Factors Affecting the Formation of Pyrazine Compounds in Sugar-Amine Reactions[1]

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Factors which affect the production of pyrazine compounds in a sugar-amino acid model system were studied. Temperature, time, reactant ratio, and acid-base effects were determined for a model system containing varying concentrations of glucose and asparagine in a diethylene glycol-water system. The changes in yield and distribution of alkylpyrazine compounds produced by utilizing different nitrogen and carbonyl compounds were also investigated.

Pyrazine compounds have been identified in the aroma fraction of coffee, cocoa, peanuts, and potato chips (Marion *et al.*, 1967; Goldman *et al.*, 1967; Rizzi, 1967; Bondarovich *et al.*, 1967; Mason *et al.*, 1966; Deck and Chang, 1965). One of the events occurring during the roasting of food products appears to be a reaction between amino acids and sugars which eventually leads to the formation of pyrazine compounds (Davidson and Wiggins, 1956; Dawes and Edwards, 1966; Koehler *et al.*, 1969). Sugar-ammonia reactions are important to ammoniated feed producers and coloring caramel manufacturers who are concerned with the formation of pyrazine and imidazole compounds in their products (Wiggins and Wise, 1955; Davidson and Wiggins, 1956). Considering the commercial interest in sugar-amine reactions, it is important to study the factors affecting the formation of pyrazine compounds in sugar-amine systems. This type of study could be used to establish factors affecting the formation of other volatile food flavor components.

PROCEDURES

Composition of Model System. The "normal" model system used in these studies utilized 0.1 mol each of D-glucose and L-asparagine heated 24 hr at 120° C ± 1° C in 200 ml of diethylene glycol and 20 ml of deionized water. This system was then altered by varying one of the "normal" reaction parameters while holding the others constant. A solvent system of low water content was chosen to more closely simulate the roasting of essentially nonaqueous food products such as peanuts. The choice of solvent reaction medium was made on the basis of reactant solubility, recovery of pyrazines formed, viscosity, and inertness in the browning reaction.

The pyrazine compounds produced were quantitatively isolated from the reaction mixtures by passing each very slowly (twice) through a falling film evaporator with a steam-heated jacket (Herz and Chang, 1966). The volatile materials were trapped on a single liquid nitrogen cold-finger trap. A smaller cold-finger trap, placed between the larger trap and the vacuum pump, prevented volatile compounds from the vacuum or oil diffusion pumps from contaminating the isolated volatiles. The collected volatiles were extracted five times with 5 ml of dichloromethane. The extract volume was reduced by removing some of the dichloromethane under reduced pressure on a rotary evaporator, and after dilution to exactly 5 ml with dichloromethane, an aliquot of the extract was chromatographed.

Identification and Quantitation of Pyrazines. Quantitative gas-liquid chromatographic analyses were performed on a Perkin-Elmer Model 801 gas chromatograph equipped with hydrogen flame ionization detectors. A 20 ft × 1/4 in. o.d. glass column containing 15% (w/w) Carbowax 20M on Gas Chrom Q (100/120 mesh) was used at 150° C isothermally with a nitrogen carrier gas flow rate of 60 ml per min. Gas chromatographic peaks were quantitated by comparison of peak areas of samples with those of standards chromatographed the same day under the same conditions. Three injections each of the sample and standard were made and the results averaged. Using a 4.0 to 1 stream-splitter, aliquots of several peaks were collected and subjected to infrared spectrometry to confirm the identifications by gas chromatographic retention times.

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RESULTS AND DISCUSSION

Before initiating quantitative work on pyrazine production in sugar-amino acid model systems, it was necessary to insure that the various pyrazine compounds could be recovered quantitatively from the reaction mixture. To determine the efficiency of the isolation procedure, 2.5 mmol each of pyrazine, 2-methylpyrazine, and 2,5-dimethylpyrazine were added to the reaction mixture solvent (200 ml diethylene glycol and 20 ml water). Using only a single pass down the falling film evaporator, 98% of the pyrazines were recovered, based on gas chromatographic analysis of the isolated pyrazines. In the actual experiments, two passes through the falling film evaporator were made to insure complete removal of all pyrazines.

The amount of alkylpyrazines produced by the model system in a 24 hr period at a variety of temperatures between 80° C and 150° C was determined (Figure 1). At temperatures below 100° C, essentially no pyrazine compounds (less than 0.1 μmol) were produced. At 100° C, pyrazine formation began and the yield increased rapidly as the temperature increased. At temperatures above 150° C, the yields were highly variable. This may have been due either to the destruction of the pyrazines after formation, or else to the loss of the compounds from the reaction mixture through the reflux condenser at the higher temperatures. The ratio of dimethylpyrazine to methylpyrazine varied with the temperature, as shown in Figure 1. This ratio reached a maximum of about 4.5 at 115° C.

The production of pyrazine compounds at 120° C (Figure 2) increased rapidly as the length of the heating period was increased up to 24 hr when pyrazine formation began to level off with only minor increases in yield for periods up to 72 hr. While methylpyrazine was the major product in the early stages of the reaction (less than 3 hr) the ratio of dimethylpyrazine to methylpyrazine produced continued to increase for about 9 hr. After this time, the ratio remained essentially constant at approximately 3.0, regardless of the length of the heating period.

The normal model system utilized asparagine and glucose reactants in a 1 to 1 molar ratio. Tripling the amount of glucose decreased the yield of 20 methylpyrazine ten-fold and that of the dimethylpyrazine about 125-fold (Figure 3).

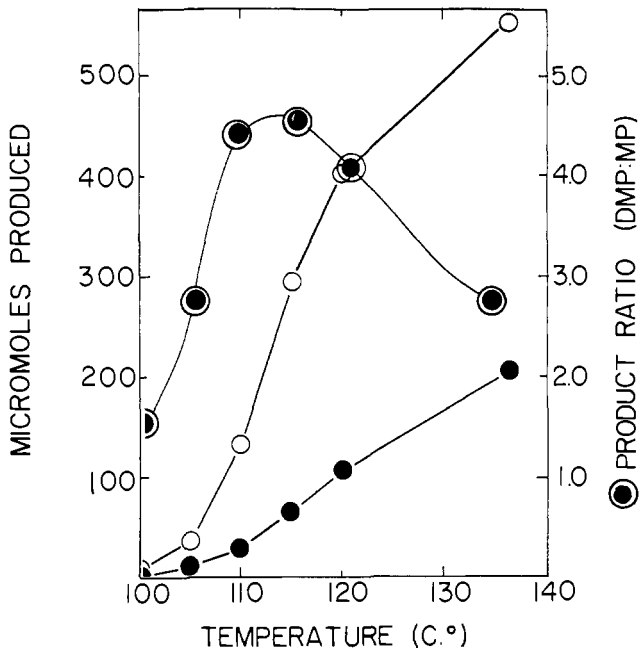

Figure 1. Temperature effect on pyrazine yield in a sugar-amino acid model system. ○ = Dimethylpyrazine; ● = methylpyrazine. 0.1 mol each of D-glucose and L-asparagine; time = 24 hr; solvent = 200 ml diethylene glycol plus 20 ml H_2O

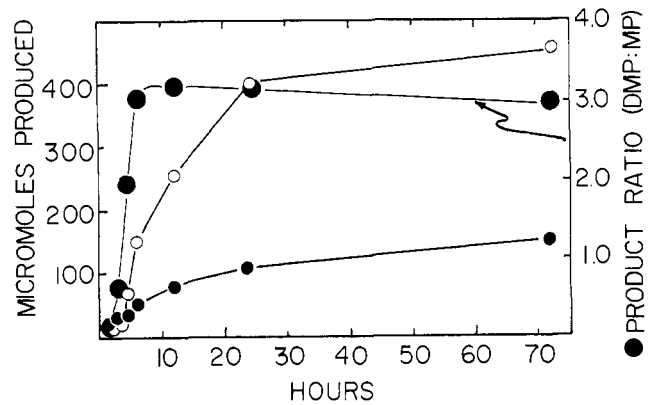

Figure 2. Methylpyrazine yields from the model system after various heating intervals. ○ = Dimethylpyrazine; ● = 2-methylpyrazine; ○ = DMP/MP ratio. 0.1 mol each of D-glucose and L-asparagine; temperature = 120° C; solvent = 200 ml diethylene glycol plus 20 ml H_2O

Tripling the amount of asparagine had very little effect on the dimethylpyrazine yields and decreased the methylpyrazine yield only about 25%.

Since the work was conducted in a nearly nonaqueous solvent, it is difficult to specify the pH of the reaction media. However, the addition of sulfuric acid or sodium hydroxide has a dramatic effect on alkylpyrazine formation (Figure 4). When both the glucose and asparagine reactants were present in amounts of 0.1 mol, the addition of an equal number of equivalents of acid lowered pyrazine formation to practically zero, while addition of an equal amount of base increased the yield of methylpyrazine ten-fold and the yield of dimethylpyrazine five-fold. This base catalysis is probably due both to the increased reactivity of the amino group of the amino acid toward the carbonyl of glucose and to the increased rearrangement and fragmentation of sugars.

The knowledge gained from the effects of time, temperature, solvent, reactant ratio, and acidity-basicity on the reaction yields was used to establish conditions which would

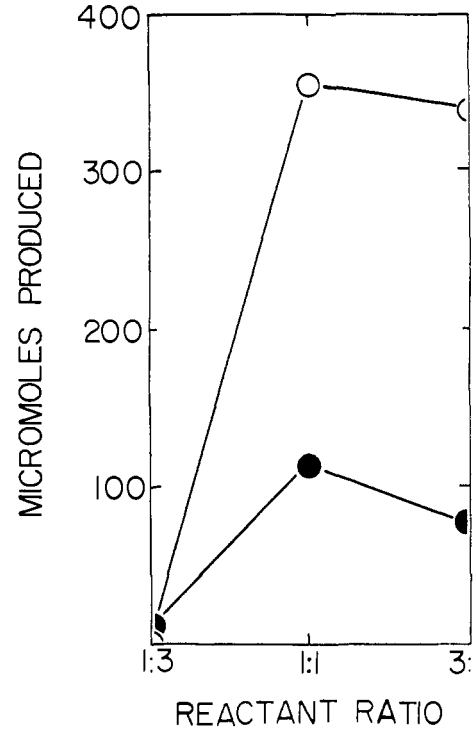

Figure 3. Effect of the asparagine:glucose reactant ratio on the yield of pyrazines in the sugar-amino acid model system. ○ = Dimethylpyrazine; ● = 2-methylpyrazine; temperature = 120° C; time = 24 hr; solvent = 200 ml diethylene glycol plus 20 ml H_2O

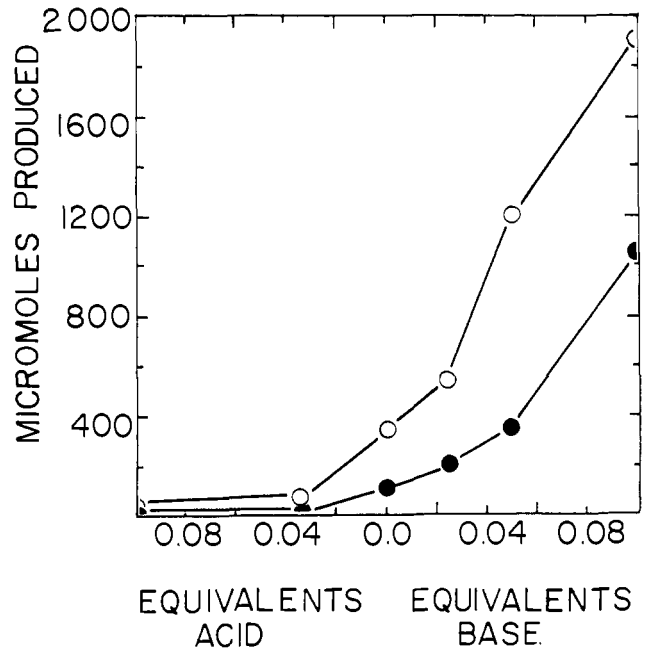

Figure 4. Acid-base effects on pyrazine production in the sugar-amino acid model system. ○ = Dimethylpyrazine; ● = 2-methylpyrazine. 0.1 mol each of D-glucose and L-asparagine; temperature = 120° C, time = 24 hr; solvent = 200 ml diethylene glycol plus 20 ml H_2O

maximize the yield of alkylpyrazines. Using diethylene glycol solvent with no water, equimolar quantities (0.1 mol) of glucose, asparagine, and sodium hydroxide, the reaction was conducted at 130° C for 48 hr. From this system, 190 mg of dimethylpyrazine and 75 mg of 2-methylpyrazine were obtained. This represents yields of 1.8 and 0.8%, respectively.

In addition to asparagine, other amino acids, glycine,

alanine, aspartate, and lysine were used in the model system, but the yields were much lower in all cases (Table I). This table also compares the yields of methyl- and dimethylpyrazine from glucose reacted with asparagine, aspartate plus base under the normal model system conditions. The results seemed to indicate that the differences between asparagine and aspartic acid were due to differences in acidity-basicity, rather than to the type or number of nitrogen atoms in the amino acid. Similar results were obtained when ammonium hydroxide and ammonium chloride were used as nitrogen sources in place of an amino acid.

Table II compares the pyrazines produced when asparagine is reacted with either glucose, fructose, sucrose, or arabinose. The alkylpyrazine yields are higher with fructose than with glucose, and the ratio of dimethylpyrazine to methylpyrazine is much higher. The higher yields could be due to the fact that fructose forms carbon fragmentation units more readily than glucose (Stadtman *et al.*, 1952). Further, a communication by Erickson (1953) indicates that ketoses react more readily with primary amines than aldoses in alcoholic aqueous solutions. The enolization, amino shift, and eliminations which follow fructose-amino acid condensations have been investigated by Heyns *et al.* (1967, 1968).

Sucrose produced good yields of alkylpyrazine compounds, although less than either glucose or fructose. Arabinose also produced pyrazines when reacted with amino acids. In this case, more methylpyrazine than dimethylpyrazine was produced, and the dimethylpyrazine to methylpyrazine ratio was very low. This is reasonable since a 5-carbon sugar must yield one 2-carbon fragment for each 3-carbon unit on fragmentation. Thus one might expect more methylpyrazine from a pentose and more dimethylpyrazine from a hexose.

In addition to sugars, several smaller compounds were used to establish their effectiveness in forming pyrazine compounds (Table III). Propionaldehyde is known to be formed in amino acid-sugar reactions (Rooney *et al.*, 1967). However, no pyrazines were detected when propionaldehyde was reacted with asparagine in the model system. Glycerol gave small amounts of each of the first three members of the alkylpyrazine series. Acrolein or glyceraldehyde was very likely the compound actually responsible for the condensation reaction with nitrogen to form the pyrazines. Acetaldehyde also occurs in amino acid-sugar reaction mixtures (Rooney *et al.*, 1967). Acetaldehyde, being a 2-carbon unit, might be expected to yield only pyrazine if it reacts. Indeed, when reacted with ammonium hydroxide, large amounts of pyrazine were produced and none of the alkylpyrazines were detected. However, when reacted with asparagine, all of the first three members of the pyrazine series were detected with dimethylpyrazine being formed in the greatest amounts (Table III). This indicates that either acetaldehyde accelerates the breakdown of the amino acid to produce 3-carbon units, or acetaldehyde itself can react to give 3-carbon units necessary for the formation of methyl- and dimethylpyrazine. Since no carbon atoms of the pyrazines arise from the amino acid in the normal glucose-amino acid reaction (Koehler *et al.*, 1969), the latter case is favored. The pH differences in the media brought about by different nitrogen compounds may produce this effect. Glyoxal, another 2-carbon unit, gives the highest yields of pyrazine of all compounds studied. It does, however, give some methylpyrazine, indicating again that some 3-carbon units must be formed. The presence of 7-carbonfuran compounds in the volatiles from heated glucose suggests that secondary recombinations and rearrangements of sugar fragments do occur (Walter and Fagerson, 1968).

Table I. Pyrazines Produced During the Reaction of Glucose with Nitrogenous Compounds in the Model System

Nitrogen source	Methylpyrazine (μmol)[a]	Dimethylpyrazine (μmol)[a]	DMP/MP
Asparagine	110	402	3.7
Glycine	ND[b]	14	...
Alanine	23	82	3.6
Lysine	11	20	1.8
Aspartate	16	81	5.1
Aspartate + NaOH	92	282	3.1
Ammonium hydroxide	1415	56	0.04
Ammonium chloride	ND	ND	...

[a] μmol/220 ml reaction mixture, 0.1 mol of D-glucose and the indicated nitrogen source, solvent = 200 ml diethylene glycol plus 20 ml H_2O, 120° C for 24 hr. [b] ND = not detected.

Table II. Methylpyrazine Yield from Reaction of Asparagine with Various Sugars in the Model System

Carbon source	Methylpyrazine (μmol)[a]	Dimethylpyrazine (μmol)[a]	DMP/MP
Glucose	110	402	3.7
Fructose	137	1142	8.4
Sucrose	85	166	2.0
Arabinose	94	48	0.5

[a] μmol/220 ml of reaction mixture, 0.1 mole of L-asparagine and the indicated sugar, solvent = 200 ml diethylene glycol plus 20 ml H_2O, 120° C for 24 hr.

Table III. Comparison of Pyrazine Yield from Various Carbon Sources

	Pyrazine (μmol)[a]	Methylpyrazine (μmol)[a]	Dimethylpyrazine (μmol)[a]	Tetramethylpyrazine (μmol)[a]
Propionaldehyde–asparagine	ND[b]	ND	ND	ND
Glycerol–asparagine	2	7	2	ND
Acetaldehyde–asparagine	29	51	291	ND
Acetaldehyde–NH_4OH	399	ND	ND	ND
Glyoxal–asparagine	527	91	ND	ND
Glyoxal–NH_4OH	253	16	ND	ND
2,3-Butanedione–asparagine	ND	ND	ND	2662
2,3-Butanedione-NH_4OH	ND	ND	ND	640
Hydroxyacetone–asparagine	ND	ND	9725	ND
Hydroxyacetone–NH_4OH	ND	ND	2208	ND
Glucosamine	107	252	27	ND

[a] μmol/220 ml of reaction mixture, 0.1 mol of each reactant, 200 ml of diethylene glycol plus 20 ml H_2O, 120° C for 24 hr. [b] ND = not detected.

The 4-carbon dicarbonyl, 2,3-butanedione, might be expected to produce the 8-carbon alkylpyrazine, 2,3,4,6-tetramethylpyrazine. Diacetyl (2,3-butanedione) has been reported as a degradation product of sugars and amino acid mixtures (Nonenzymatic Browning Conference, 1952). As seen in Table III, tetramethylpyrazine is produced in very large quantities when 2,3-butanedione is heated either with asparagine or ammonium hydroxide. No other alkylpyrazines were detected, indicating that fragmentation into smaller units probably does not occur. Again, pH differences may be responsible for the differences in the yields, particularly in view of the large pH effects shown in Figure 4.

Pyrazine compounds have been found rarely as products of microbial metabolism. Tetramethylpyrazine is believed to be responsible for the characteristic odor of fermented soybean or "natto," and is produced by a strain of *Bacillus subtilis* (Kosuge and Kamiya, 1962). This same compound has also been found in concentrations sufficient to crystallize from the

broth media containing a mutant strain of *Corynebacterium glutamicum* (Demain *et al.*, 1967). In both of these cases, the condensation of acetoin with ammonia was believed to be responsible for the formation of tetramethylpyrazine. Metabolic blocks in these organisms cause formation of unusual amounts of acetoin. Presently it is not known if the condensation and subsequent reduction of acetoin with ammonia forming tetramethylpyrazine is enzymatic.

Lento *et al.* (1960a) studied the formation of some 3-carbon carbonyl compounds from glucose and fructose heated in buffer solutions. It was suggested (Lento *et al.*, 1960b) that acetol (hydroxyacetone) might be an important intermediate in the browning reaction at high pH levels. When used as the carbon source in the model system reaction study, the 3-carbon compound, hydroxyacetone, produced only dimethylpyrazine, as might be predicted (Table III). The yield of dimethylpyrazine was very high, indicating that hydroxyacetone might be a good intermediate in the pyrazine formation reaction as well as in the browning reactions.

Glucosamine hydrochloride neutralized with an equivalent amount of sodium hydroxide produced pyrazine, 2-methylpyrazine, and dimethylpyrazine when heated in the model system (Table III). Fragmentation of glucosamine between carbon atoms number 2 and 3 would produce a 2-carbon unit with both an aldehyde and an amine group. Condensation of two of these units could produce pyrazine. Condensation of a 3-carbon unit with one of the 2-carbon sugar derived fragments could produce methylpyrazine. Thus glucosamine is capable of serving as the source of both the carbon and nitrogen atoms of a variety of alkylpyrazine molecules.

There is apparently more than one pathway whereby different compounds can form alkylpyrazines in the sugar-amine reaction system and in natural food products. In the food products, two major routes could be defined. In one, the sugar molecules would first react with amino acids and then this glucosylamine product would condense to form a di-tetrahydroxybutylpyrazine (Hough *et al.*, 1952) intermediate. This intermediate would then undergo rearrangement and cleavage to form alkylpyrazines. Alternatively, at high temperatures, sugars may immediately undergo rearrangements and cleavage into numerous smaller hydroxycarbonyl and dicarbonyl fragments. Any two of such fragments could then condense with nitrogen from amino acids to form the many alkylpyrazine compounds found in roasted food products. The pathway through the tetrahydroxybutylpyrazine intermediate (Hough *et al.*, 1952) may be important in the slow formation of alkylpyrazine compounds at low temperatures over longer intervals of time. At higher temperatures which favor rearrangement and fragmentation of the sugars, the condensation of smaller hydroxycarbonyl and dicarbonyl fragments may be of greater importance.

The production of pyrazines using a model system of glucose and asparagine in a solvent of diethylene glycol-water (10 to 1) shows the expected response to increasing temperatures. A ten-fold increase of dimethylpyrazine from 100° C to 140° C was observed with 24 hr reaction-periods, while a four-fold increase was noted for methylpyrazine. A change in product ratios over this temperature span was also noted (Figure 1). The time period of 24 hr was selected when it was shown (Figure 2) that the production of methyl and dimethylpyrazines plateaued after 20 hr. From the limited data shown in Figure 3, reactant ratios affect the amount of pyrazine formed if the glucose is present in excess. An excess of asparagine of 3 to 1 does not appreciably change the amount or the product ratio.

If the formation of a Schiff's base with the sugar-amino acid is considered as a major path for pyrazine formation, the effect of base on the reaction, as shown in Figure 4, would lend support to this hypothesis. The effect of alkali in establishing an equilibrium between the monosaccharide, glucose, and an enediol will permit the formation of a ketose. In view of the fact that fructose is an excellent reactant for pyrazine formation (Table II) the base catalyzed ketose production may be involved in the formation of pyrazines in this system. Further, with acidic conditions, a protonated amino group on asparagine would not favor a condensation reaction with an aldehyde or ketone group.

The production of pyrazines from 2,3-butanedione, hydroxyacetone, and glyoxal was as expected with an interesting effect of nitrogen source on the amount of product formed. The authors would suggest that the formation of tetramethylpyrazine from 2,3-butanedione, pyrazine from glyoxal, and dimethylpyrazine from hydroxyacetone follows the condensation and oxidation route through dihydropyrazines to pyrazines. It would also appear that aldehydes or ketones of this type, whether formed from lipids or carbohydrates, could proceed to pyrazines under the conditions used in certain cooking processes.

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