Mutagenicity of Products Obtained from Cysteamine-Glucose Browning Model Systems

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The reaction mixture obtained from a cysteamine/D-glucose/water browning model system was separated into 11 fractions, seven fractions from the methylene chloride extract by high-pressure liquid chromatography and four fractions from the residual aqueous solution by ion-exchange column chromatography. Each fraction was tested for mutagenicity by the Ames' *Salmonella* test using TA 98 and TA 100, with or without S-9 mix. The reaction mixtures obtained from cysteamine/D-glucose/$NaNO_2$ model systems were also examined for mutagenicity, with positive results. Eight thiazolidines and five *N*-nitrosothiazolidines, some of which were found in the above model systems, were synthesized and tested for mutagenicity. All thiazolidines tested showed some mutagenicity at certain concentrations.

The browning reaction occurs when a food is cooked. By 1916, L. C. Maillard had shown that brown pigments and polymers are produced after the initial reaction of the amino group of an amino acid with the carbonyl group of sugars. Since then, it has been found that sugar–amine browning reactions, commonly called the Maillard reaction, produce tremendous number of chemicals. These chemicals range from volatile alcohols, ketones, aldehydes, esters, ethers, and sulfur- and nitrogen-containing heterocyclic compounds to nonvolatile Amadori compounds and complex brown pigments of medium to high molecular weights. Because of the large variety of constituents, a mixture obtained from a browning reaction shows different esthetic chemical and biological properties: characteristic roasted or smoky odors, prooxidants, antioxidants, toxicants, and perhaps mutagens and carcinogens, or antimutagens and anticarcinogens.

Some *N*-nitrosamines are carcinogens and these are formed from the reaction of secondary amines and sodium nitrite (Freund, 1937; Lijinsky and Epstein, 1970; Druckrey et al., 1967; Magee and Barnes, 1956). Sakaguchi and Shibamoto (1978) isolated some heterocyclic compounds which contain a possible source of nitrosatable nitrogen (thiazolidine, 2-methylthiazolidine, 2-ethylthiazolidine, etc.) from a cysteamine/D-glucose model browning system. Kitamura et al. (1978) also isolated some substituted polyhydroxythiazolidines from the aqueous fraction of this reaction mixture. Sakaguchi and Shibamoto (1979) reported the formation of *N*-nitroso-2-methylthiazolidine from a cysteamine/acetaldehyde/sodium nitrite model system. Cysteamine, which is a decarboxylated compound of cysteine, is produced from cysteine by the action of the enzyme cysteine decarboxylase in an animal tissue (Yamaguchi et al., 1973). It has been accepted as a reasonable hypothesis that the decarboxylation of cysteine occurs after the formation of Schiff's base with sugar during the Maillard reaction in foods (Newell et al., 1967). We therefore chose to examine the cysteamine/D-glucose browning model system for mutagens and to assay mutagenic *N*-nitrosamine compounds with sodium nitrite added to this system.

EXPERIMENTAL SECTION

Reaction of D-Glucose and Cysteamine. An aqueous solution (200 mL) containing 0.6 mol of D-glucose and 0.6 mol of cysteamine, which had previously been converted from cysteamine hydrochloride by the addition of sodium hydroxide, was refluxed at 100 °C for 2 h. The dark-brown reaction mixture was separated into two fractions: an organic solvent extract (volatile fraction), which was expected to contain volatile thiazolidines, and a residual aqueous solution (less-volatile fraction), which was expected to contain less-volatile polyhydroxythiazolidines.

Sample Preparation of the Volatile Fraction from a Cysteamine/D-Glucose Model System. The reaction mixture obtained from the above experiment was extracted with 200 mL of methylene chloride using a liquid–liquid continuous extractor for 16 h. The extract was dried over anhydrous magnesium sulfate for 12 h, and methylene chloride was removed using a rotary flash evaporator. Approximately, 11.2 g of brown oily material was obtained. This material (2 g) was dissolved into 50 mL of acetone and the solution was filtered in order to remove brown polymers. Acetone was removed by blowing with a N_2 stream. This sample was fractionated into seven fractions (F_H-1 ~ F_H-7) by high-pressure liquid chromatography (Figure 1) and stored for mutation tests and GC/MS analysis.

Sample Preparation of the Less-Volatile Fraction from a Cysteamine/D-Glucose Model System. After the volatile fraction was removed from the above reaction mixture, the residual aqueous fraction was concentrated to syrup form by distillation under reduced pressure [50 °C (10 mmHg)]. This material (10 g) was subjected to ion-exchange column chromatography (Dowex 50-WX4, 150 mL) with gradient elution from deionized water to 5 N ammonium hydroxide solution. Two hundred and twenty fractions were obtained using a fraction collector. Each fraction was monitored with UV spectra at 220 nm (Figure 2). These fractions were gathered into four new fractions according to the UV spectra (Figure 2). Each new fraction was concentrated to dryness with a rotary flash evaporator, thoroughly evaporated to dryness under reduced pressure in a desiccator, and stored for mutation tests and GC/MS analysis.

Reaction of Cysteamine, D-Glucose, and Sodium Nitrite. *a. One Step.* An aqueous solution (100 mL) containing cysteamine (0.1 mol), D-glucose (0.1 mol), and sodium nitrite (0.03 mol) was refluxed for 2 h. The reaction mixture was extracted with 200 mL of methylene chloride using a liquid–liquid continuous extractor for 16 h. The methylene chloride extract was dried over anhydrous magnesium sulfate for 12 h and solvent removed by a rotary flash evaporator. A brown oily liquid (ca. 1.63 g) was obtained. Approximately, 0.3 g of this reaction mixture was dissolved into 10 mL of acetone, and the undissolved materials were filtered off. The acetone was removed with a N_2 stream and the sample obtained (volatile fraction) was stored for mutation tests. After the

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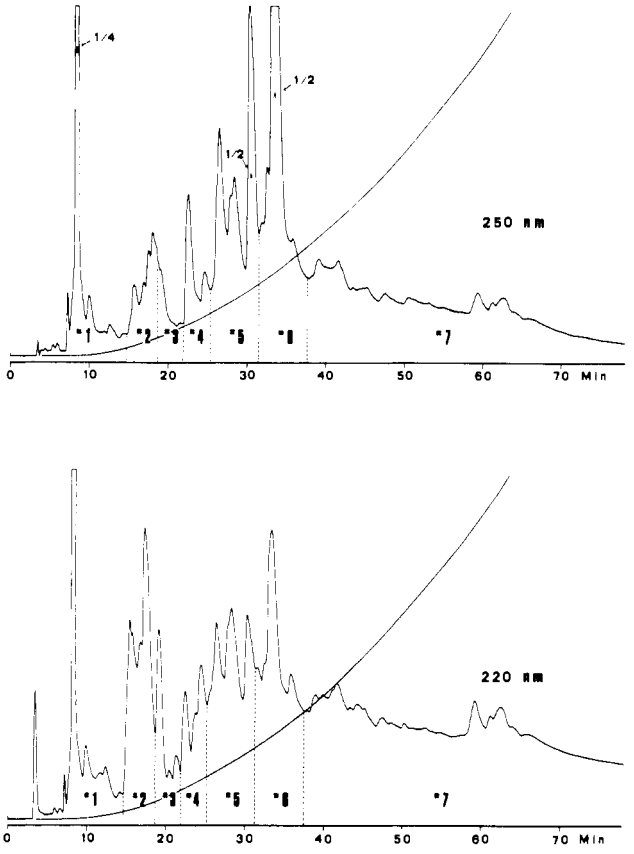

Figure 1. High-pressure liquid chromatogram of a methylene chloride extract (volatile fraction) of cysteamine/D-glucose reaction mixture.

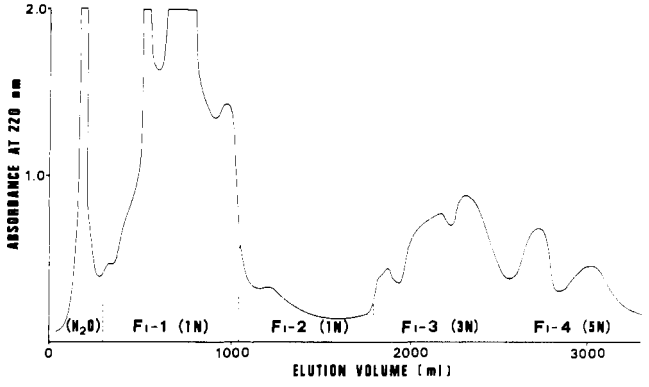

Figure 2. Elution curve of ion-exchange column chromatography of an aqueous fraction (less-volatile fraction) obtained from the reaction of cysteamine and D-glucose.

volatile fraction was removed, the residual aqueous fraction was concentrated to syrup form by distillation under reduced pressure [50 °C (10 mmHg)]. The syrup (10 g) was then subjected to ion-exchange column chromatography (Dowex 50-WXr, 70 mL; mobile phase, 1 N NH_4OH) in order to remove excess nitrite ions. The eluate was concentrated to dryness with a rotary flash evaporator and thoroughly evaporated to dryness under reduced pressure in a desiccator. The sample obtained (less-volatile fraction) was stored for mutation tests and GC/MS analysis.

b. Two Steps. An aqueous solution (100 mL) containing cysteamine (0.1 mol) and D-glucose (0.1 mol) was refluxed for 2 h. After the reaction, the reaction mixture was cooled in an ice–water bath and the pH of the reaction solution was adjusted to 2 with 6 N hydrochloric acid in order to optimize *N*-nitrosamine formation. Ten milliliters of sodium nitrite solution (0.3 M) was added to the above solution, stirring with a magnetic stirrer in a flask. Stirring was continued for 5 h at room temperature. The volatile and less-volatile fractions from the above reaction mixture were prepared by the same method as described in step a. The two samples obtained were stored for mutation tests and GC/MS analysis.

High-Pressure Liquid Chromatography (LC). All LC analyses were performed on a Hitachi HPLC System Model 635 A equipped with a multi-wavelength UV absorbance detector for simultaneous monitoring of both 220 and 250 nm. Samples were injected using a Rheodyne syringe loading sample injector Model 712 D with a 20-μL loop. The LC column (25 cm × 4 mm i.d.) was slurry packed with Merck silica gel Lichrosorb SI-100 (particle size, 10 μm). A precolumn (4 cm × 4 mm i.d.), which was dry packed with the same silica gel as was used in the main column, was installed in front of the main column. All LC analyses were conducted using gradient elution from mobile phase A (98% hexane–2% methylene chloride) to mobile phase B (78% ethanol–20% hexane–2% methylene chloride). The eluant flow rate was 1.0 mL/min. The mobile phases were filtered and degassed with an ultrasonic device prior to use.

Gas–Liquid Chromatography–Mass Spectrometry (GC/MS). A Hewlett-Packard Model 5710A gas chromatograph equipped with a flame ionization detector and a 40 m × 0.28 mm i.d. glass capillary column coated with Carbowax 20M was used for routine GLC analyses. The oven temperature was programmed from 80 to 200 °C at 1 °C/min. The nitrogen carrier gas flow rate was 13 cm/s (ca. 0.68 mL/min). The injector and detector temperatures were 250 °C. The injector split ratio was 1:100. A Hewlett-Packard Model 3385-A reporting integrator was used to determine the peak area. The Hitachi Model RMU-6M combination mass spectrometer–gas chromatograph (Hitachi Model M-5201) equipped with Hitachi Model M-6010 and 10 II/A data system was used under the following conditions: ionization voltage, 70 eV; ion source temperature, 200 °C; emission current, 80 μA; ion accel voltage, 3100 V. The gas chromatographic column and oven conditions were as described for the Hewlett-Packard instrument. Identification of products obtained in this study was made by comparison of their mass spectra and Kovats Indices to those of authentic compounds.

Preparation of Standard Thiazolidines and *N*-Nitrosothiazolidines. All alkylthiazolidines were synthesized from the reaction of cysteamine (obtained commercially from Nakari Chemical, Ltd.) and corresponding fatty aldehydes following the method reported by Tondeur et al. (1964). 2-(1,2,3,4,5-Pentahydroxy)-*n*-pentylthiazolidine was obtained following the method reported by Bonner and zu Reckendorf (1961). All *N*-nitrosothiazolidines were synthesized by the general procedure described in Ray (1978).

Mutation Test. The methods reported by Sugimura et al. (1976) and Nagao et al. (1977), a modification of the Ames test (Ames et al., 1973, 1975), were used in the mutation tests. Histidine-requiring strains of *Salmonella typhimurium* TA 98 and TA 100 were used as indicator organisms for mutagenic activity. The degree of mutagenicity was expressed as follows in Tables I–VI: X = (number of colonies per plate of sample)/(number of colonies per plate of control); (++) $X \geq 5$, (+) $5 > X \geq 1.5$, (–) $X < 1.5$.

RESULTS AND DISCUSSION

Mutagenicity of Fractions Obtained from the Reaction Mixture of Cysteamine and D-Glucose. Figure 1 shows the high-pressure liquid chromatogram of the

Table I. Mutagenicities of Fractions from Methylene Chloride Extract

fraction no.	concn, μg/mL	TA 98 without S-9	TA 98 with S-9	TA 100 without S-9	TA 100 with S-9
F_H-1	1			+	−
	10		[a]	+	−
	100			+	−
F_H-2	1			−	−
	10		[a]	−	−
	100			+	−
F_H-3	1			−	−
	10		[a]	−	−
	100			−	−
F_H-4	1			+	−
	10		[a]	+	+
	100			+	+
F_H-5	1			−	−
	10		[a]	−	−
	100			−	+
F_H-6	1	−	−	−	−
	10	−	+	−	−
	100	+	+	+	−
F_H-7	1	−	−	+	−
	10	−	−	+	−
	100	+	+	+	+

[a] No data because of lack of samples.

Table II. Mutagenicities of Fractions from Aqueous Solution

fraction no.	concn μg/mL	TA 98 without S-9	TA 98 with S-9	TA 100 without S-9	TA 100 with S-9
F_I-1	1	−	−	+	+
	10	−	−	+	+
	100	+	−	+	++
F_I-2	1	−	−	−	−
	10	+	+	−	−
	100	+	+	−	−
F_I-3	1	−	−	−	−
	10	−	−	−	−
	100	+	−	−	−
F_I-4	1	−	−	−	−
	10	−	−	−	−
	100	−	−	+	−

volatile fraction. A curve on the chromatogram indicates a gradient of solvent from nonpolar to polar. Each fraction shown on this figure was tested for mutagenicity. The mutagenicities of the fractions (F_H-1 ~ F_H-7) obtained from the methylene chloride extract are summarized in Table I. Each fraction showed some mutagenicity at the level of 100 μg/mL by TA 100. Thiazolidine and 2-methylthiazolidine were found in F_H-4 and F_H-6.

Figure 2 shows the elution curve of ion-exchange column chromatography of the aqueous fraction obtained from the reaction of cysteamine and D-glucose. The fractions shown in Figure 2 were tested for mutagenicity, and the results are shown in Table II. 2-(1,2,3,4,5-Pentahydroxy)-*n*-pentylthiazolidine was identified in F_I-1 which showed fairly strong mutagenicity.

Mutagenicity of the Fractions Obtained from the Cysteamine/D-Glucose/Sodium Nitrite Model Systems. The high pressure liquid chromatograms of the methylene chloride extracts of cysteamine/D-glucose and cysteamine/D-glucose nitrite model systems are shown in Figure 3. It is obvious that different reactions occurred upon nitrite addition. The reaction with nitrite by the two-step method resulted in greater changes than by the one-step method. Two *N*-nitrosothiazolidines were identified in these model systems: they were *N*-nitroso-2-methylthiazolidine and N-nitroso-2-ethylthiazolidine. The mutagenicities of each fraction are shown in Table III. Each sample showed fairly strong mutagenicity by TA 100. It is interesting that the addition of nitrite did not change the mutagenicity much. It is suspected that some antimutagens were formed in these reaction mixtures because *N*-nitrosothiazolidines are much stronger mutagens than thiazolidines, which will be discussed later (refer to Tables V and VI).

The mutagenicities of the less-volatile fractions (aqueous fractions) are shown in Table IV. Each fraction showed positive results by TA 100.

Mutagenicities of Standard Thiazolidines and *N*-Nitrosothiazolidines. Series of thiazolidine derivatives were tested for mutagenicity in order to find the constituents which give mutagenicity to the reaction mixture of cysteamine and D-glucose. Table V shows the results of

Table III. Mutagenicities of Volatile Fractions Obtained from D-Glucose/Cysteamine and D-Glucose/Cysteamine/$NaNO_2$ Model Systems

model system	concn, μg/mL	TA 98 without S-9	TA 98 with S-9	TA 100 without S-9	TA 100 with S-9
D-glucose/cysteamine	1	−	+	++	+
	10	−	+	++	+
	100	−	+	++	+
D-glucose/cysteamine/$NaNO_2$ (two steps)	1	−	−	++	+
	10	−	+	++	+
	100	+	+	++	+
D-glucose/cysteamine/$NaNO_2$ (one step)	1	−	+	++	+
	10	−	+	++	+
	100	+	+	++	+

Table IV. Mutagenicities of Less-Volatile Fractions Obtained from D-Glucose/Cysteamine and D-Glucose/Cysteamine/$NaNO_2$ Model Systems

model system	concn, μg/mL	TA 98 without S-9	TA 98 with S-9	TA 100 without S-9	TA 100 with S-9
D-glucose/cysteamine	1	−	−	+	+
	10	−	−	+	+
	100	+	−	+	++
D-glucose/cysteamine/$NaNO_2$ (two steps)	1	−	−	++	+
	10	−	−	++	+
	100	+	+	++	+
D-glucose/cysteamine $NaNO_2$ (one step)	1	−	−	++	−
	10	−	−	++	+
	100	+	+	++	+

Table V. Mutagenicities of Standard Thiazolidines

compound	concn, μg/mL	TA 98 without S-9	TA 98 with S-9	TA 100 without S-9	TA 100 with S-9
unsubstituted thiazolidine	1	–	–	–	–
	10	+	–	–	–
	100	+	–	+	+
2-methylthiazolidine	1	–	–	–	+
	10	–	–	+	+
	100	+	+	+	+
2-ethylthiazolidine	1	–	–	+	+
	10	+	–	+	+
	100	+	+	+	+
2-*n*-propylthiazolidine	1	–	–	+	+
	10	–	–	+	+
	100	+	+	+	+
2-isopropylthiazolidine	1	–	–	+	+
	10	–	–	+	+
	100	+	–	+	+
2-*n*-butylthiazolidine	1	–	–	–	+
	10	–	–	+	+
	100	+	–	+	+
2-isobutylthiazolidine	1	–	+	–	+
	10	+	+	+	+
	100	+	+	+	+
2-(1,2,3,4,5-pentahydroxy)-*n*-pentylthiazolidine	1	–	–	–	+
	10	+	+	+	+
	100	+	+	+	+ +

the mutagenicity test on thiazolidine derivatives. Unsubstituted, 2-methyl-, 2-ethyl-, 2-*n*-propyl-, and 2-(1,2,3,4,5-pentahydroxy)-*n*-pentylthiazolidine have been identified in the cysteamine/D-glucose model system (Sakaguchi and Shibamoto, 1978; Kitamura et al., 1978). One of the less-volatile, water-soluble components, 2-(1,2,3,4,5-pentahydroxy)-*n*-pentylthiazolidine, showed strong mutagenicity. The mutagenicity of the aqueous fraction of this model system (Table IV) may be due to the presence of this type of polyhydroxythiazolidines.

Table VI shows the results of the mutagenicity test on *N*-nitrosothiazolidines. It is obvious that they are mutagens. Thiazolidines easily form *N*-nitroso derivatives with nitrite under acidic conditions. Thiazolidines have not been reported in foods. Nevertheless many thiazoles and thiazolines, which are dehydrogenated compounds of thiazolidines, have been found in foods (Maga, 1975). Piperazines, which give pyrazines by dehydrogenation, also have not been found in natural foods. It is well known that *N*-nitrosopiperazines are strong mutagens (Zeiger and Sheldon, 1978) and pyrazines are typical browning products (Hodge, 1972). Those saturated heterocyclic compounds, such as thiazolidines and piperazines, may not form in foods, but this is not certain yet.

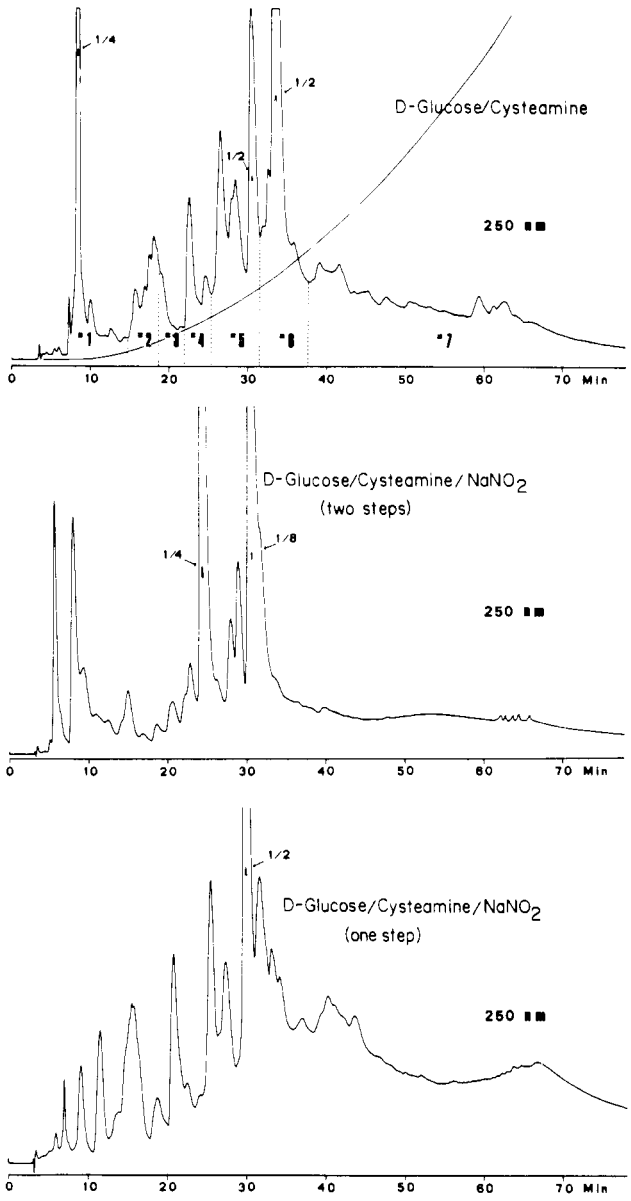

Figure 3. High-pressure liquid chromatogram of the methylene chloride extracts of cysteamine/D-glucose and cysteamine/D-glucose/sodium nitrite model systems.

We have now shown that several fractions of the browning reaction products of cysteamine and D-glucose contains mutagens. Some unidentified compounds among the browning reaction products apparently repress muta-

Table VI. Mutagenicities of Standard *N*-Nitrosothiazolidines

compound	concn, μg/mL	TA 98 without S-9	TA 98 with S-9	TA 100 without S-9	TA 100 with S-9
N-nitroso-2-ethylthiazolidine	1	–	–	++	–
	10	–	–	++	+
	100	–	–	++	+
N-nitroso-2-*n*-propylthiazolidine	1	–	+	++	+
	10	–	+	++	+
	100	–	+	++	+
N-nitrosoisopropylthiazolidine	1	–	–	++	+
	10	–	–	++	+
	100	–	+	++	+
N-nitroso-*n*-butylthiazolidine	1	–	–	++	+
	10	–	+	++	+
	100	–	+	++	+
N-nitrosoisobutylthiazolidine	1	–	+	++	+
	10	–	+	++	+
	100	–	+	++	+

genic activity. It is necessary to study further to determine their structures and the correlations between them and the mutagens in browning reaction systems.

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Chemistry of Baked Potato Flavor. 1. Pyrazines and Thiazoles Identified in the Volatile Flavor of Baked Potato

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The volatile flavor was isolated from 540 lb of Idaho Russet Burbank baked potatoes. Extensive gas chromatographic analysis yielded 420 fractions. The odor of each chromatographic fraction was evaluated, and the fractions were identified by infrared and mass spectrometry. Thirty-one pyrazines and three thiazoles were identified. The results of this work indicate that a natural baked potato flavor is not due to a single compound, but is the result of the mixture of a number of components.

Of all the forms of cooked potato, the flavor produced by baking potatoes is perhaps the most interesting because of its weak but distinct quality.

Baked potatoes are unique because they are cooked as is, only in air at high temperatures without the use of cooking oil, which has been shown to play a vital part in flavor development (Deck et al., 1973). Therefore, the volatile flavor of baked potatoes originates from the potato not from an interaction between the oil and the food, or from the oil. Also, a potato with a higher solids content is used for baking and the outer layer and skin of the potato are retained during preparation. These unique features of the potatoes used and the method employed for preparing baked potatoes play a significant role in the development of the very mild but distinct flavor.

With the identification of 2,5-dimethylpyrazine in the volatiles of potato chips (Deck and Chang, 1965) and roast peanuts (Mason, et al. 1966), pyrazines were uncovered as an extremely important class of flavor compounds in potatoes and other foods (Maga and Sizer, 1973a, b). Pyrazines have also been reported in the volatile flavor of baked potato. Buttery et al. (1973a, b) reported the identification of 45 compounds, mostly pyrazines and aliphatic aldehydes, as volatile flavor components of Washington Russet Burbank potatoes. The authors consider the following compounds to be the most important to baked potato aroma: 2-ethyl-3,6-dimethylpyrazine, methional, deca-*trans*,*trans*-2,4-dienal, and possibly, 2-ethyl-3,5-dimethylpyrazine. In the most recent report, Pareles and Chang (1974) identified 16 compounds in baked potato flavor. Among the compounds identified it was believed that a combination of 2-isobutyl-3-methylpyrazine, 2,3-diethyl-5-methylpyrazine, and 3,5-diethyl-2-methylpyrazine had an odor closer in character to baked potato aroma than did any single compound.

Baked potato flavor has not been extensively studied because of the difficulties involved in obtaining a natural flavor isolate collected from the headspace of baked potatoes in sufficient concentration for chemical analysis. The present paper reports on the identification of pyrazine and thiazole compounds isolated and fractionated from a genuine baked potato flavor.

EXPERIMENTAL SECTION

Isolation of the Volatile Flavor. The volatile flavor was isolated from Idaho Russet Burbank potatoes (with skins) baked at 205 °C for 105 min, the apparatus previously described by Chang et al. (1977). The principle of the apparatus is removal and subsequent condensation of the volatile flavor in the headspace of the baked potatoes. The flavor was not isolated from a water slurry of baked potatoes but from the food as it exists under normal conditions. Thirty pounds of baked potatoes was used for each isolation which lasted 48 h. After 48 h there was a definite decrease in the quality of the flavor in the headspace of the baked potatoes. Due to the low concentration of the flavor in the headspace of the food and

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