

The Mechanism of Pyrrole, Pyrazine and Pyridine Formation in Non-Enzymic Browning Reaction

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ABSTRACT

Seven pyrroles, twenty pyrazines and 2-methyl-5-hydroxy-6-propylpyridine have been identified in the CH_2Cl_2 extract of a heated reaction mixture of D(+)-glucose and aminobutyric acid isomer model systems by gas chromatography-mass spectrometry (GC/MS). Pyrazines, as the main constituent of these reactions, are responsible for the odour properties.

Based on an electron spin resonance (ESR) study, the shape and g values of the splitting constant of the ESR spectra of $a^H = 2.81$ Gs, and $a^N = 8.73$ Gs for D(+)-glucose and the 2-aminobutyric and 3-aminobutyric acid model systems, respectively, and of $a^H = 2.81$ Gs, $a^{Ha} = 5.44$ Gs and $a^N = 8.23$ Gs for D(+)-glucose and the 4-aminobutyric acid model system, it can be concluded that the 1,4-dialkylpyrazine cation radicals formed are responsible for pyrazine formation in the initial stage of the non-enzymic browning reaction.

The mechanisms of formation of pyrroles, pyrazines and pyridine derivatives are proposed.

INTRODUCTION

The elucidation of the mechanism of reaction between aldoses and amino acids, including the mechanism of formation of pyrrole, pyrazine and some flavour constituents, is one of the most important problems in the chemistry of non-enzymic browning and that of food flavour.

Many pyrrole derivatives have been isolated from many foods whose flavours had been improved or impaired by non-enzymic browning (Shigematsu *et al.*, 1972). They are believed to be formed in the non-enzymic browning reaction between sugars and amino acids, or between the Strecker degradation products of amino acids, present in the reaction mixture, and a hexosulose intermediate.

Since many pyrroles have powerful flavours and aromas, more knowledge of their origin might permit control of their formation in order to either enhance desirable flavours or minimize off-flavours.

The presence of alkylpyridines in foods has been investigated by many researchers (Ferretti & Flanagan, 1971; Watanabe & Sato, 1971; Yajima *et al.*, 1978). Recently, Buttery *et al.* (1977) and Suyama & Adachi (1980) reported that many pyridines were formed by the reaction of aldehydes, ketones, α,β -unsaturated carbonyl compounds, or various derivatives of such compounds with ammonia or amino acids.

A large and very important group of flavour and aroma constituents of foods consists of pyrazine derivatives. Buttery *et al.* (1971) concluded that, because of its low threshold, 2-ethyl-3,6-dimethyl-pyrazine could be considered important to potato chip aroma. Sheldon *et al.* (1972) reported that 2-methyl-, 2,3-dimethyl-, 2,5-dimethyl-, 2-ethyl-, 2,6-dimethyl-3-ethyl- and 2,5-diethyl-3-methyl-pyrazine, which they isolated and identified as volatile flavour compounds from roasted filbert, could be formed from the reaction between the constituents of the various ingredients used.

The most complete reports on this field were published by Koehler & Odell (1970), Maga & Sizer (1973), Mussinan *et al.* (1973), Vitzthum & Werkhoff (1975), Valisek *et al.* (1976), Shibamoto *et al.* (1979, 1981), Coleman & Chi-Tang Ho (1980), Nishimura *et al.* (1980) and Kitamura *et al.* (1981). A detailed list, of about 70 to 115 different pyrazines and other volatile compounds, identified from many common foods and different model systems, can be found in their reports. Pao-Shui Wang & Odell (1973) reported on the formation of pyrazines from the thermal treatment of some aminohydroxy compounds.

Namiki *et al.* (1974), Namiki & Hayashi (1975) and Hayashi *et al.* (1977) were the first to study the electron paramagnetic resonance spectra of free radicals in the reaction mixtures of some simple carbonyl compounds and amines or amino acids. Shibamoto & Russell (1976, 1977) published a paper speculating on the mechanism of pyrazine formation of a sugar-ammonia model system. Recently, the appearance

and kinetics of free radicals in the initial stages of the carbonyl–amino acid reaction and the D(+)-glucose–aminobutyric acid isomer model systems have been studied by Milić *et al.* (1978, 1979, 1980a, b).

In an effort to clarify the effect of the positions of the amino group in amino acid molecules on the mechanisms of formation, yields and distributions of flavour and aroma constituents, the present paper examines the formation of pyrazine-cation radicals, as intermediates, and pyrroles, pyrazines and pyridines, as final compounds, in the initial stage of non-enzymic browning reactions between D(+)-glucose and 2-, 3- and 4-aminobutyric acids.

EXPERIMENTAL

Model systems were obtained by mixing equimolar amounts of 2-, 3- and 4-aminobutyric acid with D(+)-glucose separately in 0.01M acetic buffer at pH 9.0. The final concentrations of reactants were 1.5M. The reaction mixtures were heated at 368 K in ESR-quartz test tubes (Varian E-248) for the water solution for a 0 to 3.6×10^3 s reaction period.

Electron spin resonance (ESR) spectra of free radicals were determined with a Varian ESR-spectrometer 109 E set as follows: scan range, 100 Gs; receiver gain, 1.25×10^3 ; time constant, 1.0 s; modulation amplitude, 0.63×10^1 Gs; microwave power, 16 mW; field set, 3400 Gs; scan time, 1.8×10^3 s; modulation frequency, 100 kHz; microwave frequency, 9515 GHz; and temperature, 293 K, for free radical determination. The g values were determined by means of a Varian double cavity E-232 with PPADS (potassium peroxyamine disulfonate; $g = 2.0036 \pm 0.0002$ Gs) as a standard. Isotropic hyperfine splitting constants of ESR spectra were determined with a Varian Data System E-900 in relation to isotropic splitting of Mn^{2+} ions ($I = 5/2$), with $a_{Mn} = 51.23$ Gs, which were brought in ZnS as an 'impurity'.

The reaction mixtures of D(+)-glucose–aminobutyric acid isomer, heated at 368 K for 3.6×10^3 s, were cooled to room temperature. The dark brown solution was saturated with sodium chloride and extracted with diethyl ether using a Universal Extraction Unit, Büchi Rotavapor EL. The extracts were dried over anhydrous sodium sulphate, filtered and concentrated to approximately 0.5 ml by a careful distillation of the solvent through a Büchi Spinning Column. The concentrates of volatile constituents were analysed by gas chromatography–mass spectrometry

on a Hitachi GC, M-5201/RMU-6E MS combination with a Hitachi 10 II/A Data System. The chromatography column used was a 90 m × 0.45 mm inside diameter high resolution stainless steel capillary coated with UCON HB 5100. Helium flow rate was 5 ml min⁻¹. The chromatograph oven temperature was held at 303 K for 5 min, then programmed at 2 K min⁻¹ to 473 K. The injection port and the flame ionizing detector were both at 473 K. The mass spectrometer was set as follows: ionization voltage, 70 eV; ion source temperature, 473 K; ion acceleration voltage, 3100 V; and emission current, 80 μA.

RESULTS AND DISCUSSION

Seven pyrroles (Table 1), twenty pyrazines and 2-methyl-5-hydroxy-6-propylpyridine (Table 2) were identified in slightly different quantities in CH₂Cl₂ extracts of heated D(+)-glucose-aminobutyric acid isomer model systems.

Although the formation of pyrrole derivatives is feasible in slightly acidic and neutral solution (Nakayama *et al.*, 1980), the presence of OH ions affects proton rearrangement in sugar molecules and forms 3-deoxyhexosulose and hexosulos-3-ene. These intermediates react with the amino acids present, with simultaneous Strecker degradation, according to mechanisms A and B (Scheme I), producing pyrrole derivatives. The obtained pyrrole derivatives, at the base catalytic condition, are relatively stable and thus not readily available as intermediates for the formation of

TABLE 1
Pyrroles Identified in D(+)-Glucose-Aminobutyric Acid Isomer Model Systems

<i>Compound*</i>	<i>Odour</i>	<i>Reference</i>
1-Ethylpyrrole	Burnt	Takei (1977)
1-Propionylpyrrole	Peanut	Flath <i>et al.</i> (1978)
1-Ethylpyrrole-2-aldehyde	Roasted	McLeod & Coppock (1977)
1-Propylpyrrole-2-aldehyde	Caramel	Frattini <i>et al.</i> (1977)
1-Butylpyrrole-2-aldehyde	Minty	Harding <i>et al.</i> (1977)
1-Butylpyrrole-3-aldehyde	Bready	Tressl <i>et al.</i> (1977)
2-Methyl-5-acetylpyrrole	Sweet	Thomas (1973)

* All listed compounds were identified in all the examined model systems in very small amounts.

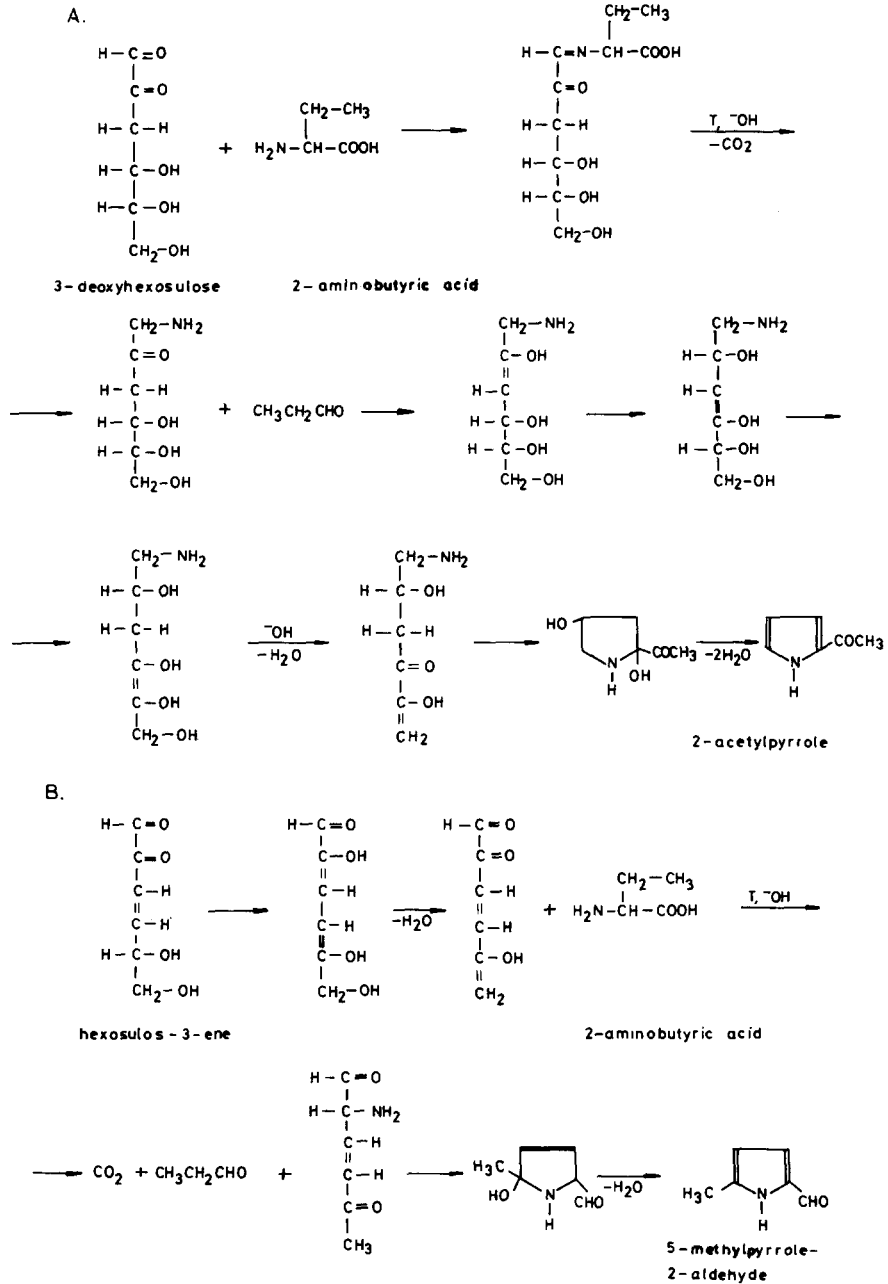
TABLE 2
Compounds Identified in CH₂Cl₂ Extracts of Heated D(+)-Glucose-Aminobutyric Acid Isomer Model Systems

Compound	R_f $\times 10^3 s$	Peak area (%)			Reference (see footnote)
		2- aminobutyric acid	3-	4-	
Pyrazine	0.45	1.58	1.90	1.42	<i>a</i>
Methylpyrazine	0.54	1.33	1.30	1.50	<i>a</i>
2,3-Dimethylpyrazine	0.60	1.60	1.63	1.90	<i>a</i>
2,5-Dimethylpyrazine	0.96	1.82	2.04	3.24	<i>a</i>
2,6-Dimethylpyrazine	1.14	0.41	0.20	0.28	<i>a</i>
2,3,5-Trimethylpyrazine	1.32	4.52	4.54	5.42	<i>b</i>
2-Ethyl-3-methylpyrazine	1.80	1.63	1.67	1.00	<i>c</i>
2-Ethyl-5-methylpyrazine	1.84	0.97	0.83	1.40	<i>d</i>
2-Ethyl-6-methylpyrazine	1.90	0.92	1.07	1.54	<i>a</i>
2,3-Diethylpyrazine	2.22	1.42	1.67	1.48	<i>e</i>
2-Ethyl-3,6-dimethylpyrazine	2.40	3.34	3.93	3.42	<i>f</i>
2-Ethyl-3,5-dimethylpyrazine	2.70	5.24	4.83	4.20	<i>a</i>
2,3-Diethyl-5-methylpyrazine	3.00	4.52	4.46	4.12	<i>a</i>
3,5-Diethyl-2-methylpyrazine	3.31	6.44	8.12	10.04	<i>g</i>
2-Ethyl-6-propylpyrazine	3.45	7.48	9.03	11.18	<i>f</i>
2-Ethyl-3,5,6-trimethylpyrazine	3.90	5.02	5.48	5.20	<i>f</i>
2,3-Dimethyl-5-butylpyrazine	4.52	2.84	3.06	3.30	<i>f</i>
2,5-Dimethyl-3-butylpyrazine	4.84	3.05	3.20	3.08	<i>e</i>
2,6-Dimethyl-3-butylpyrazine	5.10	3.32	3.42	3.35	<i>e</i>
2,5-Dimethyl-3-isobutylpyrazine	5.35	2.96	3.10	2.20	<i>h</i>
2-Methyl-5-hydroxy-6-propylpyridine	3.78	1.63	2.25	1.62	<i>ij</i>
Total peak area (%)		61.14	67.73	71.89	

a Kinlin *et al.* (1972); *b* Shibamoto *et al.* (1981); *c* Coleman & Chi-Tang Ho (1980); *d* Pittet & Hruza (1974); *e* Maga (1981); *f* Friedel *et al.* (1971); *g* Bondarovich *et al.* (1967); *h* Vitzthum & Werkhoff (1975); *i* Peterson *et al.* (1975); *j* Stenhagen *et al.* (1974).

other flavour-related compounds. On the other hand, a high degree of sugar fragmentation at pH 9.0 gave priority to the formation of pyrazines, not of pyrroles, which are usually formed in weakly acetic and neutral solutions.

According to the results listed in Table 2, it can be concluded that pyrazines, which are mainly responsible for earthy, nutty, baked, cinnamon-like and caramel-like odours, were obtained predominantly in the heated alkaline sugar-amino acid model systems.



Scheme I. Mechanism of pyrrole derivatives formation.

TABLE 3
Mass Spectra of Pyrazines in GC Fractions of a CH_2Cl_2 Extract of the Heated D(+)-Glucose-4-Aminobutyric Acid Model System

<i>Compound</i>	<i>Ions (%)</i>
Pyrazine	80(76), 79(34), 52(100), 25(43)
Methylpyrazine	94(60), 93(78), 78(24), 66(13), 52(100), 25(40)
2,3-(2,5- or 2,6-)Dimethylpyrazine	108(30), 107(81), 92(63), 81(100), 80(16), 67(73), 66(16), 41(80), 27(63)
2,3,5-Trimethylpyrazine	122(28), 121(66), 107(48), 94(65), 80(100), 62(70), 53(82), 27(13)
2-Ethyl-3-(5- or 6-)methylpyrazine	122(40), 121(60), 107(53), 94(72), 91(60), 80(100), 67(70), 62(30), 52(80), 27(13)
2,3-(3,5-)Diethyl-5-(2-)methylpyrazine	150(33), 149(48), 135(71), 123(100), 121(63), 108(43), 84(100), 67(62), 52(72), 27(40)
2-Ethyl-6-propylpyrazine	150(36), 149(42), 135(82), 123(100), 121(66), 114(16), 107(47), 99(12), 84(100), 67(43), 55(73), 43(11), 29(16), 27(42)
2-Ethyl-3,5,6-trimethylpyrazine	150(33), 149(63), 135(96), 121(92), 108(100), 82(29), 80(68), 67(24), 55(48), 53(21), 41(62), 28(31), 27(11)
2,3-(2,5- or 2,6-)Dimethyl-5-(3-)butylpyrazine	164(42), 163(43), 149(82), 136(100), 122(100), 101(63), 80(100), 57(14), 41(37), 27(32)
2,5-Dimethyl-3-isobutylpyrazine	164(37), 163(52), 149(92), 136(100), 122(100), 101(75), 80(100), 57(22), 41(30), 27(40)

Table 3 summarises the mass spectra of the pyrazine derivatives identified. Possible fragmentation of the molecular ion of 2-ethyl-3,5,6-trimethylpyrazine, for example, according to values given in Table 3, is presented in Fig. 1.

The ESR spectra obtained, shown in Figs 2 and 3, for the D(+)-glucose-2- and 3-aminobutyric acid model systems, respectively, and g values of the splitting constants, $a^{\text{N}} = 8.73$ Gs, for the quintet due to two equivalent N atoms ($I = 1$), and $a^{\text{H}} = 2.81$ Gs, for the quintet of the

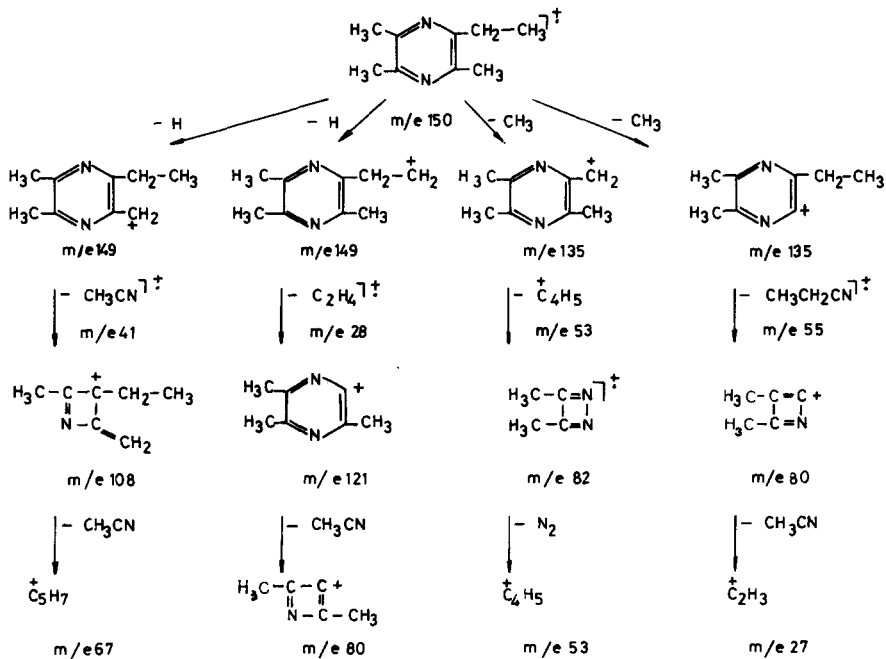


Fig. 1. Fragmentation of molecular ion of 2-ethyl-3,5,6-trimethylpyrazine.

Derivative: 1st
 Temperature: RT
 Total number of lines: 19
 Total line width: 50 G
 Relative line intensities:
 1:2:3:2:1

Splitting constants:

$$a^{\text{H}} = 2.81 \text{ G}$$

$$a^{\text{N}} = 8.73 \text{ G}$$

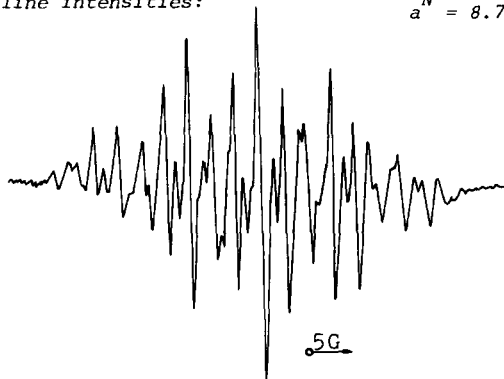


Fig. 2. ESR spectrum of free radical in heated reaction mixture of D(+)-glucose and 2-aminobutyric acid.

Derivative: 1st
 Temperature: RT
 Total number of lines: 19
 Total line width: 50 G
 Relative line intensities:
 1:2:3:2:1

Splitting constants:
 $a^H = 2.81 \text{ G}$
 $a^N = 8.73 \text{ G}$

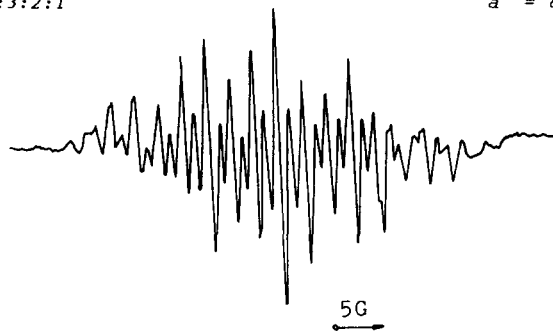


Fig. 3. ESR spectrum of free radical in heated reaction mixture of D(+)-glucose and 3-aminobutyric acid.

isotropic splitting constant for four equivalent H atoms ($I = \frac{1}{2}$), indicated the presence of 1,4-dialkylpyrazine cation radicals in the reaction mixtures.

The ESR spectrum of the D(+)-glucose and 4-aminobutyric acid model system is shown in Fig. 4.

The structure of the free 1,4-dialkylpyrazine cation radical, formed in the heated reaction mixture of D(+)-glucose and 4-aminobutyric acid, was deduced from the splitting constants: $a^N = 8.23 \text{ Gs}$, for the two equivalent N atoms ($I = 1$) and $a^H = 2.81 \text{ Gs}$ for the quintet of four equivalent H atoms ($I = \frac{1}{2}$) bound to the pyrazine ring. The splitting constant $a^{H\alpha} = 5.44 \text{ Gs}$ indicated that two α -H-atoms of the alkyl group, linked to the N-atom of the pyrazine ring, possess a stereo arrangement which enables magnetic interaction with the π -orbital of an unpaired electron and they split ESR-spectrum lines with g values of 5.44 Gs. Such a stereo effect was not determined from the ESR spectra of heated reaction mixtures of the D(+)-glucose-2- and 3-aminobutyric acid model systems, respectively, because their alkyl groups, linked to the N-atom of the pyrazine ring, possess just one α -H-atom.

When a reaction mixture of D(+)-glucose and aminobutyric acid isomers was heated at pH 9.0 and 368 K, the development of the ESR signal was observed as soon as the reaction was started. The relative intensity of the signal increased rapidly. The ESR spectra showed a characteristic hyperfine structure, as shown in Figs 2 to 4. After the first

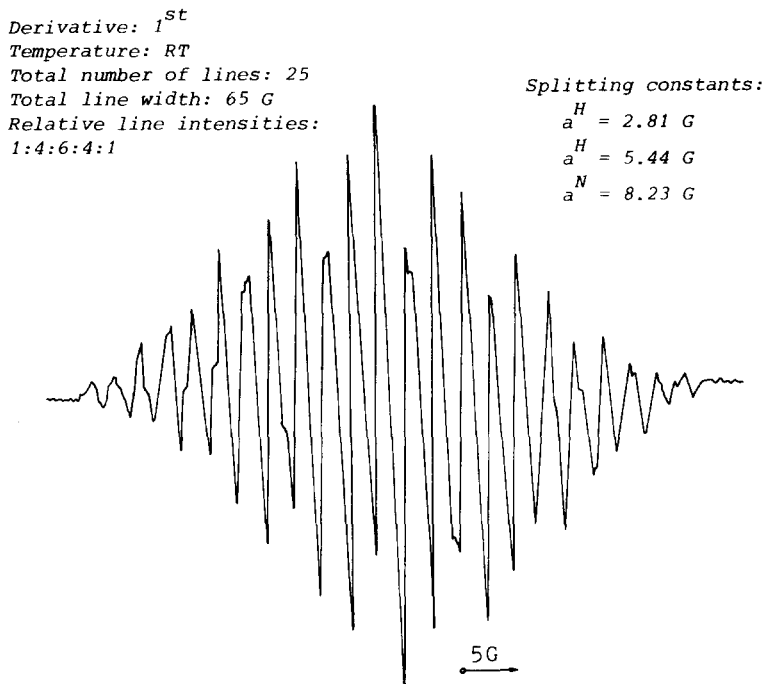
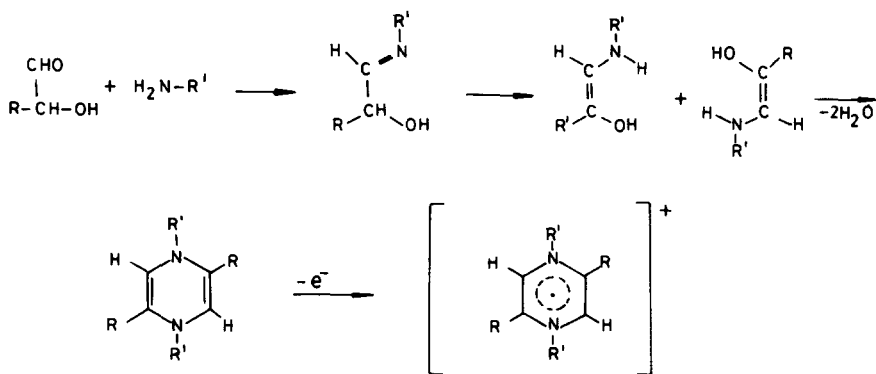


Fig. 4. ESR spectrum of free radical in heated reaction mixture of D(+)-glucose and 4-aminobutyric acid.

15 min, the intensity of the ESR signal started to fall with further heating and decreased gradually to a constant level and, finally, to a broad singlet, after 35 min.

Based on the ESR spectra and the hyperfine splitting constants determined, it is possible to conclude that the free 1,4-disubstituted pyrazine cation radicals were formed in the initial stage of the reaction between glucose and amino acid. This stage included the nucleophile attack of the amino group of the amino acid on the electrophile C-atom of the carbonyl group of the aldose and Strecker degradation of the amino acid moiety where ketosylamines were also formed. The ketosylamines are assumed to be very important precursors for further cyclisation and formation of pyrazine cation radicals, as shown in Scheme II.

On the other hand, the sugar in the reaction mixture at pH 9.0 underwent alkaline dehydration to provide 1,2-enolic products, glycol-aldehyde, glyceraldehyde, lactic acid and other diketo and glucosone derivatives. The dicarbonyl compound formed from sugar reacts readily



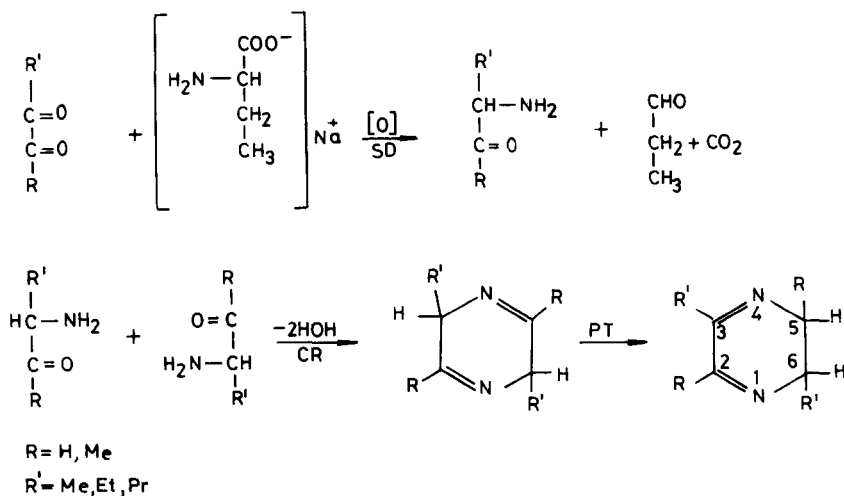
$R = \text{H}, \text{Me}, \text{Et}, \text{Pr}, \text{ and Bu}$

$R' = \text{Pr}, \text{ and iso-Pr}$

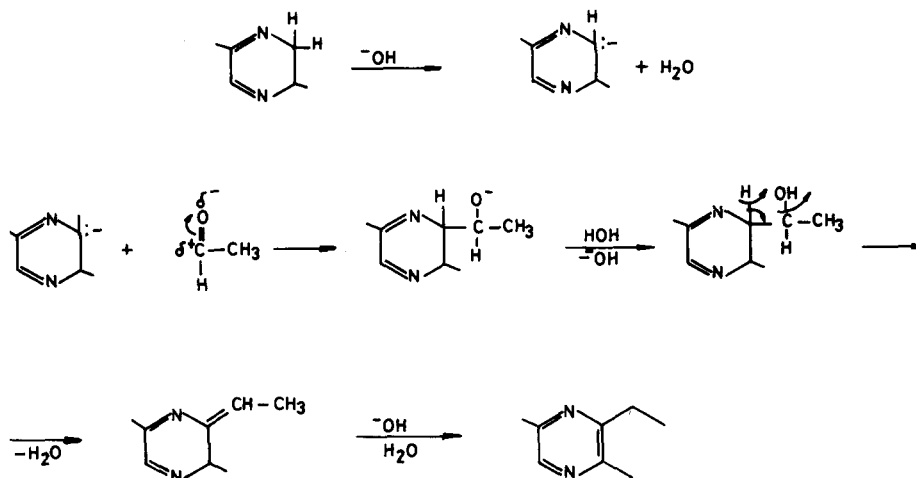
Scheme II. Mechanism of pyrazine cation radical formation.

with amino acids, which, at the same time, undergo Strecker degradation (SD), yielding aminoketo derivatives. These intermediates lose one molecule of water and react with another molecule of the same structure by cyclisation reaction (CR) to give dialkyl- or tetraalkyl-5,6-dihydropyrazines on subsequent proton transfer (PT), as shown in Scheme III.

The dialkyl- or tetraalkyl-5,6-dihydropyrazine formed represents a



Scheme III. Formation of dialkyl- or tetraalkyl-pyrazines from Strecker degradation products.



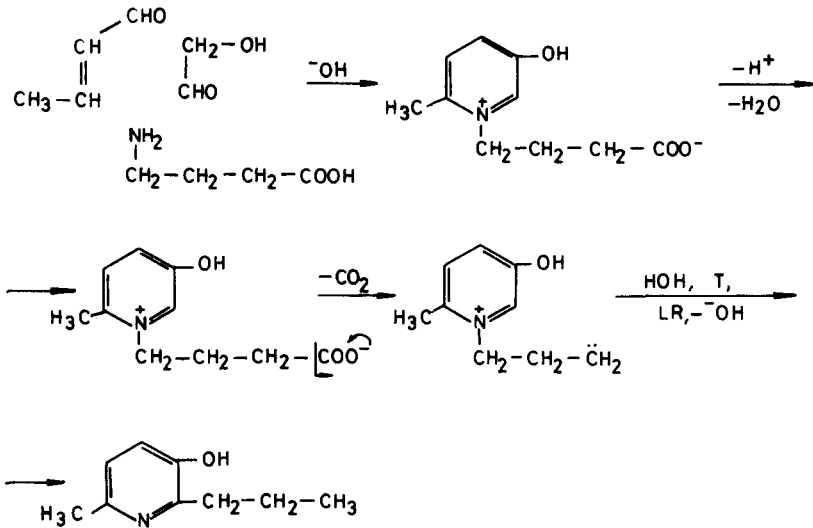
2-ethyl-3,6-dimethylpyrazine

Scheme IV. Formation of 2-ethyl-3,6-dimethylpyrazine from 3,5-dimethyl-5,6-dihydropyrazine.

very important intermediate for dialkyl- or tetraalkyl-pyrazine formation. The dehydrogenation of 3,6-dimethyl-5,6-dihydropyrazine, for example, if R is H, and R' is Me, can occur under basic conditions (Akiyama *et al.*, 1978) to yield the dihydropyrazine carbanion, which can react with a small amount of acetaldehyde, present in the reaction mixture, as shown in Scheme IV.

Comparing the total peak areas of the pyrazine derivatives identified from three model systems (Table 2), 4-aminobutyric acid yields the largest amount, 3-aminobutyric acid less, and 2-aminobutyric acid least. 4-Aminobutyric acid, as the amino acid with a terminal amino group, is probably able to take a greater part in carbonyl-amine reactions relative to its isomers.

The assumed mechanism of 2-methyl-5-hydroxy-6-propylpyridine formation is based mainly on the fact that, in the reaction mixtures of aldoses and amino acids at pH 9.0, D(+)-glucose give rise to carbonyl compounds by alkaline transformation and fragmentation of the open-aldehyde form. Crotonal and glyoxal, which are easily formed (Suyama & Adachi, 1980), react with amino acid molecules by condensation reaction to give 1-(3-carboxypropyl)-2-methyl-5-hydroxypyridine cation radicals. The cation radicals obtained are stabilized by decarboxylation, and by



Scheme V. Mechanism of 2-methyl-5-hydroxy-6-propylpyridine formation.

Ledenburg rearrangement (LR) to give 2-methyl-5-hydroxy-6-propylpyridine, according to the mechanism presented in Scheme V.

The proposed mechanisms of pyrrole, pyrazine and pyridine derivative formation are not the only ones and are still speculative because of the complexity of the non-enzymic browning reaction, even in very simple model systems.

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