Formation of Pyrazine Compounds in Sugar-Amino Acid Model Systems

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The pathway for the formation of alkylated pyrazine compounds in amino acid–carbohydrate model systems of low water content was investigated. Radioisotopic labeling studies indicated that sugars were the principal source of the carbon atoms, while amino acids mostly furnished only nitrogen to the pyrazine molecule. Ammonium ions were not the common intermediate through which nitrogen entered the pyrazine ring. Possible pathways for fragmentation of hexoses into two- and three-carbon units and their incorporation into pyrazines were developed.

Newell *et al.* (1967) suggested that amino acids and carbohydrates are among the precursors of typical peanut flavor. A number of simple alkylated pyrazine compounds have been shown to occur among the volatile compounds isolated from roasted peanuts (Mason *et al.*, 1966). It was suggested (Johnson, 1966; Mason *et al.*, 1966) that these heterocyclic compounds were responsible for the "roasted-nutty" character of roasted peanuts. Alkylated pyrazines have also been found among the volatile aroma compounds from potato chips, coffee, and cocoa (Bondarovich *et al.*, 1967; Deck and Chang, 1965; Goldman *et al.*, 1967; Marion *et al.*, 1967; Rizzi, 1967). All of these foods are characterized by treatment at high temperatures during their processing. Thus it becomes important to demonstrate that amino acid and carbohydrate precursors give rise to alkylated pyrazines on heating and to investigate the pathway by which this occurs, which is at present uncertain. The purpose of this study was to obtain data from a model system which would demonstrate the sources of the carbon and nitrogen atoms of the pyrazine molecule and suggest the probable pathway for their incorporation.

PROCEDURES

Production and Isolation of Pyrazines from Model System. Amino acid–carbohydrate model systems were routinely prepared by dissolving 10 mmoles each of an amino acid and a sugar in 20 ml. of deionized water and 200 ml. of diethylene glycol. This mixture was refluxed with stirring at 120° C. for 24 hours, then passed slowly over a falling film evaporator maintained at 98° C. (Hertz and Chang, 1966). The volatile materials were trapped on a single cold-finger trap cooled with liquid nitrogen. This material was then extracted with methylene chloride and aliquots were analyzed by gas chromatography to determine qualitatively and quantitatively the distribution of the various alkylated pyrazine products. Experiments showed that recovery of the pyrazines formed in the model system was better than 90%.

Identification and Quantitation of Pyrazines. Analytical gas chromatography was conducted on a Perkin-Elmer Model 801 dual hydrogen flame gas chromatograph using a 20-foot by 1/4-inch O.D. glass column packed with 15% (w./w.) Carbowax 20M on Gas Chrom Q. Flow rate of the nitrogen carrier gas was 48 ml. per minute. The temperature was programmed linearly from 75° to 190° C. at 4° per minute. A prototype of the LKB 9000 combination mass spectrometer–gas chromatograph was used for mass spectral identification of the gas chromatographic components. Agreement of relative retention times of compounds from the model system with those of standards was routinely used for identification, after mass spectra of components from the glucose-asparagine model system indicated that identifications made from relative retentions alone were valid. Gas chromatographic peaks were quantitated by comparison of peak areas of samples with those of known weights of standards chromatographed the same day under the same conditions.

Estimation of Total Yield from Various Carbohydrate–Amino Acid Mixtures. Aliquots of the volatiles removed from the reaction mixture with the falling film evaporator were diluted and the ultraviolet absorbance at the absorption maximum was determined. The absorption maximum (Table I) was always near that expected for a mixture of alkylated pyrazines—e.g., 2-methylpyrazine (MP) $\lambda_{max} = 278$ mμ. The extinction coefficient of a sample of commercial 2,5-dimethylpyrazine (DMP) was determined. This value ($E_{max} = 6.97$ absorbance units per μmole) was used to estimate the

Table I. Yield of Pyrazines Produced from Heated Sugar–Amino Acid or Sugar–Ammonium Chloride Model Systems

Model System	Total Yield, μmoles	Absorption Maximum, Mμ
Asparagine-glucose	411	272
Glutamine-glucose	205	272
Aspartic acid–glucose	198	274
Glutamic acid–glucose	114	274
NH_4Cl-glucose	59	262
NH_4Cl-fructose	195	280
Asparagine-sucrose	268	274
Asparagine-glucose-acetaldehyde	11643	280

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total pyrazine concentration in the volatiles from the various amino acid–sugar mixtures. This method was used routinely after several analyses by gas chromatography showed it to be a reliable estimate of total pyrazine concentration. The over-all yield of pyrazines (Table I) in the glucose-asparagine system was about 4% of 10 mmoles of glucose used as reactant.

Radioisotope Labeling Studies. The model systems were prepared as described previously, except that 2 μcuries of either a radioactive amino acid or glucose labeled in various positions was added to the reaction mixture. The methylene chloride extract of the condensate containing the volatiles was subjected to preparative gas chromatography using a 20-foot × 1/4-inch aluminum column packed with Carbowax 20M (15% w./w.) on Gas Chrom Q in a F & M Model 500 gas chromatograph equipped with a thermal detector. The helium flow rate was 60 ml. per minute. The column was maintained at 120° C. The MP and DMP peaks were collected in traps filled with methylene chloride. Each of the collected compounds was then quantitatively estimated by gas chromatography and its radioactivity determined with a Packard Tricarb Model 3003 liquid scintillation spectrometer. From this information, the specific activity of each pyrazine was calculated.

RESULTS AND DISCUSSION

To determine the origin of the carbon atoms in the pyrazine molecule, companion experiments were run, first utilizing ^{14}C-labeling in the sugar molecule and unlabeled amino acid, and then repeating the experiment using ^{14}C-labeling in the amino acid with unlabeled sugar. The ratios of the specific activities of the product pyrazine (P) to the labeled reactant (R) are compared in Table II for two different mixtures. In each mixture, the labeling of the product pyrazines was dramatically lower when the ^{14}C-label was in the amino acid. Thus, the major source of the carbon atoms of the pyrazine molecule was the sugar. The amino acid served primarily as the nitrogen source.

Table II. Origin of Pyrazine Carbon Atoms in Sugar–Amino Acid Model Systems

Labeled Reactant	Unlabeled Reactant	Ratio Specific Activities (P/R) Methylpyrazine	Dimethylpyrazine
Experiment 1			
Glucose-1-^{14}C	Asparagine	1.290	0.970
Asparagine-UL-^{14}C	Glucose	0.002	0.007
Experiment 2			
Glucose-1-^{14}C	Alanine	1.200	0.950
Alanine-2-^{14}C	Glucose	0.026	0.007

Two possible pathways for the formation of pyrazines in sugar–amino acid systems were considered. In the first, the amino group of each amino acid would ultimately be converted to ammonia before reacting with the carbohydrate. If this were the case, and ammonia were the common intermediate, each amino acid would be expected to give the same distribution of alkylated pyrazine products and also the same product distribution as an ammonium salt heated with glucose. In a second possible pathway involving condensation of the amino acid–bound nitrogen with the carbonyl of the sugar or a six-carbon intermediate, the ease of the

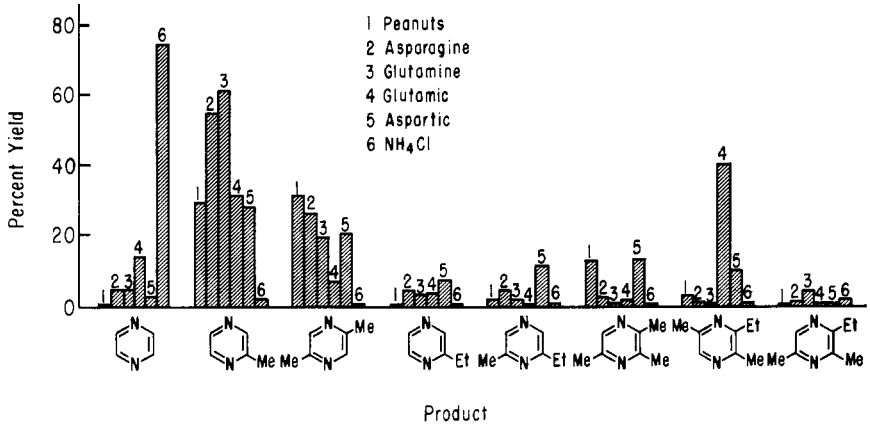

Figure 1. Distribution of pyrazine compounds produced by heating glucose with various amino acids

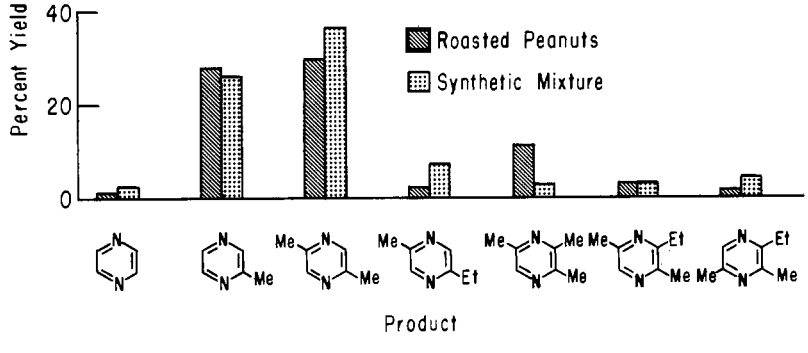

Figure 2. Pyrazines produced upon heating a mixture of amino acids and reducing sugars resembling good-flavored peanuts

nucleophilic attack of the amino acid molecule on the sugar would be influenced by the structure of the amino acid reactant. Each amino acid would be expected to give a different product distribution and also one different from an ammonium salt. Figure 1 shows that upon heating some of the amino acids found in raw peanuts with glucose in the model system, different distributions of alkylated pyrazine products were obtained. Per cent yield in the figures indicates the per cent that each peak contributed to the total yield of pyrazines. Ammonium chloride yielded mostly pyrazine and only traces of alkylated pyrazines, while the amino acids gave mostly alkylated pyrazines with very small amounts of pyrazine. Thus ammonia or the ammonium ion must not be the common intermediate through which amino acid nitrogen enters the pyrazine molecule.

No single amino acid when heated with glucose gave a distribution of pyrazines identical to that obtained from a falling film distillate of homogenized, roasted peanuts (Figure 1). However, a mixture of amino acids and monosaccharides which approximated the amino acid and carbohydrate content of good flavored raw peanuts (Newell *et al.*, 1967) did give a distribution of pyrazines more closely resembling that of roasted peanuts (Figure 2).

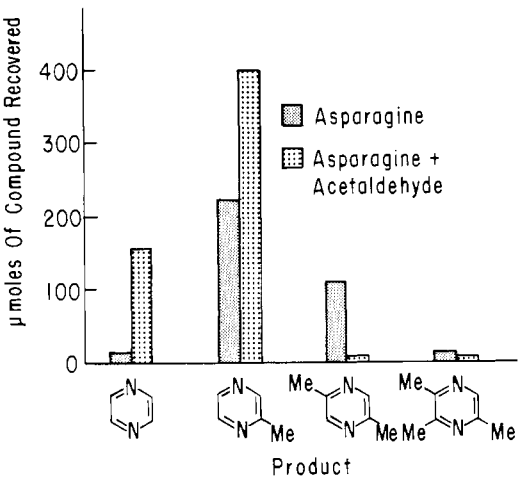

Figure 3. Effect of adding excess acetaldehyde to a model system containing glucose and asparagine

The probable pathway of incorporation of carbon atoms into pyrazine molecules was also investigated. The formation of all of the alkylated pyrazines could be thought of as involving condensation of two- and three-carbon fragments from sugars with nitrogen from amino acids (Dawes and Edwards, 1966). Pyrazine itself could arise from condensation of two two-carbon fragments with nitrogen, methylpyrazine from condensation of one two-carbon and one three-carbon fragment, and dimethylpyrazine from condensation of two three-carbon fragments.

To determine if two-carbon fragments would indeed take part in the pyrazine formation reactions, a large excess of two-carbon fragment in the form of acetaldehyde was added to the normal glucose-asparagine model system (Stoehr, 1895). The results (Figure 3) indicated that two-carbon fragments did take part in the reaction, in that the relative concentration of pyrazines made up of two-carbon fragments increased markedly while those containing three-carbon fragments were not increased as much. In fact, those made up of two three-carbon fragments (DMP) actually decreased, suggesting that two-carbon fragments competed effectively for three-carbon fragments to form relatively more of the pyrazines made up of a combination of two- and three-carbon fragments—e.g., MP.

Two possible pathways by which a hexose or hexose intermediate could fragment into two- and three-carbon units, which would then combine with nitrogen to form pyrazines, were considered. In either pathway, the hexose can break into two equivalent three-carbon fragments which on recombination and incorporation of nitrogen would yield dimethylpyrazine. The two pathways differ as follows: Pathway I (Figure 4) assumes

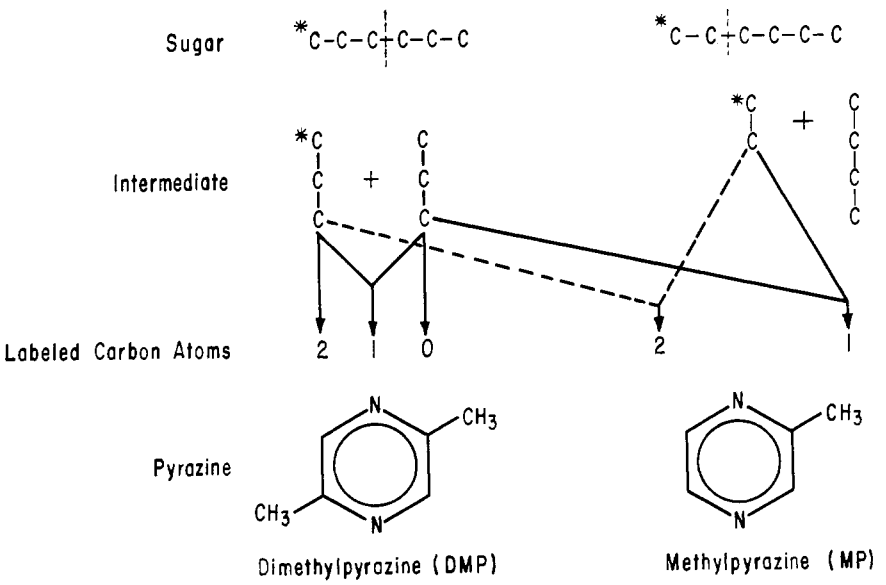

Figure 4. Fragmentation of hexose to form pyrazine molecule via pathway I

Table III. Ratio of Specific Activity of Product (Pyrazine) to Reactant (Glucose) for Hexose Fragmentation Pathways I and II

Labeled Reactant	Glucose-1-^{14}C		Glucose-6-^{14}C		Glucose-3,4-^{14}C	
Product	DMP	MP	DMP	MP	DMP	MP
Pathway I (theoretical)	1.00	1.50	1.00	0.50	1.00	0.50
Experimental results	0.97 ± 0.01	1.29 ± 0.01	1.14 ± 0.20	0.63 ± 0.002	1.10	0.63 ± 0.005
Pathway II (theoretical)	1.00	0.83	1.00	0.83	1.00	0.83

the breakdown into only one two-carbon fragment per hexose, with this fragment being formed from carbon atoms 1 and 2 of the hexose. The possible recombinations of pathway I hexose fragments with nitrogen are shown by lines which indicate the alkylated pyrazine produced and the predicted specific activity of the resultant molecule for the case of glucose-1-^{14}C (illustrated). In the alternate pathway (II), three equivalent two-carbon units would be produced per hexose molecule. The predicted and experimentally determined values for the ratio of specific activity of product (alkylated pyrazine) to reactant (glucose labeled in the 1, 3-4, or 6 position) are shown in Table III for each of the two pathways considered. Each experimental value in Table III represents an average of either two or three experiments. The average deviation from the mean value is shown in the table.

The breakdown into a single two-carbon unit per hexose (pathway I) gives a better correlation of predicted and experimental labeling values than pathway II (Table III). The slightly lower than predicted ratio for methylpyrazine from glucose-1-^{14}C in the preferred pathway (I) could be explained by assuming a small amount of cleavage of the remaining four-carbon unit into two-carbon units, thus diluting the activity of the two-carbon unit pool. The slightly higher than predicted values for MP from glucose-6-^{14}C and glucose-3,4-^{14}C confirm this explanation, since further breakdown in these situations would add ^{14}C-labeled two-carbon units to the otherwise unlabeled combined two-carbon units. Slight fragmentation of the four-carbon unit into a three-carbon and a one-carbon unit would explain the slightly higher than predicted specific activity ratios for DMP from glucose labeled in the 3-4 and 6 positions.

Thus, these data provide experimental support for the hypothesis of Dawes and Edwards (1966) that the carbon atoms of pyrazines arise from sugar degradation products. The results obtained are difficult to resolve in terms of compounds known or proposed in the pathway for amine-catalyzed breakdown of sugars (Hodge, 1963, 1965). The fact that both 1- and 6-^{14}C-glucose produced DMP labeled to the same extent seemed to indicate that fragmentation into three-carbon fragments took place at a symmetrical intermediate. However, the dissimilar labeling of MP from glucose-1-^{14}C from that of MP from glucose-6-^{14}C indicated that the split into two- and four-carbon fragments apparently takes place almost exclusively between carbons 2 and 3 and suggested a dissymmetrical intermediate.

Knowledge of the position of the labeling in the pyrazines (exocyclic *vs.* endocyclic) and whether splitting of the intermediate is by hydrolysis, aminolysis, or retroaldolization will be essential to formulating a logical mechanism for pyrazine production. Since the structure of the nitrogen source influences the quantitative distribution of pyrazines formed (Figure 1), and since any proposed mechanism must account for incorporation of nitrogen into the ring, a mechanism involving direct ammonolysis of the six-carbon intermediate is favored. Alternatively, dealdolization of the six-carbon intermediate followed by condensation of amines (ammonia) with the resulting carbonyl compounds might explain the results. Thus, the variable distribution of pyrazines might be explained by the differing rates at which various amines present would react with various two-, three-, and four-carbon fragments.

Studies are now under way to determine the position of labeling in MP and DMP when the various ^{14}C-labeled glucoses are the precursors.

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