

# Mechanistic Studies of Tetramethylpyrazine Formation under Weak Acidic Conditions and High Hydrostatic Pressure

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A significant enhancement of the tetramethylpyrazine (TMP) formation at high pressure was observed in the 3-hydroxy-2-butanone/ammonium acetate model system. In a water system, the activation volume of TMP formation under high pressure was found to be  $-6.82$  mL/mol. A mechanism was proposed to elucidate the formation of TMP under a weak acidic condition and high hydrostatic pressure. Solvents such as propylene glycol (PG), glycerol, methanol, ethanol, propanol, and butanol were found to enhance TMP formation. Kinetic analyses indicated that TMP formation in aqueous, 80% PG, and ethanol systems followed pseudo-zero-order reaction kinetics. The activation energies were found to be  $18.84 \pm 1.3$ ,  $14.19 \pm 7.1$ , and  $13.09 \pm 4.7$  kcal/mol, respectively. The intermediate of TMP formation was characterized as tetramethyldihydropyrazine using gas chromatography–mass spectrometry. A  $^{15}\text{N}$ -labeled ammonium acetate/3-hydroxy-2-butanone model system was used to confirm the incorporation of a nitrogen atom in the molecule of tetramethyldihydropyrazine. Hydrogen acceptors such as nicotinamide adenine dinucleotide and flavin adenine dinucleotide were found to increase TMP formation, and the formation of TMP from tetramethyldihydropyrazine through dehydrogenation was shown.

**Keywords:** *Pyrazine formation; high hydrostatic pressure; kinetics*

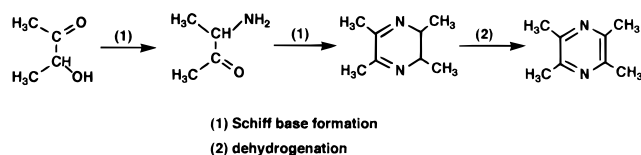
## INTRODUCTION

Pyrazines are nitrogen-containing heterocyclics that are potent and characteristic flavorants found in a wide range of raw and processed foods. The odor of tetramethylpyrazine (TMP) has been described as pungent, walnut, and green.

Most of the alkylpyrazines are formed in foods that are processed at high temperatures. The activation energies for pyrazine formation are relatively high. Rizzi (1988) postulated that 3-amino-2-butanone is a reaction intermediate in the formation of TMP. Aminocarbonyls, such as 3-amino-2-butanone, are known to dimerize to dihydropyrazine and to oxidize to TMP as shown in Figure 1. Schiff base formation is believed to be the critical step in pyrazine synthesis. However, the detailed mechanistic steps of Schiff base formation remain unclear.

Dihydropyrazine has been reported to be an important intermediate in pyrazine formation. According to the generally accepted mechanism for pyrazine formation in the Maillard reaction, pyrazines are formed in an oxidation step from their dihydropyrazine precursors. Shibamoto and Bernhard (1977) summarized the formation of pyrazines via the oxidation of dihydropyrazine. At least two possible mechanisms to convert dihydropyrazine to pyrazine without oxygen were proposed. One is the disproportionation of dihydropyrazine, and the second mechanism involves the dehydrogenation of dihydropyrazine.

Solvent effect on Schiff base formation is another controlling factor that could promote Schiff base forma-



**Figure 1.** Proposed overall Schiff base formation mechanism for TMP synthesis.

tion dramatically. In reactions, though solvents have been used to replace water as a medium for many years, the reason for using organic solvents has not been discussed.

Pressure can influence most chemical reactions, since they often involve a change in volume. Volume-increasing reactions will tend to be inhibited by pressure, while reactions leading to a decrease in volume will tend to be promoted. High hydrostatic pressure is of great value in organic synthesis and is especially useful for those compounds that cannot be synthesized at ambient pressure due to steric hindrances or other reasons. Numerous papers have been published concerning the pressure effect on organic synthesis, many of which have been reviewed by Matsumoto et al. (1985). It has been reported that high pressure could increase organic synthesis reactions, such as aldol condensation, Michael reaction, alkylation of aldehydes, Diels–Alder reaction, and Mannich reaction.

Most of the published pyrazine formation mechanisms are studied at high temperatures using a model system. However, Rizzi (1988) reported that alkylpyrazines were formed in reactions of acylloins and ammonia at acidic pH and low temperature. Pyrazine formation at ambient temperature under high hydrostatic pressure has not been previously studied. Therefore, the objectives of this research were to study the effects of temperature and pressure on TMP formation and to identify the intermediate of the reaction. The mechanism of TMP

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formation in a weak acid condition and under high hydrostatic pressure is discussed.

## EXPERIMENTAL PROCEDURES

**Materials.** 3-Hydroxy-2-butanone and tetramethylpyrazine (TMP) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Ammonium salts (acetate, formate, oxalate, hydroxide, carbonate, bicarbonate, sulfate, and chloride), propylene glycol (PG), and the solvents for HPLC were of chemical grade and obtained from Fisher Chemical Co. Ammonium glutamate was obtained from Ajinomoto Co. Nicotinamide adenine dinucleotide (NAD<sup>+</sup>) and flavin adenine dinucleotide (FAD) were purchased from Sigma Chemical Co. (St. Louis, MO). [<sup>15</sup>N]Ammonium acetate was purchased from Isotec, Inc. (Miami, OH).

**Reaction Procedure.** Mixtures were composed of 0.01 mol of 3-hydroxy-2-butanone and 0.03 mol of ammonium salts dissolved in 4 mL of deionized water or solvent. The vials were shaken regularly to ensure that all of the reactants were dissolved. The reactions were run in a water bath at the required constant temperature. The amount of TMP was analyzed by HPLC.

**HPLC Analysis.** Reaction mixtures were routinely analyzed by HPLC using a Waters Associate liquid chromatograph Model 6000A and a Model 440 absorbance detector (280 nm) with a 25 × 0.46 cm RP Partisphere C<sub>18</sub> column (Whatman) under isocratic conditions at ambient temperature. Solvent was 50/50 (v/v) methanol–water (1.0 mL/min) for all separations. Tetramethylpyrazine standards were prepared at 0.02, 0.04, 0.06, 0.08, and 0.10 mg/mL in methanol. Quantitation was done on base-line-resolved peaks vs external standards on a Varian 4270 integrator.

**High Hydrostatic Pressure Experiments.** The reactions were done by putting the reactants into small plastic vials (polyethylene bottles with a screwed lid with approximately 4.5 mL capacity) which were filled with water or propylene glycol. High pressure was applied to the plastic vials with a hand-type pressure generator (Type K-P5-B, Hikari, Koatsu Co., Hiroshima, Japan) using water as the pressure medium. The temperature was maintained by immersing the pressure vessel in the water bath.

**Effect of Different Nitrogen Sources on TMP Formation.** Different ammonium salts (acetate, bicarbonate, carbonate, hydroxide, oxalate, glutamate, chloride, and sulfate) were set up to study the influence of nitrogen sources on TMP formation.

**Effect of Water Content on TMP Formation.** Different percentages of PG (0, 20, 40, 60, 80, and 100% in water) were used to investigate the water content on TMP formation at 1 and 5000 kg/cm<sup>2</sup>, respectively.

**Effect of Solvents on TMP Formation.** Solvents such as methanol, ethanol, propanol, butanol, PG, and glycerol were used to investigate the solvent effect on TMP formation at 25 °C for 1 h.

**Kinetic Analysis.** The rate of TMP formation in 3-hydroxy-2-butanone/ammonium acetate in water, 80% PG, and ethanol systems at the four reaction temperatures (25, 35, 45, and 55 °C) was analyzed by HPLC in the reaction mixture every hour. All kinetic studies were carried out in duplicate. The kinetics of pyrazine formation was determined using the basic equation for the rate of change of *A* with time:  $dA/dt = KA^n$ , where *A* is concentration, *t* is time, *K* is a rate constant, and *n* is the reaction order. Slopes and intercepts were calculated by the linear least-squares method. The activation energies for formation of the TMP were calculated by the Arrhenius equation from the slope of the line generated by plotting the log value of the rate constant versus the reciprocal of the absolute temperature (Labuza, 1983).

**Effect of Pressure on Pyrazine Formation.** The rate of TMP formation in 3-hydroxy-2-butanone/ammonium acetate in the water system was obtained at 1, 3000, 4000, and 5000 kg/cm<sup>2</sup> and 25 °C for 1, 3, or 5 h. The activation volume was calculated according to the basic relationship between pressure and rate. The equation originally proposed by van't Hoff is as follows:  $\Delta V^\ddagger = -RT \ln K/P$ , where  $\Delta V^\ddagger$  is the change in

**Table 1. Effect of Different Ammonium Salts on TMP Formation**

| ammonium salt | TMP concn (mg/mL) | ammonium salt | TMP concn (mg/mL) |
|---------------|-------------------|---------------|-------------------|
| acetate       | 5.40              | glutamate     | 0.06              |
| bicarbonate   | 0.56              | chloride      | undetectable      |
| carbonate     | 0.21              | oxalate       | a                 |
| formate       | 0.14              | sulfate       | a                 |
| hydroxide     | 0.06              |               |                   |

<sup>a</sup> No product was detected due to the poor solubility of the ammonium salts.

volume per mole when the activated complex is formed from the reactants.  $\Delta V^\ddagger$  can be obtained from the plot of pressure versus rate constant. Various methods have been proposed and used to calculate  $\Delta V^\ddagger$ . The most realistic one is fitting by the least-squares method. Among all of the equations proposed and used, the most popular is the parabolic one,  $\ln K = a + bp + cp^2$ , so that then, at  $p = 0$ ,  $\Delta V^\ddagger = -bRT$ , where *R* is the gas constant, 82 cm<sup>3</sup> atm K<sup>-1</sup> mol<sup>-1</sup> (Asano and Le Noble, 1978). The pressure units used are 1 atm = 1.01325 bar = 1 kg/cm<sup>2</sup> = 0.101325 MPa. These equations are similar to those classically calculated for temperature as a variable at constant pressure.

**Gas Chromatography.** A Varian gas chromatograph equipped with a fused silica column (60 m × 0.32 mm i.d., film thickness, 0.25 nm; DB-1; J&W Scientific) and a flame ionization detector was used to analyze the volatile compounds. The operating conditions were as follows: injector and detector temperatures, 270 and 300 °C, respectively; helium carrier flow rate, 1.0 mL/min; temperature program, 40–260 °C at 2 °C/min followed by an isothermal hold at 260 °C for 10 min.

**Gas Chromatography–Mass Spectrometry.** GC–MS analysis was accomplished by using a Varian 3400 gas chromatograph coupled to a Finnigan MAT 8230 high-resolution mass spectrometer equipped with an open split interface. Mass spectra were obtained by electron ionization at 70 eV and a source temperature of 250 °C. The filament emission current was 1 mA, and spectra were recorded on a Finnigan MAT SS 300 data. The operating conditions were the same as those used in the GC analysis described above.

**Effect of Hydrogen Acceptors on TMP Formation.** The hydrogen acceptors NAD<sup>+</sup> and FAD were added to the 3-hydroxy-2-butanone/ammonium acetate model system in the amount of 15, 75, and 150 μmol. The reactions were run at 25 °C for 24 h. GC was used to analyze the amount of TMP.

## RESULTS AND DISCUSSION

### TMP Formation in Different Ammonium Salts.

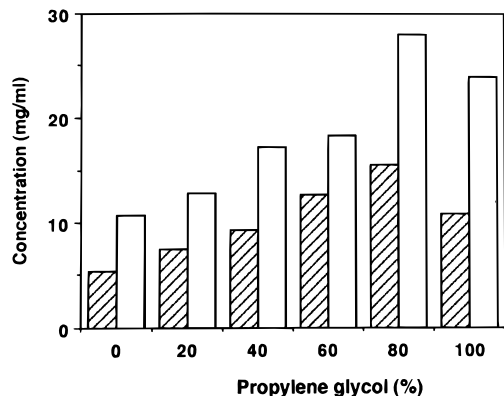
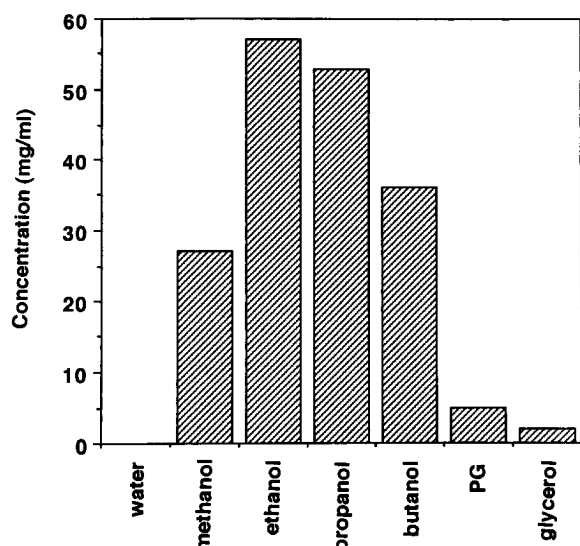
At ambient pressure, the amount of TMP was influenced by the type of ammonium salt. Among the nitrogen sources, as shown in Table 1, ammonium acetate produced the most TMP. The *pK<sub>a</sub>* values for acetic acid and ammonium are 4.76 and 9.25, respectively. An equimolar solution of ammonia and acetic acid had the maximum buffering capacity at pH 7.0.

According to Scudder (1992), Schiff base formation could be separated into four sequential steps. These steps include trimolecular addition (AdE3), proton transfer, and general acid catalyzed β-elimination (EgA), followed by another proton transfer. The first proton transfer increased the polarization of the carbonyl group, resulting in an increase of nucleophilic activity of ammonia which led to the formation of a positively charged transition state. For Schiff base formation to occur, both the hydroxyl group and the hydrogen on the nitrogen must be released. The second proton transfer was necessary to remove the hydroxyl group, which is converted to a molecule of water. The proton transfer not only made the hydroxyl group a better leaving group but also made the lone nitrogen pair push the hydroxyl group out. The third proton transfer led to the elimina-

**Table 2. Effect of Different Ammonium Salts on TMP Formation at 22 °C under Different Pressures**

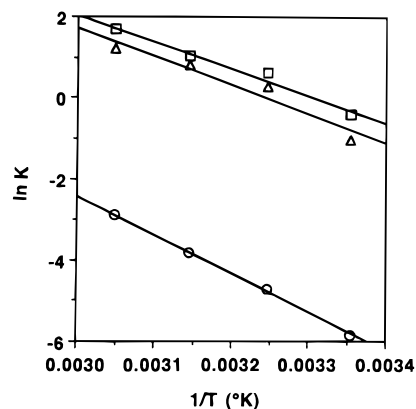
| ammonium salt | concn (mg/mL) at pressure of |                        |                         |                         |
|---------------|------------------------------|------------------------|-------------------------|-------------------------|
|               | 1 kg/cm <sup>2</sup>         | 500 kg/cm <sup>2</sup> | 1000 kg/cm <sup>2</sup> | 2000 kg/cm <sup>2</sup> |
| acetate       | 12.44                        | 14.35                  | 14.29                   | 22.05                   |
| carbonate     | 0.27                         | 0.34                   | 0.37                    | 0.45                    |
| hydroxide     | 0.01                         | 0.04                   | 0.04                    | 0.03                    |
| oxalate       | a                            | a                      | a                       | a                       |

<sup>a</sup> Undetectable.

**Figure 2.** Effect of propylene glycol on TMP formation at pressures of 1 (slashed bars) and 5000 (open bars) kg/cm<sup>2</sup>.**Figure 3.** Solvent effect on TMP formation.

tion of the hydrogen atom on the nitrogen atom. The acetic acid/ammonia buffer systems served as either a proton donor or an acceptor, which led to the completion of the Schiff base formation. The important role of buffer systems in promoting Schiff base formation in the ammonium acetate/3-hydroxy-2-butanone model system reconfirmed the concept discussed by de Kok and Rosing (1994). Similar evidence has been reported by Miyashita et al. (1991) and Lin et al. (1992). However, in their studies no detailed mechanistic elucidation of the role of buffer salts on Schiff base formation was discussed.

**Effects of High Pressure on TMP Formation.** As shown in Table 2, in the 3-hydroxy-2-butanone/ammonium hydroxide system, the amount of TMP was 0.01 mg/mL at ambient pressure, increasing to 0.04 mg/mL at a pressure of 500 kg/cm<sup>2</sup>. However, when the pressure was increased to 1000 or 2000 kg/cm<sup>2</sup>, the amount of TMP did not increase. This may be due to the high pH of the reaction system.

**Figure 4.** Arrhenius plot of TMP formation in water (○), 80% PG (△), and ethanol (□) systems.**Table 3. Zero-Order Constants (*K*) and Corresponding Regression Coefficients (*R*<sup>2</sup>) of TMP Formation from 3-Hydroxy-2-butanone/Ammonium Acetate in Water (1), 80% PG (2), and Ethanol (3)**

| conditions  | temp (°C) | <i>K</i> (mg/mL h) | <i>R</i> <sup>2</sup> |
|-------------|-----------|--------------------|-----------------------|
| (1) water   | 25        | 0.0028             | 0.948                 |
|             | 35        | 0.0089             | 0.989                 |
|             | 45        | 0.0216             | 0.953                 |
|             | 55        | 0.0550             | 0.989                 |
| (2) 80% PG  | 25        | 0.3495             | 0.983                 |
|             | 35        | 1.3309             | 0.989                 |
|             | 45        | 2.2264             | 0.972                 |
|             | 55        | 3.3000             | 0.995                 |
| (3) ethanol | 25        | 0.6570             | 0.989                 |
|             | 35        | 1.8760             | 0.995                 |
|             | 45        | 2.6520             | 0.965                 |
|             | 55        | 5.5000             | 0.990                 |

**Table 4. Activation Energies of TMP Formation from 3-Hydroxy-2-butanone/Ammonium Acetate in Water (1), 80% PG (2), and Ethanol (3)**

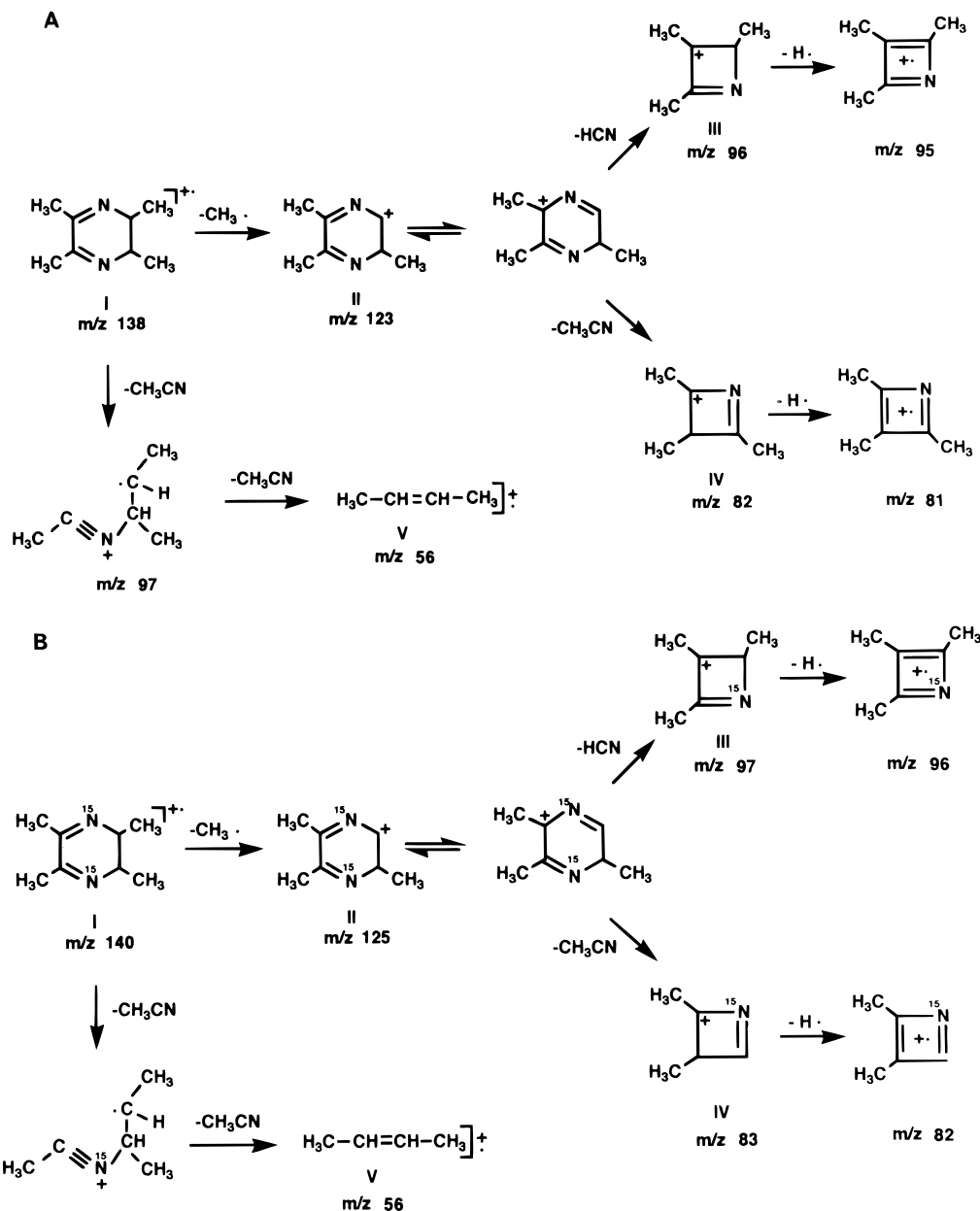
| conditions  | <i>E</i> <sub>a</sub> (kcal/mol) | linearity ( <i>R</i> <sup>2</sup> ) |
|-------------|----------------------------------|-------------------------------------|
| (1) water   | 18.84 ± 1.3                      | 0.999                               |
| (2) 80% PG  | 14.19 ± 7.1                      | 0.929                               |
| (3) ethanol | 13.09 ± 4.7                      | 0.967                               |

In the 3-hydroxy-2-butanone/ammonium carbonate system, the amount of TMP increased with increasing pressure. The amounts of TMP were 0.27 and 0.34 mg/mL at 1 and 500 kg/cm<sup>2</sup>, respectively. When the pressure increased to 1000 kg/cm<sup>2</sup>, the amount of TMP increased to 0.37 mg/mL. When the pressure of 2000 kg/cm<sup>2</sup> was applied, the amount of TMP increased to 0.45 mg/mL. These results showed that high pressure could enhance TMP formation. No TMP was detected in the oxalate system due to the low solubility of ammonium oxalate.

A significant pressure effect on TMP formation was observed in the 3-hydroxy-2-butanone/ammonium acetate system. The amounts of TMP at pressures of 500 and 1000 kg/cm<sup>2</sup> were 14.35 and 14.29 mg/mL, respectively. When the pressure was increased to 2000 kg/cm<sup>2</sup>, the amount of TMP increased to 22.05 mg/mL. The amount of TMP increased 1.2-fold under 1000 kg/cm<sup>2</sup> and 1.8-fold under 2000 kg/cm<sup>2</sup> for the sample pressurized at 22 °C for 24 h.

Hamman (1957) proposed that those reactions in which the transition state is more highly ionic, and hence more extensively solvated, than the initial state are greatly accelerated by pressure; those in which the transition state is less ionic and less solvated than the initial state are retarded by pressure. The positive





**Figure 7.** Fragmentation pathways of tetramethyldihydropyrazine from  $^{14}\text{N}$ -labeled (A) and  $^{15}\text{N}$ -labeled (B) ammonium acetate.

**Mechanism of TMP Formation at a Weak Acidic Condition.** As shown in Figure 5, a mechanism for the formation of TMP under a weak acidic condition was proposed. The proton from acetic acid protonated the carbonyl carbon of 3-hydroxy-2-butanone, which facilitated the formation of an ionic intermediate. This was confirmed by the high-pressure experiments. Subsequent rearrangement of the intermediate was catalyzed by the acetate ion and led to the formation of an amino ketone. Condensation of two molecules of the amino ketone produced one molecule of TMP.

**Characterization of Tetramethyldihydropyrazine.** An unknown compound with a molecular weight of 138 was formed in the reaction mixture of ammonium acetate. This compound was suspected to be tetramethyldihydropyrazine, an intermediate compound toward the formation of TMP. The reaction mixture of 3-hydroxy-2-butanone/ $^{15}\text{N}$ ammonium acetate or  $^{14}\text{N}$ ammonium acetate was then analyzed by GC and GC-MS to confirm the incorporation of a nitrogen atom in this unknown molecule. Parts A and B of Figure 6 show the electron impact mass spectra of this intermediate

from  $^{14}\text{N}$ - and  $^{15}\text{N}$ ammonium acetate, respectively. The mass ranges of these two spectra were, unfortunately, different. The mass range of the spectrum shown in Figure 6A started at  $m/z$  40 and that for Figure 6B started at  $m/z$  50. In Figure 6A, a molecular ion  $[\text{M}]^+$  at  $m/z$  138 (20%), which corresponded to a possible elemental composition of  $\text{C}_8\text{H}_{14}\text{N}_2$ , was similar to the fragmentation pattern of 2,3-dimethyl-5,6-dihydropyrazine (Porter and Baldas, 1972), which is less stable to electron impact than the fully aromatic pyrazines. The proposed fragmentation pathway is summarized in Figure 7A. The peak observed at  $m/z$  123 (45%) was the loss of the  $\text{CH}_3$  group from the parent molecule. Peaks at  $m/z$  96 (10%) and  $m/z$  82 (15%) represented a loss of  $\text{HCN}$  and  $\text{CH}_3\text{CN}$  fragments, respectively, from the  $m/z$  123 ion. The other pathway, yielding the ion  $m/z$  56 (48%), is due to expulsion of two molecules of  $\text{CH}_3\text{CN}$ . The compound was, therefore, tentatively characterized as tetramethyldihydropyrazine.

For the reaction mixture of 3-hydroxy-2-butanone/ $^{15}\text{N}$ ammonium acetate, a molecular ion  $[\text{M}]^+$  at  $m/z$

**Table 5. Comparison of the Fragments of Tetramethyldihydropyrazine Derived from [<sup>14</sup>N]- and [<sup>15</sup>N]Ammonium Acetate**

| fragment | <i>m/z</i>      |                 |
|----------|-----------------|-----------------|
|          | <sup>14</sup> N | <sup>15</sup> N |
| I        | 138             | 140             |
| II       | 123             | 125             |
| III      | 96              | 97              |
| IV       | 82              | 83              |
| V        | 56              | 56              |

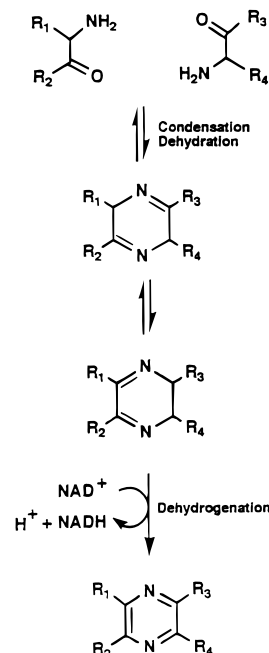
**Table 6. Effect of Hydrogen Acceptors on TMP Formation**

|                  | $\mu\text{mol}$ added | TMP concn (mg/mL) |
|------------------|-----------------------|-------------------|
| control          |                       | 0.97              |
| NAD <sup>+</sup> | 150                   | 2.57              |
|                  | 75                    | 2.02              |
|                  | 15                    | 1.60              |
| FAD              | 150                   | 1.80              |
|                  | 75                    | 1.60              |
|                  | 15                    | 1.20              |

140 (35%) rather than *m/z* 138 was observed in Figure 6B. This could be interpreted as the replacement of two <sup>14</sup>N atoms with two <sup>15</sup>N atoms. The fragmentation scheme is shown in Figure 7B. The base peak observed at *m/z* 125 (100%) corresponded to a loss of CH<sub>3</sub> from the parent molecule. This revealed that two <sup>15</sup>N atoms remained in this fragment. Further loss of the neutral fragments, CH<sub>3</sub>CN and HCN, led to the fragments *m/z* 83 and 97, respectively. Comparison of the fragmentation pattern of the intermediate with <sup>14</sup>N showed that only one nitrogen remained in these fragments. The sequential loss of two neutral fragments corresponding to CH<sub>3</sub>CN from the parent molecule resulted in the formation of a *m/z* 56 fragment. Apparently, there was no nitrogen atom in the *m/z* 56 fragment in either fragmentation pattern. A comparison of the two suggested fragmentation patterns of the intermediates derived from [<sup>14</sup>N]ammonium acetate and [<sup>15</sup>N]ammonium acetate is summarized in Table 5.

**Effect of Hydrogen Acceptor on TMP Formation.** NAD<sup>+</sup> functions as an oxidizing agent in the formation of TMP. The oxidized forms of NAD<sup>+</sup> and FAD engage in a two-electron transfer by accepting a hydride ion H<sup>-</sup> from the reduced substrate AH (Abeles et al., 1992). A series of experiments was developed to examine the possible effects of oxidative factors on the formation of pyrazine from dihydropyrazine. In our experiments a model system of 3-hydroxy-2-butanone/ammonium acetate in a water system was used which consisted of NAD<sup>+</sup> and FAD selected as hydrogen acceptors. The results of the quantitative analyses of TMP are presented in Table 6. Both FAD and NAD<sup>+</sup> showed a significant effect on the transformation of dihydropyrazine to the corresponding TMP. The amount of TMP increased with increasing NAD<sup>+</sup> concentration linearly, in the range of 15–150  $\mu\text{mol}$ . FAD showed a similar effect on TMP formation. When hydrogen acceptors such as FAD or NAD<sup>+</sup> were added to the reaction system, no peak for the intermediate dihydropyrazine was observed.

These results confirmed the mechanism proposed by Turksma, which was cited in a paper by Weenen and Tjan (1991). It was reported that pyrazines could be formed from dihydropyrazines in a redox reaction. A pathway similar to the previous reaction which shows

**Figure 8.** TMP formation from tetramethyldihydropyrazine through dehydrogenation.

the formation of TMP through the intermediate, tetramethyldihydropyrazine, is presented in Figure 8.

**Conclusion.** Application of high hydrostatic pressure was found to enhance the formation of TMP in a 3-hydroxy-2-butanone/ammonium acetate system. Solvents such as PG, glycerol, methanol, ethanol, propanol, and butanol were found to enhance TMP formation. The use of high hydrostatic pressure in combination with an adequate solvent may lead to a new way to prepare flavor compounds.

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