compounds.

No clear-cut dependence of the type of activity on electronic (ν), lipophilicity (π), or steric factors (MR) was observed.

LITERATURE CITED

Clifford, D. P.; Jackson, D.; Edwards, R. V.; Jeffrey, P. *Pestic. Sci.* **1976**, *7*, 453–458.

Holan, G. British Patent 1 087 779, 1967.

Holan, G.; Samuel, E. L. U.S. Patent 3 520 898, 1970.

Holan, G.; Samuel, E. L.; Ennis, B. C.; Hinde, R. W. *J. Chem. Soc. C* **1967**, 20–25.

Stephens, F. F.; Bower, J. D. *J. Chem. Soc.* **1950**, 1722–1726.

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Mutagenicity of Products Obtained from a Maltol–Ammonia Browning Model System

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The methylene chloride extract obtained from a reaction mixture of a maltol and ammonia browning model system was separated into five fractions by thin-layer chromatography (TLC). Each fraction was examined for mutagenicity by the Ames' *Salmonella* test using strains TA 98 and TA 100, both with and without S-9 mix. The fraction which exhibited the strongest mutagenicity toward strain TA 98 with S-9 mix was further fractionated into three fractions by TLC. The new fractions were also tested for mutagenicity, with positive responses. 2-Ethyl-3-hydroxy-6-methylpyridine and acetamide isolated from one of new fractions, however, exhibited no mutagenicity. Twenty-two compounds were positively identified in the methylene chloride extract by GC/MS. 2-Ethyl-3-hydroxy-6-methylpyridine was identified by NMR and IR in addition to GC/MS.

In the beginning of this century, Maillard (1912) proposed the browning reaction to account for the brown pigments and polymers produced from the reaction of the amino group of an amino acid and the carbonyl group of a sugar. Hodge (1953) summarized the nonenzymatic browning reactions and indicated that simple model systems could be used to learn about complex food systems. Model systems consisting of an amino acid and a sugar have been most widely used to study the formation of food flavors (Koehler et al., 1969; Shigematsu et al., 1975). More simplified browning systems such as a sugar–ammonia (protein or amino acid decomposition product) have also been used to investigate the formation mechanism of flavor volatiles (van Praag et al., 1968; Shibamoto and Bernhard, 1976).

Certain browning reaction mixtures have exhibited some mutagenic activities toward *Salmonella typhimurium* strains (Mihara and Shibamoto, 1980; Spingarn and Garvie, 1979; Shibamoto, 1980; Toda et al., 1981). In order to investigate the formation and biological nature of browning products, we chose a simple system: maltol [sugar degradation products (Hodge, 1967)] and ammonia. There is much evidence that maltol forms from certain sugars as a product of a nonenzymatic browning reaction (Hodge and Nelson, 1961). Patton (1950) reported that maltol was obtained from lactose and maltose upon heating aqueous solutions of these sugars with glycine. Maltose gave maltol with piperidine salts in nearly dry reactions (Hodge et al., 1963).

In an actual food system, maltol can be formed from a carbohydrate or sugar constituent; it is possible that maltol contacts ammonia formed from protein or amino acid during heat treatment (cooking). Maltol itself showed some mutagenic activities toward *S. typhimurium* strain TA 100 with metabolic activation (Bjeldanes and Chew, 1979). It is, therefore, interesting to observe the behavior of maltol in a browning reaction system.

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Table I. Results of TLC of the Methylene Chloride Extract

fraction no.	R_f values	amount of sample recovered, %[a]
1	0.78–0.87	2.9
2	0.56–0.78	2.3
3	0.48–0.56	3.5
4	0.10–0.48	16.1
5	0.00–0.10	11.7

[a] [(Amount of sample recovered)/(amount of sample applied)] × 100.

Table II. Results of TLC of Fraction 4

fraction no.	R_f values	amount of sample recovered, %[a]
4a	0.35–0.45	17.2
4b	0.23–0.35	41.4
4c	0.11–0.23	14.7

[a] [(Amount of sample recovered)/(amount of sample applied)] × 100.

EXPERIMENTAL SECTION

Sample Preparation. Maltol (0.1 mol) was dissolved into 60 mL of deionized water in a Kjeldahl flask. Ammonium hydroxide solution (0.5 mol as NH_3) was then added. The neck of the flask was flame-sealed and the flask placed in an oven at 100 °C for 5 h. The reaction solution was extracted with 300 mL of methylene chloride by using a liquid–liquid continuous extractor for 16 h. The

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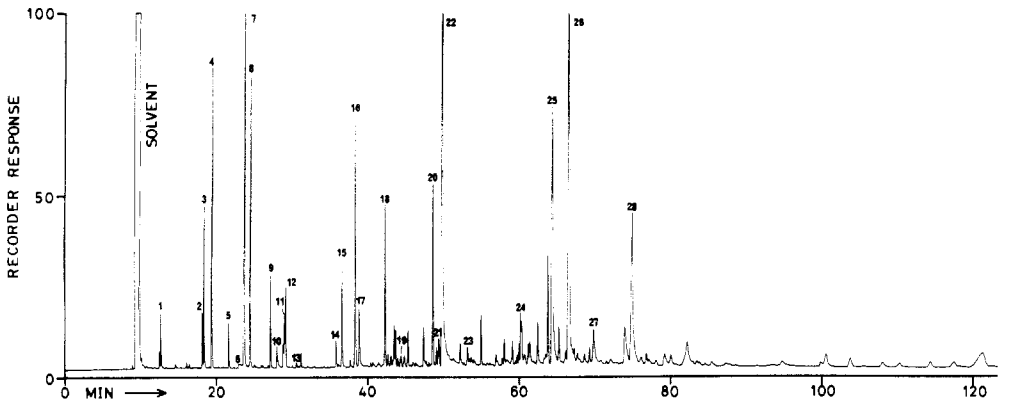

Figure 1. A typical chromatogram of the methylene chloride extract. See Table IV for peak identification and see Experimental Section for gas chromatographic conditions.

methylene chloride solution was dried over anhydrous sodium sulfate and concentrated to yield 3 g of brown liquid.

One gram each of this sample was fractionated into five fractions (fractions 1–5) by thin-layer chromatography, and corresponding fractions were combined. The R_f values of the fractions and the amount of sample recovered from each fraction are shown in Table I. Fraction 4 was further fractionated into three fractions (fractions 4a, 4b, and 4c) by thin-layer chromatography. The R_f values of the new fractions and the amount of sample recovered from each fraction are shown in Table II.

Thin-Layer Chromatography (TLC). Merck silica gel plates (F_{254}; 20 × 20 cm; 2 mm thickness) were used to fractionate the methylene chloride extract (Table I). An acetone–methylene chloride solution (40:60 in v/v) was applied as the developing solvent. Fraction 4 was fractionated under exactly the same conditions (Table II).

Isolation of 2-Ethyl-3-hydroxy-6-methylpyridine. The brown material obtained from fraction 4a (R_f = 0.35–0.45) was recrystallized from ethyl ether. The light brown crystal obtained was further purified by the sublimation at 80–85 °C/60 mmHg. The white crystal obtained was identified as 2-ethyl-3-hydroxy-6-methylpyridine by MS, NMR, and IR. The spectral data of the authentic 2-ethyl-3-hydroxy-6-methylpyridine synthesized by the method of Smirnov and Sholina (1963) are shown in Table III.

Gas–Liquid Chromatography/Mass Spectrometry (GC/MS). A Hewlett-Packard Model 5710A gas chromatograph equipped with a flame ionization detector and a 50 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 2 °C/min. The nitrogen carrier gas flow rate was 13 cm/s. 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 acceleration 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.

Mutagenicity Test. The mutagenicity of the various samples was tested following the method of Ames et al.

Table III. Spectral Data of 2-Ethyl-3-hydroxy-6-methylpyridine

spectrometer	data
MS	M^+ = 137 (88), 136 (100), 135 (5), 122 (10), 109 (11), 94 (8), 81 (10), 80 (6), 53 (9)
NMR (CD_3OD)	δ 1.21 (3 H, t, $-CH_3$), 2.40 (3 H, s, $-CH_3$), 2.80 (2 H, q, $-CH_2-$), 7.00 (2 H, AB-q, -CH-)
IR	2963 (m), 2926 (m), 2870 (w), 1580 (s), 1498 (m), 1450 (m), 1350 (m), 1290 (s), 1270 (s), 1227 (m), 1159 (m), 1124 (m), 1042 (w), 820 (m), 762 (w), 702 (m), 619 (w), 578 (w)

(1975) with minor modifications. Liver homogenate (S-9) was prepared from male Sprague–Dawley rats, which were treated with polychlorinated biphenyl to activate liver enzymes. *S. typhimurium* strains TA 98 and TA 100 were used throughout the experiments. The samples were dissolved in dimethyl sulfoxide and preincubated with a strain at 30 °C for 30 min (prior to plating) with or without S-9 mix. A buffer was substituted when S-9 was not used. After the test samples were added, the plates were incubated at 37 °C for 2 or 3 days. The assay was performed in triplicate for each sample.

RESULTS AND DISCUSSION

The compounds identified in the methylene chloride extract obtained from the reaction mixture of maltol and ammonia are listed in Table IV. A typical gas chromatogram of a methylene chloride extract is shown in Figure 1. The results of the mutation test were interpreted by the method recommended by de Serres and Shelby (1979). A positive (+) result is defined as a reproducible, dose-related increase in number of histidine-independent colonies exceeding twice the number of spontaneous revertant colonies. A negative (−) result is defined as the absence of a reproducible increase in the number of histidine-independent colonies. The results of mutation tests are shown in Table V. All samples which showed positive results required metabolic activation (S-9 mix). A standard deviation/mean ($SD/\bar{X}$) of the number of colonies from triplicate runs varied between 0.01 and 0.19.

Fraction 4, which exhibited the greatest mutagenicity of the fractions obtained toward TA 98 with S-9 mix, was further fractionated into three fractions (4a, 4b, and 4c) in order to search for the specific mutagenic components. Fractions 3 and 5 also showed mutagenicity toward TA 98 with S-9 mix. Neither further fractionation nor analysis was performed on these fractions in this study. The major

Table IV. Compounds Identified from the Maltol-Ammonia Browning Model System

peak no. (Figure 1)	compound	peak[a] area %	MS ref
1	2-methylpyrazine	0.60	Bondarovich et al. (1967)
2	2,5-dimethylpyrazine	0.70	Mussinan and Walradt (1974)
3	2,6-dimethylpyrazine	2.01	Mussinan and Walradt (1974)
4	2,3-dimethylpyrazine	4.02	Mussinan and Walradt (1974)
5	trimethylpyrazine	0.57	Bondarovich et al. (1967)
6	acetic acid	0.04	Mussinan and Walradt (1974)
7	2-ethyl-3,6-dimethylpyrazine	6.11	Kinlin et al. (1972)
8	2-ethyl-3,5-dimethylpyrazine	3.93	Kinlin et al. (1972)
9	2,6-diethyl-3-methylpyrazine	1.23	Kinlin et al. (1972)
10	2-methylpyrrole	0.36	Budzikiewicz et al. (1964)
11	diethyldimethylpyrazine	0.74	Kinlin et al. (1972)
12	triethylpyrazine	1.16	Kinlin et al. (1972)
13	2,4-dimethylpyrrole	0.03	Stenhagen et al. (1974)
14	2,5-dimethyl-6,7-dihydro-5*H*-cyclopentapyrazine	0.37	Kinlin et al. (1972)
15	3,5-dimethyl-6,7-dihydro-5*H*-cyclopentapyrazine	1.65	Kinlin et al. (1972)
16	5-methyl-5,6,7,8-tetrahydroquinoxaline	4.16	Pittet et al. (1974)
17	acetamide	1.17	Ferretti et al. (1970)
18	pyridine derivate, M = 137	2.50	
19	4-ethyl-5-methyloxazole	0.25	Vitzthum and Werkhoff (1974)
20	trimethyloxazole	3.12	Peterson et al. (1975)
21	4,5-dimethyl-2-ethyloxazole	0.04	Vitzthum and Werkhoff (1974)
22	maltol	11.34	
23	unknown	0.26	
24	2,4-dimethylimidazole	0.81	Stenhagen et al. (1974)
25	imidazole derivate, M = 124	8.66	
26	2-ethyl-3-hydroxy-6-methylpyridine	12.99	
27	pyridine derivate, M = 137	1.26	
28	pyridine derivate, M = 137	6.17	

[a] Including 23.75% of solvent (methylene chloride) peak.

Table V. Results of Mutation Tests with Strains TA 98 and TA 100

sample	TA 98 with S-9	TA 98 without S-9	TA 100 with S-9	TA 100 without S-9
methylene chloride extract	+	–	+	–
fraction 1	–	–	–	–
fraction 2	–	–	–	–
fraction 3	+	–	+	–
fraction 4	+	–	–	–
fraction 5	+	–	–	–
fraction 4a	+	–	+	–
fraction 4b	+	–	–	–
fraction 4c	+	–	–	–
maltol	–	–	+	–
2-ethyl-3-hydroxy-6-methylpyridine	–	–	–	–
acetamide	–	–	–	–

Table VI. Major Components Identified in Fractions 4a and 4b

compound	peak area %[a] fraction 4a	peak area %[a] fraction 4b
2-ethyl-3-hydroxy-6-methylpyridine	27.17	2.98
acetamide	5.47	trace
2,3-dimethylpyrazine	4.06	
maltol (unreacted)	7.46	2.51
unknown pyridine derivate	12.11	7.12

[a] Obtained from the gas chromatogram of each fraction, not from the gas chromatogram of the total methylene chloride extract.

components identified in fractions 4a and 4b are listed in Table VI. Fraction 4c did not yield a sufficient amount of constituents to identify.

The dose–response curves of the important samples are shown in Figures 2 and 3. Fractions 4a, 4b, and 4c showed similar mutagenic responses toward tester strain TA 98 with S-9 mix. Only fraction 4a, however, showed dose-related activity against tester strain TA 100. Even though fraction 4a exhibited strong mutagenicity, authentic sam-

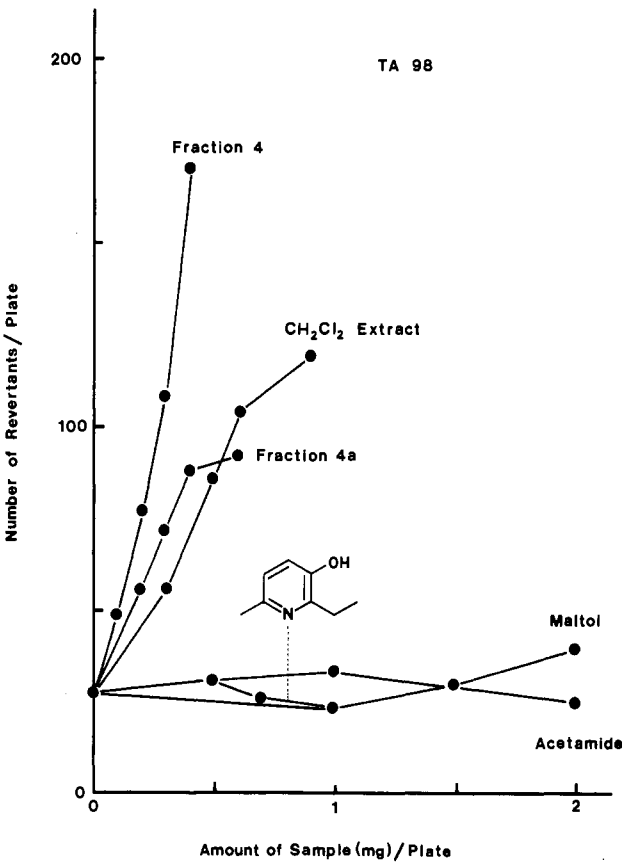

Figure 2. Mutagenicity of methylene chloride extract, fraction 4, fraction 4a, maltol, 2-ethyl-3-hydroxy-6-methylpyridine, and acetamide against *S. typhimurium* strain TA 98 with S-9 mix. Each point is the average of triplicate runs.

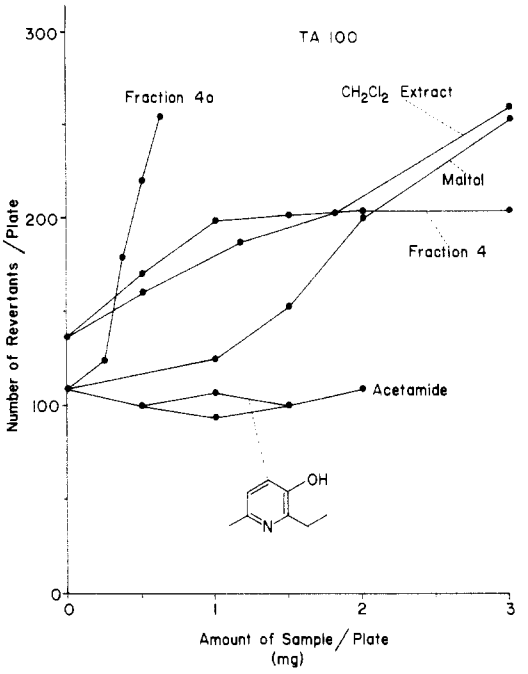

Figure 3. Mutagenicity of methylene chloride extract, fraction 4, fraction 4a, maltol, 2-ethyl-3-hydroxy-6-methylpyridine, and acetamide against *S. typhimurium* strain TA 100 with S-9 mix. Each point is the average of triplicate runs.

ples of its major components, 2-ethyl-3-hydroxy-6-methylpyridine and acetamide, did not show any mutagenic activity toward tester strains TA 98 or TA 100 (Figures 2 and 3).

Fraction 4a contained some unreacted maltol (peak area % = 7.46) which showed mutagenicity toward TA 100 with S-9 mix. The mutagenicity of fraction 4a was, however, stronger than that of maltol toward TA 100, and fraction 4a also responded positively toward TA 98 but maltol did not. The mutagenicity of fraction 4a cannot, therefore, be due only to the presence of maltol. No chemicals which could be determined to be responsible for the mutagenicity of the reaction mixture were isolated; it is, however, obvious that certain mutagenic materials were produced from the reaction of maltol and ammonia. Fractionating the mixture reduced mutagenicity. The mechanism of losing mutagenicity is not well understood yet. The total loss of mutagenicity could, however, be produced by several means, including loss of comutagens, reaction and decomposition of mutagens in the presence of silica, action of heat or light, etc.

The major components of the methylene chloride extract were 2-ethyl-3-hydroxy-6-methylpyridine (peak area % = 12.99) and 2-ethyl-3,6-dimethylpyrazine (peak area % = 6.11). A certain amount of unreacted maltol was also recovered (peak area % = 11.34). It was expected that the main products of this model system would be pyrazines, which comprised 27.61% of the total extract. The formation of a hydroxypyridine in a browning model system was previously been reported by Shibamoto and Russell (1977), although the quantity of 2-hydroxypyridine isolated from their D-glucose–H_2S–NH_3 model system was rather small (peak area % = 0.46). The maltol–NH_3 model system, however, produced a hydroxypyridine as a major product. It is difficult to construct possible formation mechanisms for this compound from maltol and ammonia. All the carbon atoms come from maltol. It is possible, then, that maltol fragments into smaller carbon units and that these fragments recombine to form larger carbon units, producing 2-ethyl-3-hydroxy-6-methylpyridine.

LITERATURE CITED

Ames, B. N.; McCann, J.; Yamasaki, E. *Mutat. Res.* **1975**, *31*, 347.
Bjeldanes, L. F.; Chew, H. *Mutat. Res.* **1979**, *67*, 367.
Bondarovich, H. A.; Giammarino, A. S.; Renner, J. A.; Shepard, F. W.; Shingler, A. J.; Gianturco, M. A. *J. Agric. Food Chem.* **1967**, *15*, 36.
Budzikiewicz, H.; Djerassi, C.; Jackson, A. H.; Kenner, G. W.; Newman, D. J.; Wilson, J. M. *J. Chem. Soc.* **1964**, 1949.
de Serres, F. J.; Shelby, M. D. *Mutat. Res.* **1979**, *64*, 159.
Ferretti, A.; Flanagan, V. P.; Ruth, J. M. *J. Agric. Food Chem.* **1970**, *18*, 13.
Hodge, J. E. *J. Agric. Food Chem.* **1953**, *1*, 928.
Hodge, J. E. *Symp. Foods: Chem. Physiol. Flavors*, [*Proc.*], *1965* **1967**, 474.
Hodge, J. E.; Fisher, B. E.; Nelson, E. C. *Proc. Am. Soc. Brew. Chem.* **1963**, 84.
Hodge, J. E.; Nelson, E. C. *Cereal Chem.* **1961**, *38*, 207.
Kinlin, T. E.; Muralidhara, R.; Pittet, A. O.; Sanderson, A.; Walradt, J. P. *J. Agric. Food Chem.* **1972**, *20*, 1022.
Koehler, P. E.; Mason, M. E.; Newell, J. A. *J. Agric. Food Chem.* **1969**, *17*, 393.
Maillard, L. C. *C. R. Hebd. Seances Acad. Sci.* **1912**, *154*, 66.
Mihara, S.; Shibamoto, T. *J. Agric. Food Chem.* **1980**, *28*, 62.
Mussinan, C. J.; Walradt, J. P. *J. Agric. Food Chem.* **1974**, *22*, 827.
Patton, S. *J. Biol. Chem.* **1950**, *184*, 131.
Peterson, R. T.; Izzo, H. J.; Jungermann, E.; Chang, S. S. *J. Food Sci.* **1975**, *40*, 948.
Pittet, A. O.; Muralidhara, R.; Walradt, J. P.; Kinlin, T. E. *J. Agric. Food Chem.* **1974**, *22*, 275.
Shibamoto, T. *J. Agric. Food Chem.* **1980**, *28*, 883.
Shibamoto, T.; Bernhard, R. A. *J. Agric. Food Chem.* **1976**, *24*, 847.
Shibamoto, T.; Russell, G. F. *J. Agric. Food Chem.* **1977**, *25*, 109.
Shigematsu, H.; Shibata, S.; Kurata, T.; Kato, H.; Fujimaki, M. *J. Agric. Food Chem.* **1975**, *23*, 233.
Smirnov, L. D.; Sholina, S. E. *Izv. Akad. Nauk. SSSR, Otd. Khim. Nauk.* **1963**, 890.
Spingarn, N. E.; Garvie, C. T. *J. Agric. Food Chem.* **1979**, *27*, 1319.
Stenhagen, E.; Abrahamson, S.; McLafferty, F. W. "Registry of Mass Spectral Data"; Wiley: New York, 1974; Vol. I.
Toda, H.; Sekizawa, J.; Shibamoto, T. *J. Agric. Food Chem.* **1981**, *29*, 381.
van Praag, M.; Stein, H. S.; Tibbetts, M. S. *J. Agric. Food Chem.* **1968**, *16*, 1005.
Vitzthum, O. G.; Werkhoff, P. *J. Food Sci.* **1974**, *39*, 1210.

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