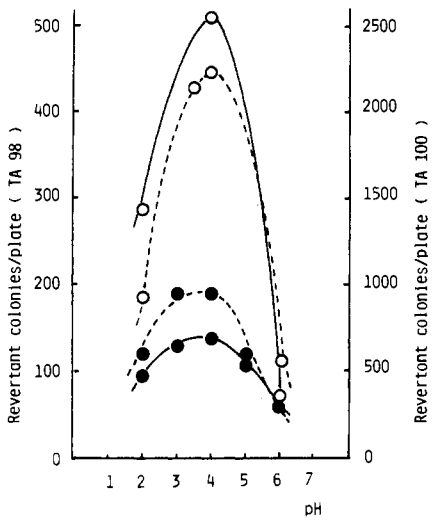

Figure 4. Effect of reaction pH on the formation of mutagenicity. Reaction procedure as shown Figure 1 except for pH. Ames test (–S9 mix): (- - -) TA 98; (—) TA 100. (○) Pepper–nitrige; (●) nutmeg–nitrite.

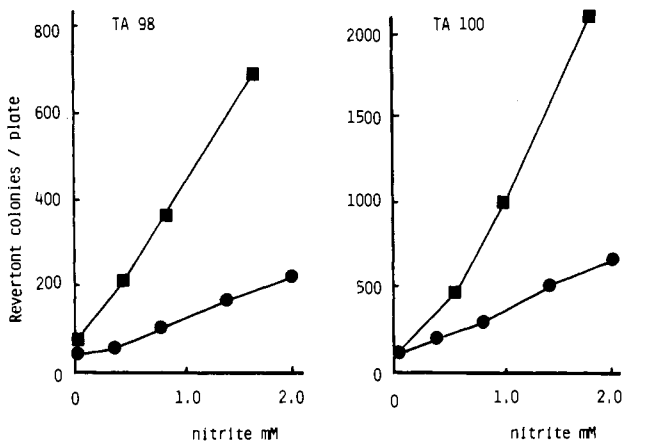

Figure 5. Effect of nitrite concentration in the reaction on the formation of mutagenicity. The amount of the spice was 1 g/1000 mL of sodium nitrite solution of 0.4–2.0 mM. The reaction was continued for 20 h at 40 °C pH 3.5. (■) Pepper–nitrite; (●) nutmeg–nitrite.

Heat Stability of Mutagenic Products. The stability of the products of pepper– and nutmeg–nitrite reactions toward heat was examined, roughly simulating the conditions of actual food cooking. As shown in Figure 8, the mutagenic activities of pepper–nitrite and nutmeg–nitrite decreased considerably on heating, especially at 180 °C.

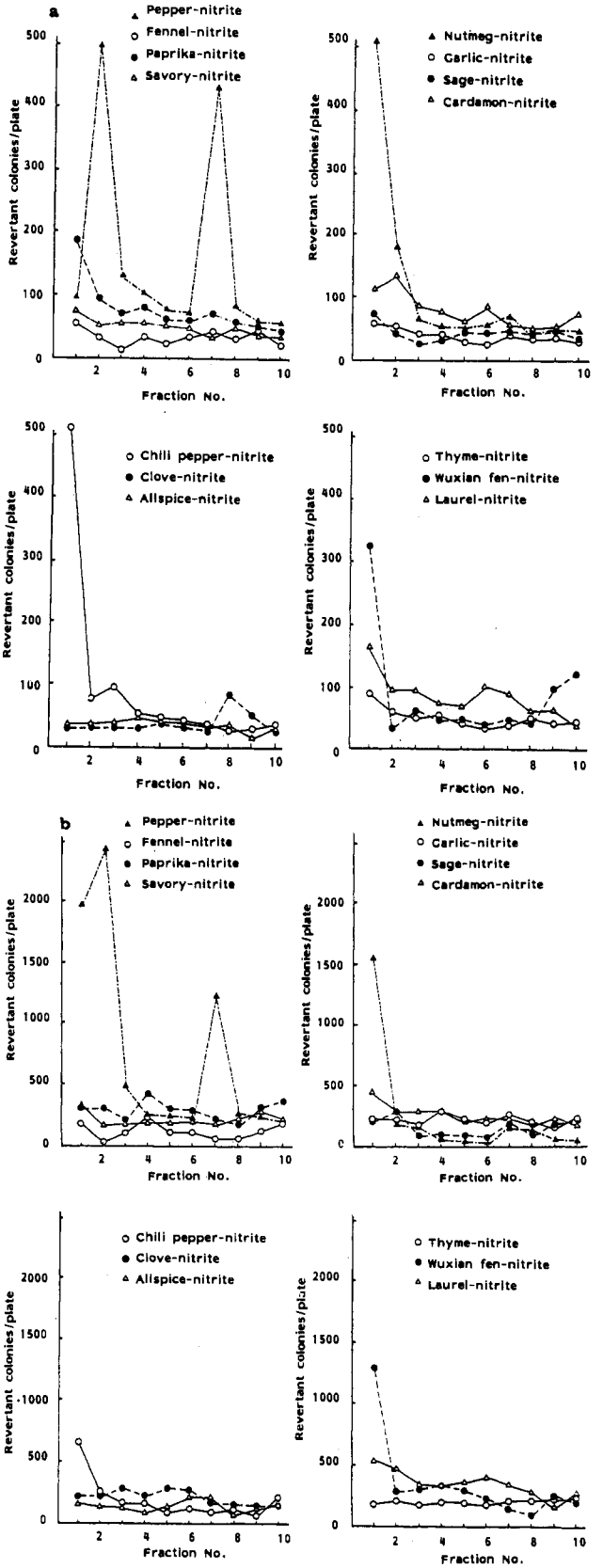

Figure 6. Fractionation of the reaction mixture on TLC plate. The concentrated extract of the reaction mixture obtained by the procedure described in Figure 1 was developed on a silica gel plate (Wakogel FM) with a solvent system *n*-hexane–acetic acid–ethyl acetate–chloroform (90:9:12:2). The fractions scraped off from the plates were assayed by the Ames method with S. *typhimurium* TA 98 and TA 100. (a) TA 98; (b) TA 100.

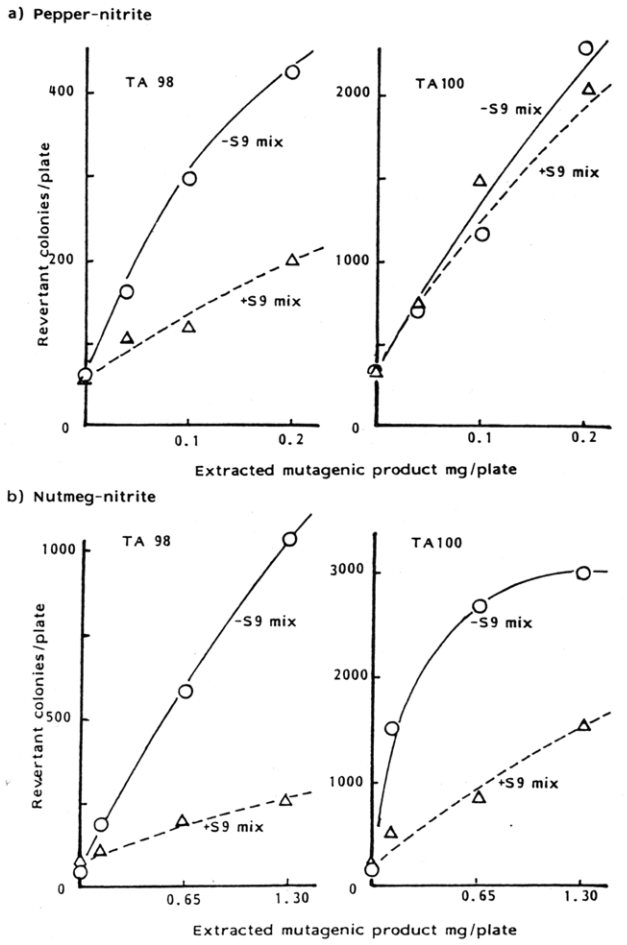

Figure 7. Effect of metabolic activation by S9 mix. S9 mix (Oriental Yeast Co.) was added (0.3 mL/plate). (a) Pepper–nitrite; (b) Nutmeg–nitrite.

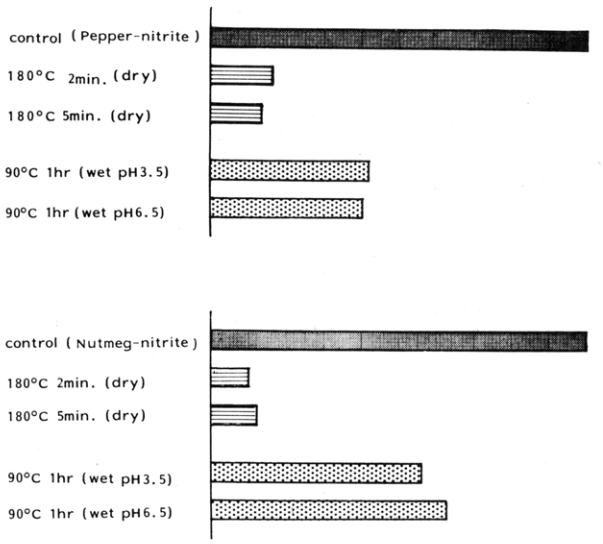

Figure 8. Heat stability of mutagenic products. The products obtained were either (a) heated as they were for 2 and 5 min at 180 °C(dry) or (b) the suspensions of the products were heated in MacIlvaine buffer, pH 3.5 and 6.5, for 1 and 2 h at 90 °C.

CONCLUSION

Among the 14 kinds of spices tested, pepper, nutmeg, chili pepper, and laurel produced significant mutagenic activities upon reaction with nitrite under the conditions indicated, even at low levels of nitrite permitted by legal regulations.

The active principles of the mutagenic products present in the ethyl acetate extracts from those spices were crudely fractionated on TLC plates, and it was found that, among many others, the fractions 2 and 7 of the pepper–nitrite product and the fraction 1 of the nutmeg–nitrite product showed espectially strong mutagenicity.

For the results obtained in our experiments on spice–nitrite products, we have to consider two sources of mutagenicity, i.e., the well-known *N*-nitroso compounds, on whose formation and activity so many works have been done, and the *C*-nitroso and *C*-nitro compounds. One of our previous works (Osawa et al., 1981), however, showed that N-nitrosation of the piperidine moiety of piperine was far exceeded by the formation of *C*-nitro type products under the conditions employed, which were nearly identical with those in the present work. It looks, therefore, reasonable to assume that the main cause of mutagenicity produced by spice–nitrite reactions comes from *C*-nitro type reaction products similar to those of the sorbic acid–nitrite reaction. Chemical identification of these products is in progress.

As has been shown, especially in Figure 4, the favorable pH conditions for the formation of *C*-nitro type products are around 3.5. This makes the formation of the mutagenic products during food processing rather unlikely or less probable, where the most foods are processed at near-neutral pH. However, it seems appropriate to take into consideration of the conditions under which the ingested foods are present with gastric juice in the digestive tract, which are exactly the conditions the present study is trying to simulate.

The inactivation of the mutagenic products by S9 mix is of enough interest from the viewpoint of the detoxification systems in liver, but it calls for more detailed study to justify this supposition. The effects of the presence of other constituents of foods on the mutagen-forming reaction and on the already formed mutagenic products by spice–nitrite reactions have also been investigated. These results indicated that the mutagen formation and mutagenicity seem to be counteracted by the addition of common food constituents such as ascorbic acid and vegetable juices, and details will be reported elsewhere.

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Registry No. Nitrite, 14797-65-0.

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