Genotoxicity Study of Reaction Products of Sorbic Acid

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Sorbic acid (E200) and its salts (potassium and calcium sorbate: E202 and E203) are allowed for use as preservatives in numerous processed foods. Sorbic acid has a conjugated system of double bonds which makes it susceptible to nucleophilic attack, sometimes giving mutagenic products. Under conditions typical of food processing (50−80 °C), we analyzed the cyclic derivatives resulting from a double addition reaction between sorbic acid and various amines. Mutagenesis studies, involving the Ames test and genotoxicity studies with HeLa cells and plasmid DNA, showed that none of the products studied presented either mutagenic or genotoxic activities.

Keywords: *3D test; amines; reaction products; Salmonella typhimurium; sorbic acid*

INTRODUCTION

Sorbic acid and sorbates, initially used for their activity as mold and yeast growth inhibitors are now known to exert the same effect on a wide range of bacteria. In the European Union, the use of sorbic acid (E200) and its potassium (E202) and calcium (E203) salts is authorized in many foods to lengthen their shelf life (margarine, various fruit preserves and desserts, wines and other drinks, moist cheeses, etc.). The levels at which they are used cover the range 100 to 2000 mg/L or mg/kg. The acceptable daily intake has been fixed for many years at 25 mg/kg body-weight (JECFA, 1973).

Sorbic acid, which is the trans-trans form of hexa-2,4-dienoic acid, has a system of conjugated double bonds susceptible to the attack by nucleophiles such as thiols (Wedzicha and Brook, 1989; Khandelwal and Wedzicha, 1990a), amines (Leraux and Vauthier, 1970; Verbiscar and Campbell, 1974; Kheddis et al., 1981), and nitrites (Khandelwal and Wedzicha, 1990b; Kito et al., 1970). But no study was performed on these interaction products directly in food. Earlier studies by our group (Ferrand et al., 1998a) have shown the occurrence of interactions between sorbic acid and the $R-NH_2$ functional groups naturally present in foods. New products are formed, and the process was analyzed by means of a model system based on sorbic acid, sorbates, and various simple amines such as methylamine, ethylamine, propylamine, butylamine, and benzylamine in relatively mild conditions of temperature and pressure. At 50 and 80 °C, both with sorbic acid and potassium sorbate, two isomeric cyclic reaction products (enantiomers of each other) were formed whatever the amine used. Their chemical structures, deduced by mass spectrometry and ^{1}H and ^{13}C NMR (Ferrand et al., 1998a), their yield, and their purity are presented in Table 1. The effects of various parameters on the reaction products formation were observed in our previous works (Ferrand et al., 1998).

These compounds result from the double addition of amines onto the double bonds of the sorbate followed by cyclization with dehydration and loss of an amine, following the reaction described by Kheddis et al. (1981) who studied similar chemicals in more drastic reaction conditions.

Some reports indicate (Hayastu et al., 1975; Namiki and Kada, 1975; Namiki et al., 1980) that, at high concentrations and at temperatures between 60 and 90 °C, sorbic acid reacts with nitrites to produce mutagenic substances. Using the Ames test with *Salmonella typhimurium* strain, Namiki and Kada (1975) showed that ethylnitrolic acid had a weak mutagenic activity but using the rec assay test with *Bacillus subtilis* strain, the activity was much higher. Another product that has been identified, 1,4-dinitro-2-methylpyrrole (DNMP), is renowned as the most mutagenic compound known: its mutagenic power is at the top of the scale in the Ames test toward *Salmonella typhimurium* strains TA98 and TA100 with no metabolic activation and in the rec assay test (Namiki and Kada, 1975; Namiki et al., 1980). The third compound produced by sorbate−nitrite interactions is a derivative of furoxan. It is deprived of mutagenic activity and a compound E—who's structure has not yet been determined—has a weak mutagenic activity in the rec test assay (Namiki et al., 1980). Mutagenic activity has also been shown by Lafont and Lafont (1979) in wine containing sulfur dioxide and sorbic acid after 45 days of storage.

Considering this general background, we decided to pursue our studies on the interactions between sorbic acid and amines (Ferrand et al, 1998a) with the evaluation of the genotoxicity and mutagenesis of the products that are likely to be present in food. In this way

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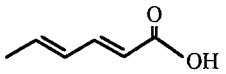
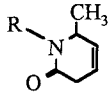
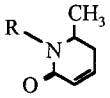
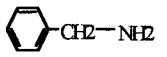
Table 1. Cyclic Reaction Products Structures Formed at 50 °C and 80 °C

		reaction products		purity
reagents :  sorbic acid		majority 	minority 	
CH_3NH_2 methylamine	50°C	MWt = 125 g/mole R = $-CH_3$ N-methyl-6-methyl -3,6-dihydro-2-pyridone 72 %	N-methyl-6-methyl -5,6-dihydro-2-pyridone 25.1%	97.1 %
$CH_3CH_2NH_2$ ethylamine	50°C	MWt = 139 g/mole R = $-CH_3CH_2$ N-ethyl-6-methyl -3,6-dihydro-2-pyridone 75.2%	N-ethyl-6-methyl -5,6-dihydro-2-pyridone 22.2%	97.4 %
$C_3H_7NH_2$ propylamine	50°C	MWt = 153 g/mole R = $-C_3H_7$ N-propyl-6-methyl -3,6-dihydro-2-pyridone 77.9%	N-ethyl-6-methyl -5,6-dihydro-2-pyridone 18.9%	96.8 %
$C_4H_9NH_2$ butylamine	50°C	MWt = 167 g/mole R = $-C_4H_9$ N-butyl-6-methyl -3,6-dihydro-2-pyridone 67.1%	N-butyl-6-methyl -5,6-dihydro-2-pyridone 30.8%	97.9 %
 benzylamine	80°C	MWt = 201 g/mole R =  N-benzyl-6-methyl -3,6-dihydro-2-pyridone 98.2%		98.2 %

their safety can be proven since biological activity has not yet been evaluated.

In the present study we investigated the mutagenic activity of the reaction products with the Ames test using *Salmonella typhimurium* strains TA98 and TA100 (Maron and Ames, 1983) and the genotoxic activity using the damaged DNA detection assay (3D test), with plasmid DNA and genomic DNA (Salles and Provot, 1999). For the 3D test, the products were incubated in microwells in which DNA molecules had been previously absorbed. Any lesions caused by the compounds are then repaired by adding a mixture containing a human-derived enzyme system (rH repairase + nucleotides) and a marker. The marker enables the amount of repair to be quantified through a chemoluminescence reaction. The genotoxicity of a compound is therefore proportional to the amplitude of the repair in the 3D test.

MATERIALS AND METHODS

Compounds Studied. During 15 days, 0.33 M sorbic acid and 0.33 M amines were put together in water at 50 °C or 80 °C, and the genotoxic and mutagenic activities of interaction cyclic compouds so obtained were tested. The formation conditions of neoproducts were close to those of actual food processing and storage. The amines used were methylamine, ethylamine, propylamine, butylamine, and benzylamine (model for aromatic amines). They were chosen for their simple molecular structure and their natural existence in food (Bauza at al., 1995). The resulting products were isolated by ether extraction, purified on alumin column, and studied by GC, RMN, and GC-MS (Ferrand et al., 1998a).

Study of the Direct Genotoxic Effects of the Reaction Products: 3D Tests. *Materials.* The apparatus (luminometer and microtitration plates) and all the reagents for the 3D test came form the SFRI Co (France), except for methyl methanesulfonate (MMS) which came from Sigma (France). The plasmids were produced by a bacterium, *Escherichia coli* pBluescript KS+, 2959 bp, (Stratagene, La Jolla, CA). The cells used were adherent HeLa cells of human origin (European Molecule Biology Laboratory, Heidelberg, Germany).

3D Test for DNA Damage Using Plasmid DNA. The method used was adapted and modified from the reports of Salles et al. (1999). The samples were diluted in DMSO (dimethyl sulfoxide) at 400 mg/mL; then, the following dilutions were made in ultrapure water: 10, 1, 0.1, 10^{-2}, and 10^{-3} mg/mL (the control used for the test was MMS).

Ultrapure, undamaged plasmid DNA was placed in contact with the sensitized wells for 30 min at 30 °C under gentle shaking. The dilutions of the samples were incubated on the DNA for 30 min at 30 °C. Each test was performed in duplicate. A positive repair control consisting of UV-damaged plasmid DNA was included along with two further controls, one with the solvent alone and one with a known genotoxic compound (2 and 10 mM MMS).

The wells were then rinsed twice with phosphate-buffered saline (PBS) containing 0.1% Tween 20 after which 50 μL of DNA repair mix was added. The repair solution was composed of protein repair complexes (P2) and nucleotides, one of which was labeled (dUTP-biotin (solution E of the 3D kit from SFRI).

After 3 h of incubation at 30 °C, the plate was washed three times, and 50 μL of conjugate solution was placed in each of the wells. The conjugate solution was composed of modified bovine serum albumin and a solution of concentrated avidine−peroxidase conjugate (solution c1 of the SFRI 3D kit). Incubation at 30 °C was then continued for 15 min under gentle stirring.

The plate was washed five times, and 50 μL of developing solution (chemoluminescent substrate: flask D form the SFRI 3D kit) was added to each well under total darkness. After 5 min of incubation at 30 °C under gentle stirring in the dark, the light emitted was measured with a luminometer. The signal—in relative light units (RLU)—is directly proportional to the number of breaks repaired. The ability of a drug to cause strand breaks in DNA is measured from the ratio of the signal at a given level (mean RLU adduct of two wells) to the signal of the solvent alone (RLU DMSO) = repair ratio. Each well was performed in duplicate, and the mean of the relative light units obtained was calculated.

3D Test for DNA Damage Using Genomic DNA. The adherent HeLa cells were cultured for 20 h at 37 °C in SFRI 4 medium containing 10% foetal calf serum and 2 mM glutamine under a CO_2-enriched atmosphere. The 96-well culture plates were seeded with 10^4 cells per well. The sample was added to the culture supernatant. The cells were incubated for 1 h with the various doses. The culture supernatant then was eliminated and 100 μL of lysis solution (Solycel, SFRI) added to each well. Incubation was then continued for 90 min at 30 °C under gentle shaking. Genomic DNA was recovered and analyzed with the 3D test. Aliquots of 50 μL of each cell lysate were placed in contact with the sensitized wells (washed twice beforehand) for 30 min at 30 °C under gentle shaking. The other steps of the test were the same as those for the 3D test on plasmid DNA (Salles and Provot, 1999).

Mutagenicity Test. *Bacterial Strains*. *Salmonella typhimurium* strains TA98 and TA100 were provided by Dr. Bruce N. Ames (Departement of Biochemistry, University of California, CA 94720).

Mutagenesis Assay. The procedure described by Maron and Ames (1983) was followed. The strains of *Salmonella typhimurium* TA100 and TA98 were grown for 10 h at 37 °C in liquid broth (8 g of Difco nutrient and 5 g of NaCl per liter). A 100 μL aliquot of the substance to be tested at the concentrations of 10 ng/plate, 0.1 μg, 1 μg, 10 μg, 100 μg, 1 mg, 2 mg, 3 mg, 4 mg, and 5 mg/plate was added to 100 μL of TA100 or TA98 strains and 500 μL of S9 mix (when the reaction products were activated). The mixtures were then mixed in 2.5 mL of top agar and poured onto the Vogel−Bonner minimal glucose agar plates which were scored for histidine revertants after 48 h incubation at 37 °C. Triplicate plates were done for each determination. The mutagenic activity was defined as the number of bacterial-induced revertants his^+ per plate. The assays were run both with a 20-min preincubation of the Salmonalla thiphymurium strains, and without preincubation

We ran a viability test on bacteria in the presence of the products to be tested in a histidine added environment (12 mL/L of liquid broth, for the TA his− to develop) in order to check that the reaction products themselves did not affect the growth.

RESULTS AND DISCUSSION

Genotoxicity of the Chemical Reaction Products. For each concentration the repair ratio was determined, and results of genotoxicity on plasmid DNA and genomic DNA are shown in Tables 2 and 3.

Any ratio greater than one indicates an increase of the repair signal and thus an increase in the number of lesions on the DNA. If its value is greater than two, the molecule tested has a significant genotoxic effect

Whatever the concentrations of the different samples tested, the repair ratios obtained were close to 1 and statistical comparisons were not justified. The samples were not found to be genotoxic with the 3D tests; none reached the threshold of significance for genotoxicity, i.e., 2.

This absence of direct toxicity does not exclude the possibility of a progenotoxicity; it therefore appeared advisable to pursue the study on that previously me-

Table 2. Test 3D Results on Plasmid DNA in Vitro[a]

compounds	concn mg/mL	yield: R^b
reaction products	10	0.70 ± 0.03
from	1	0.79 ± 0.01
methylamine + sorbic acid	0.1	0.814 ± 0.04
	10^{-2}	0.91 ± 0.02
	10^{-3}	0.96 ± 0.04
reaction products	10	0.75 ± 0.03
from	1	0.84 ± 0.01
ethylamine + sorbic acid	0.1	0.85 ± 0.02
	10^{-2}	1.06 ± 0.06
	10^{-3}	1.07 ± 0.06
reaction products	10	0.78 ± 0.02
from	1	0.84 ± 0.03
propylamine + sorbic acid	0.1	0.89 ± 0.02
	10^{-2}	1.06 ± 0.10
	10^{-3}	0.94 ± 0.05
reaction products	10	0.78 ± 0.05
from	1	0.71 ± 0.05
butylamine + sorbic acid	0.1	0.95 ± 0.04
	10^{-2}	0.97 ± 0.10
	10^{-3}	1.19 ± 0.12
reaction products	10	0.70 ± 0.09
from	1	0.86 ± 0.02
benzylamine + sorbic acid	0.1	0.90 ± 0.09
	10^{-2}	0.96 ± 0.07
	10^{-3}	1.00 ± 0.15
MMS	10 mM	3.64 ± 0.21
	2 mM	2.82 ± 0.11

[a] Each test was performed in duplicate. [b] *R*: repair ratio = ratio of the signal at a given level (mean RLU adduct of two wells) to the signal of the solvent alone. If its value is greater than 2, the molecule tested has a significant genotoxic effect.

Table 3. 3D Test Results on Genomic DNA from HeLa Cells[a]

compounds	concn, mg/mL	yield: R^b
reaction products	10	0.71 ± 0.05
from	1	0.75 ± 0.09
methylamine + sorbic acid	0.1	0.87 ± 0.10
	10^{-2}	1.16 ± 0.15
	10^{-3}	0.92 ± 0.09
reaction products	10	0.72 ± 0.03
from	1	0.75 ± 0.03
ethylamine + sorbic acid	0.1	0.81 ± 0.10
	10^{-2}	0.82 ± 0.14
	10^{-3}	0.9 ± 0.08
reaction products	10	1.06 ± 0.17
from	1	1.09 ± 0.17
propylamine + sorbic acid	0.1	1.42 ± 0.13
	10^{-2}	1.34 ± 0.09
	10^{-3}	0.91 ± 0.07
reaction products	10	0.75 ± 0.01
from	1	01.00 ± 0.10
butylamine + sorbic acid	0.1	0.91 ± 0.06
	10^{-2}	1.16 ± 0.11
	10^{-3}	0.78 ± 0.12
reaction products	10	1.69 ± 0.12
from	1	0.99 ± 0.10
benzylamine + sorbic acid	0.1	1.08 ± 0.9
	10^{-2}	0.92 ± 0.21
	10^{-3}	0.97±0.30
MMS	10 mM	8.30±2.9
	2 mM	2.72±0.23

[a] Each test was performed in duplicate. [b] *R*: repair ratio = ratio of the signal at a given level (mean RLU adduct of two wells) to the signal of the solvent alone. If its value is greater than 2, the molecule tested has a significant genotoxic effect.

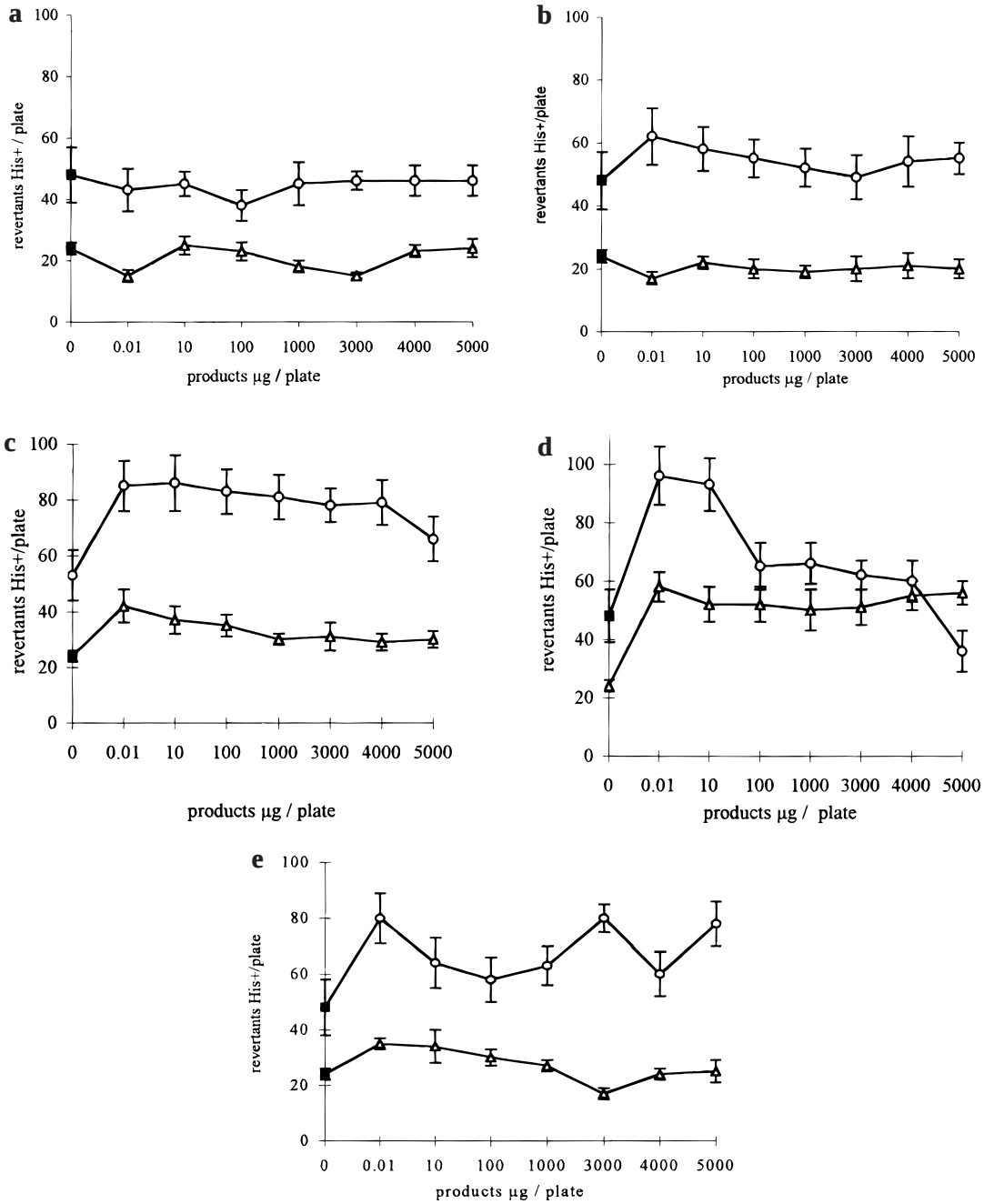


Figure 1. (a) Mutagenesis in TA98 of reaction products from methylamine + sorbic acid. (b) Mutagenesis in TA98 of reaction products from ethylamine + sorbic acid. (c) Mutagenesis in TA98 of reaction products from propylamine + sorbic acid. (d) Mutagenesis in TA98 of reaction products from butylamine + sorbic acid. (e) Mutagenesis in TA98 of reaction products from benzylamine + sorbic acid. (○): Mutagenic activity of reaction products in strain TA98 with S9 mix, (Δ): mutagenic activity of reaction products in strain TA98 without S9 mix, (■): blank (buffer): spontaneous revertants in the presence and in the absence of S9. The values are presented with the SD.

tabolized by the S9 fraction. A variation of this test consists of using hepatocytes which, due to their capacity to metabolize progenotoxics, will induce damage on their own DNA.

Mutagenicity of the Chemical Reaction Products. A viability test showed that the values of the colonies that appeared in the presence of the products to be tested were similar to the colonies that appeared on the reference plate. In all cases, bacterial growth was 10^7-10^8 bacteria/mL after 10-h-long culture. So we showed thus that the residual sorbic acid and the reaction products themselves do not affect the growth.

The results of the mutagenesis tests toward *Salmonella typhimurium* strains TA98 and TA100 are depicted in Figures 1 and 2. The revertant values being similar for whatever dose tested, the entire results were not presented.

Butylamine−sorbic acid interaction products present a lightly positive result at a weak dose on TA98 strain in absence of the S9 activation-system. For higher doses, revertants values are always inferior to twice that of the spontaneous revertants' values. Moreover, these observations were not found for the Ames test with preincubation (data not shown). We can suppose that these products are not mutagenic.

As far as all other interaction products are concerned and whatever the doses tested, the value of revertants his^+ induced by our tested samples is similar to the

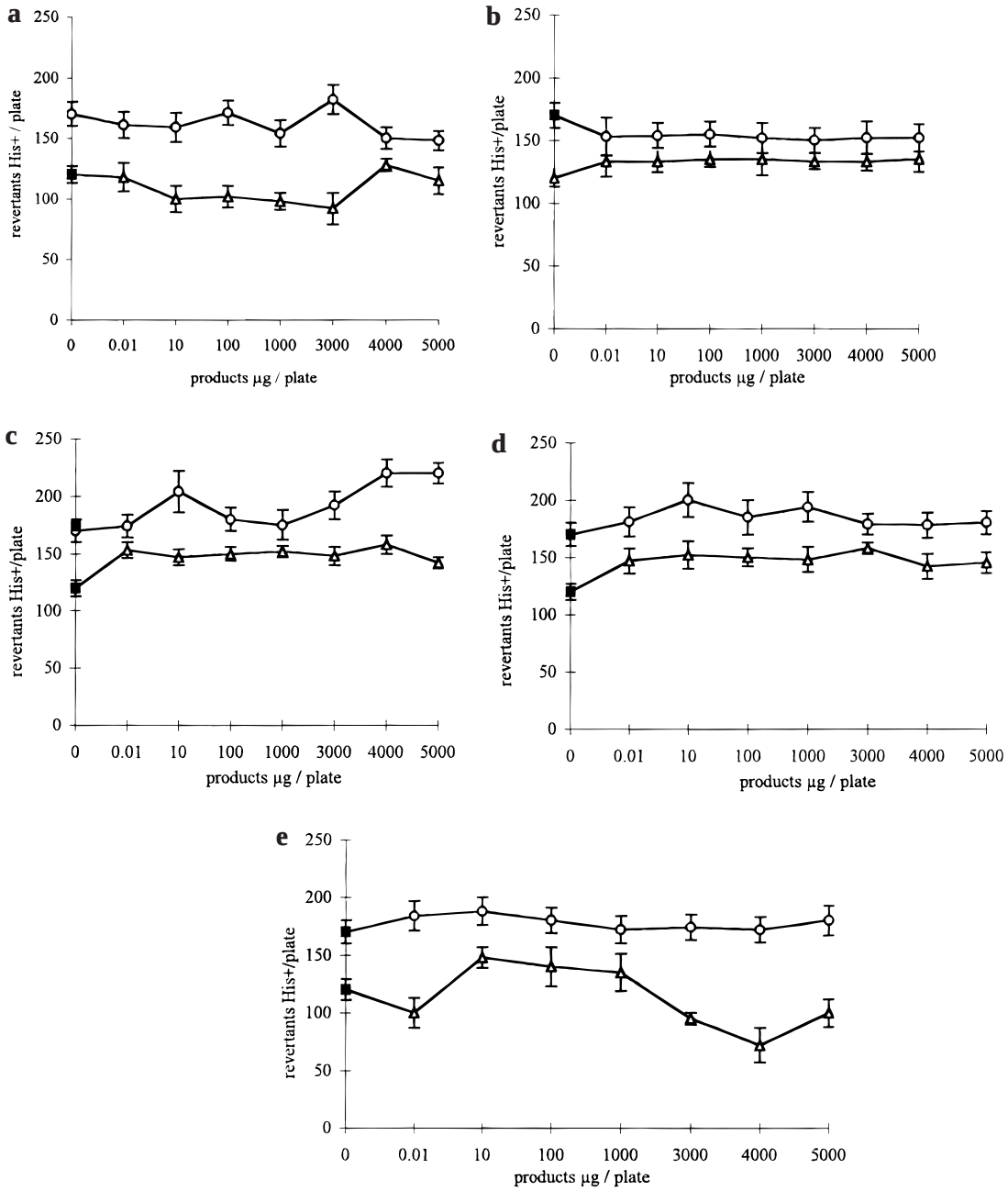


Figure 2. (a) Mutagenesis in TA100 of reaction products from methylamine + sorbic acid. (b) Mutagenesis in TA100 of reaction products from ethylamine + sorbic acid. (c) Mutagenesis in TA100 of reaction products from propylamine + sorbic acid. (d) Mutagenesis in TA100 of reaction products from butylamine + sorbic acid. (e) Mutagenesis in TA100 of reaction products from benzylamine + sorbic acid. (○): Mutagenic activity of reaction products in strain TA100 with S9 mix, (Δ): mutagenic activity of reaction products in strain TA100 without S9 mix, (■): blank (buffer): spontaneous revertants in the presence and in the absence of S9. The values are presented with the SD.

value of spontaneous revertants. The data obtained with (data not presented) and without incubation are identical. The chemical reaction products tested did not appear to be mutagenic, with or without the presence of the S9 activation system on the TA98 and TA100 strains. As the value of revertants his^+ for the compouds has never reached twice that of the value of spontaneous revertants, none of the compounds tested is to be considered as mutagenic (Chu et al., 1981).

No mutagenic effect was demonstrated for the cyclic products formed by interaction between sorbic acid and amines at 50 °C and at 80 °C, unlike the situation reported for compounds formed between sorbic acid and nitrates (Namiki and Kada, 1975; Namiki et al., 1980) or sorbic acid and sulfates (Lafont and Lafont, 1979). However, we cannot conclude that reaction products studied are totally safe. Indeed, Namiki and Kada (1975) observed that ethylnitrolic acid (addition product of nitrite and sorbic acid) only caused a low mutagenic response with the Ames test, but this compoud was classified as a strong mutagen with the rec assay test. Likewise, Lafont and Lafont (1979) showed that wine containing SO_2 and sorbic acid coexerts a mutagenic activity in the rec assay test, while Lück (1983) reported no mutagenic effect in the Ames test. It therefore appears preferable to confirm the total absence of toxicological risk of the reaction products studied by additional tests such as genotoxicity tests in vivo.

We are planning to check the absence of any oxidative lesion or any other type of lesion thanks to tests on

rodents, in particular in testing the effect of the products on liver cells and lymphocytes. The running of the comet assay is also planned to check the absence of DNA reparation.

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