Electron Spin Resonance Spectral Study on the Structure of the Novel Free Radical Products Formed by the Reactions of Sugars with Amino Acids or Amines

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The hyperfine structures of ESR spectra of the novel free radical products formed by the amino–carbonyl reactions of sugars or related carbonyl compounds with amino acids or amines could be assigned to the contributions of two equivalent nitrogens, four equivalent protons, and additional equivalent proton(s) depending on the number of α-protons of amino compounds. The results lead to the assumption of the identity of the radical products as 1,4-disubstituted pyrazine radicals. The ESR spectral analyses and the assumed structures were strongly supported by the close agreement between the observed spectra and those simulated on the analyzed splitting constants, and moreover by the striking similarity of the spectrum observed with the D-glucose–ethylamine system to that of the synthesized 1,4-diethylpyrazine cation radical.

Previous works in this series have demonstrated the development of novel free radicals in an early stage of the amino–carbonyl reactions of sugars with amino acids (Namiki et al., 1973; Namiki and Hayashi, 1975), which appeared of great interest to suggest the presence of new processes involving free radical reaction in the browning of sugar–amino acid reaction systems. These radicals showed ESR spectra of characteristic hyperfine structures and whose patterns and splitting line numbers depended mainly upon the structure of amino compounds, e.g., α- vs. β-alanine, but not upon the structure of sugars except for their three-carbon analogues (Namiki and Hayashi, 1975). Some speculation on the functional group of the free radical products has been presented but the principal structure as well as the formation mechanism remained obscure.

We have attempted to analyze the hyperfine structure of novel free radical products and, consequently, have made a proposal that the radical products would be 1,4-disubstituted pyrazine cation radicals, with which this paper is concerned.

EXPERIMENTAL SECTION

Sugars, amino acids, and other related compounds were all Guaranteed Grade reagent. Distilled water was prepared with Pyrex apparatus.

Mixtures of sugar, or carbonyl compound, with amino acid or amine were prepared with distilled water sufficient to make solutions of 1 M or more concentrated for each when solubilized. For the sake of convenience molar concentration is used in this paper.

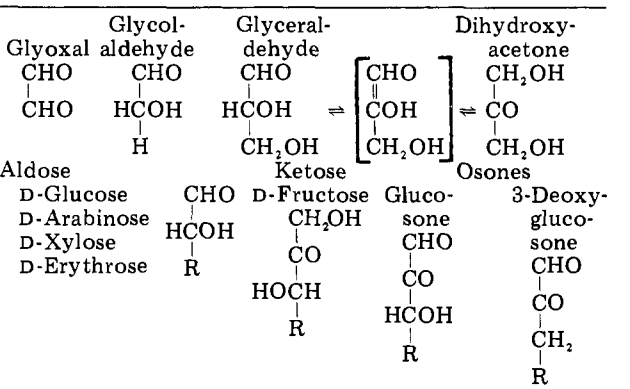
The mixtures in Pyrex test tubes were heated in a boiling water bath for a given time. A part of the reaction mixture was placed in a quartz tube (1 mm i.d.) to measure the ESR spectrum. ESR spectra were recorded at room temperature with a JES-ME-1X ESR spectrometer equipped with an X-band microwave unit. Modulation width of 0.05~0.2 G was used to observe the hyperfine structure of the spectrum. The splitting constants and g values of the spectra were determined by use of potassium peroxylamine disulfonate as a standard.

RESULTS AND DISCUSSION

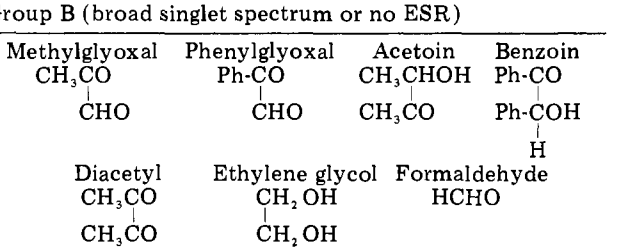
Structural Effect of the Reactions on the Development of Free Radicals Showing ESR Spectra with Hyperfine Structure. A potentiality in the novel free radical development has previously been compared among common sugars or their related compounds by the reactions with α- or β-alanine along with the development of browning (Namiki and Hayashi, 1975), and where it was demonstrated that most of the sugars and analogous compounds gave the spectra of essentially the same type in hyperfine structure by the reaction with a given amino acid except glyceraldehyde and dihydroxyacetone which showed the ESR spectra with more complicated hyperfine structures.

Table I. Structural Effect of Carbonyl Compounds on the Development of Free Radicals

Group A (ESR spectrum with hyperfine structure)



Group B (broad singlet spectrum or no ESR)



In order to elucidate what kind of carbonyl compounds or amino compounds are capable of developing ESR spectra of characteristic hyperfine structure, further ESR investigations were done on the system consisting of a variety of carbonyl and amino compounds. As to the potentiality of carbonyl compounds, various sugars or related carbonyl compounds were reacted with α-alanine, β-alanine, or *tert*-butyl amine, and they were tentatively classified into two groups according to their readiness to produce hyperfine structural ESR spectra (Table I).

Comparison of chemical structures of these groups of sugar-like compound lead to the supposition that the presence of an endiol group or a potential endiol group (R–HCOH–CHO) in the molecule is requisite for such

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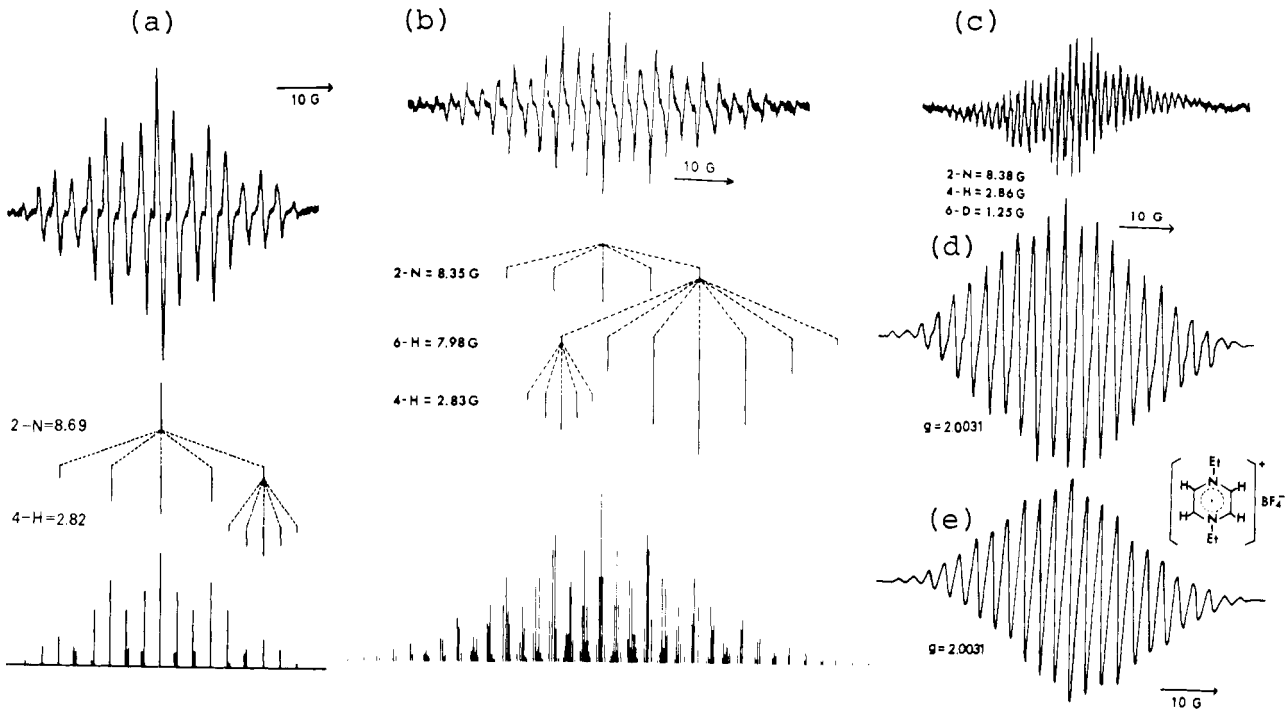


Figure 1. ESR spectra of the reaction mixtures of amines with sugar or glycolaldehyde: (a) *tert*-butylamine with glycolaldehyde; (b) methylamine with D-glucose; (c) methyl-d_3-amine with D-glucose; (d) ethylamine with D-glucose; (e) synthesized 1,4-diethylpyrazine cation radical.

radical formation. Glyoxal is an exception to this, and although no explanation can yet be given, it may conceivably give rise to endiol-like function during reaction, by dimerization, reduction, hydration, or dehydration.

Among the compounds incapable of producing the ESR signals with hyperfine structure, some of them as methylglyoxal gave a marked ESR signal along with an intense browning, but its spectrum was a broad singlet without hyperfine splitting, and the carbonyl compounds as furfural and crotonaldehyde showed a marked browning but gave neither hyperfine structural nor broad singlet ESR spectra.

On the other hand, various amino acids, amines, and other amino compounds were respectively reacted with D-xylose, D-arabinose, or D-glucose. Usually suspended mixtures adjusted at pH 8.0–8.5 were heated at 90 °C for 10 min and the ESR spectrum was measured.

With respect to the readiness to give the ESR spectrum with hyperfine structure, the amino compounds tested could be classified into two groups as listed in Table II. The ability to give the characteristic hyperfine ESR spectrum was only observed in the compounds with a primary amino group, though there were several exceptions as listed in group B, most of which seemed to belong in primary amines of special character.

Among the compounds listed in group B, tetramethylammonium hydroxide, imidazole, and indole showed broad singlet ESR signal during the reaction with sugars in alkaline solution.

Analyses of Hyperfine Structures of ESR Spectra. To elucidate the structures of the free radical products, analyses of the hyperfine structures were made mainly on the spectra observed in the mixtures of glycolaldehyde, D-arabinose, or D-glucose with several amino compounds, because the types of hyperfine structures were known to be determined mainly by the structural differences of the amino compounds.

Amines with Sugars or Glycolaldehyde. The analysis was first made on the spectrum of the *tert*-butylamine and glycolaldehyde system, because this amine has no α-proton so that influence of protons in the amino compound residue on the hyperfine structure would be negligibly small and, moreover, the reactions of *tert*-butylamine with sugars and related compounds gave essentially the same spectral pattern, including the three-carbon carbonyl compounds as glyceraldehyde and dihydroxyacetone. As shown in Figure 1a, the hyperfine structure could be resolved into the 2.82 G quartet and the 8.69 G doublet responsible for equivalent protons and two equivalent nitrogens, respectively, as indicated by the stick diagram in the figure. Here, it is to be noted that no spectral change was observed by addition of heavy water to the reaction mixture, suggesting no exchangeable proton in neighbor of the free radical.

Figure 1b shows the ESR spectrum of the reaction mixture of methylamine and D-glucose, each 2 M in distilled water, heated at 90 °C for 20 min. The hyperfine structure could be resolved into the 8.35 G quintet, the 7.98 G septet, and the 2.83 G quintet due to two equivalent nitrogens, six equivalent protons, and four equivalent protons.

When methylamine was replaced by methyl-d_3-amine (CD_3NH_2), the hyperfine structure was changed as shown in Figure 1c, which is resolved into the 8.38 G doublet, the 2.86 G quintet, and the 1.25 G septet responsible for two equivalent nitrogens, four equivalent protons, and six equivalent deuterons. The change in the splitting constant of the septet from 7.98 to 1.25 G is due apparently to the replacement of CD_3, and thus it was demonstrated that the equivalent six protons belong to two methyl groups. This fact and the presence of two equivalent nitrogens suggest the involvement of two molecules of methylamine in one molecule of free radical product.

A similar experiment with ethylamine and D-glucose gave the spectrum shown in Figure 1d, which could also be assigned to the contributions of two equivalent nitrogens, four equivalent protons, and four equivalent protons.

These assigments on various spectra reasonably lead to the assumption that the free radical products in the

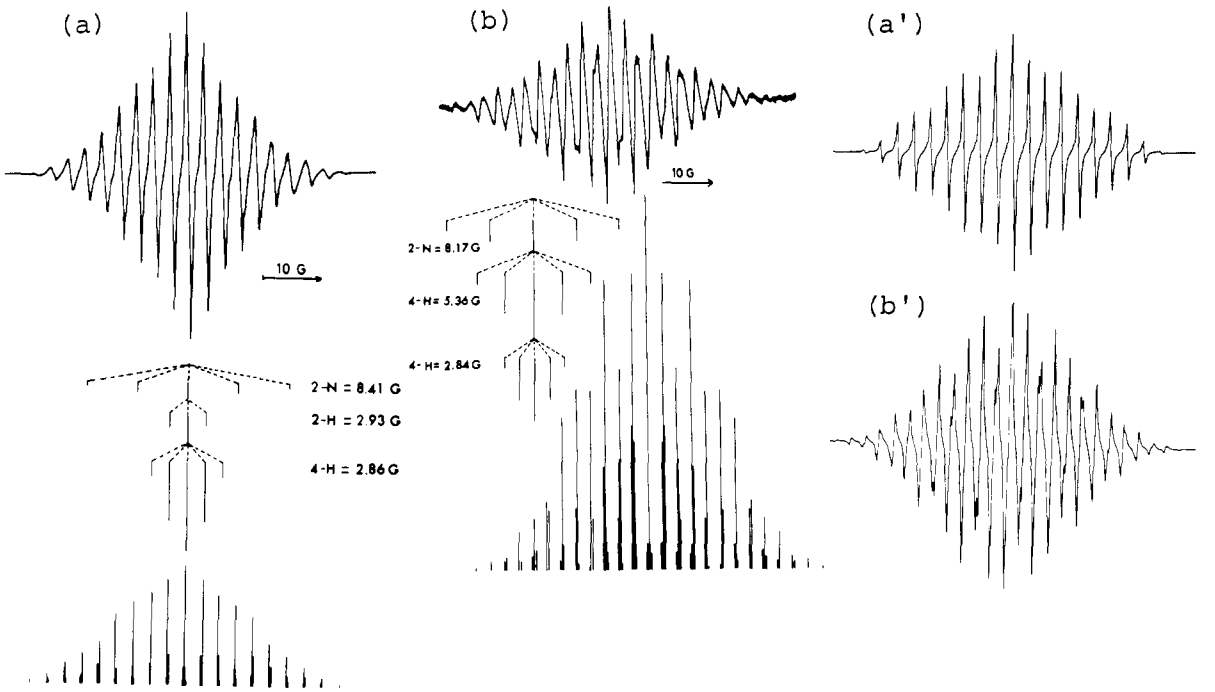


Figure 2. ESR spectra of the reaction mixtures of (a) D-glucose–α-alanine, (b) D-glucose–β-alanine, and the synthesized ESR spectra based on their spitting constants, a′ for D-glucose–α-alanine and b′ for D-glucose–β-alanine, respectively.

TABLE II. Structural Effect of Amino Compounds on the Development of Free Radicals

Group 1. ESR spectrum with hyperfine structure	
Amino acids	Threonine
Glycine	Tryptophan
α-Alanine	Lysine
β-Alanine	Glutamic acid
Valine	Glutamine
Leucine	Aspartic acid
Isoleucine	Asparagine
Phenylalanine	Amines
Histidine	n-Butylamine
Serine	Ethylamine
Methionine	Methylamine
Tyrosine	Isopropylamine
Arginine	*tert*-Butylamine

Group 2. ESR spectrum with broad singlet or no ESR signal		
	Amines	Sugars
Quarternary ammonium	Tetramethylammonium hydroxide	Xylose
Tertiary amine	Trimethylamine	Xylose
Secondary amines	Dimethylamine	Xylose, glucose
	Imidazole	Xylose, arabinose
	Indole	Xylose, arabinose
	Proline	Xylose, arabinose
	N-Acetylhistidine	Xylose, arabinose
	N-Acetyltryptophan	Xylose, arabinose
Primary amines	Urea	Xylose
	Formamide	Glucose
	Guanidine	Xylose, arabinose
	2,4-Dinitroaniline	Xylose
	Aniline	Glucose
	2,4-Dinitrophenyl-hydrazine	Xylose
	Ammonium chloride	Glucose, glyoxal
	28% Ammonia water	Glucose
	o-Methylhydroxylamine	Glucose
	Ethylenediamine	Glucose, glyoxal
	Cysteine	Glucose
	Cystine	Glucose

sugar-amine system might be a radical compound of N,-N′-disubstituted derivative, that is, 1,4-diethylpyrazine radical in the case of the ethylamine–glucose system.

The ESR pattern of 1,4-diethylpyrazine cation radical reported by Curphey (1965) shows striking similarity with that found in the present system. The spectrum of this radical prepared according to Curphey is also shown in Figure 1e. The hyperfine structure as well as *g* values agreed well with each other, strongly supporting this assumption.

Amino Acid with Sugar. As has been presented, the hyperfine structure of ESR spectra observed in the sugar–amino acid systems differed apparently between α- and β-amino acid series. The spectra of representative ones, D-glucose–α-alanine and D-glucose–β-alanine, are shown in Figure 2. The hyperfine structures of each spectra could also be analyzed by assuming pyrazine radical, that is, two pyrazine nitrogens, four equivalent pyrazine protons, and a couple of α-proton(s) in the amino acid, respectively, as indicated by the stick diagrams and splitting constants in each figure.

To confirm this analysis, these spectra were compared with those made by the simulation based on the above splitting constants. The synthesized spectra are shown in Figure 2. The excellent agreement between them and the observed ones verified the analytical data.

Among the ESR spectra observed in the reactions of various α-amino acids with a given sugar, those of phenylalanine, serine, leucine, tyrosine, and arginine gave indentical hyperfine structures, while those of valine and isoleucine are somewhat different from those of the above group in pattern of the ESR spectra though they are essentially the same in the number of splitting.

The representative spectra, those of the phenylalanine–arabinose system and the valine–arabinose system, are shown in Figure 3. Each hyperfine structure could also be analyzed by assuming two pyrazine nitrogens, four equivalent pyrazine protons, and a couple of α-protons of amino acids, respectively, as shown by the stick diagram and splitting constants in the figure.

The synthetic spectra by simulation based on analyzed data, also shown in the figure, are in good agreement with experimental spectra in their hyperfine structure.

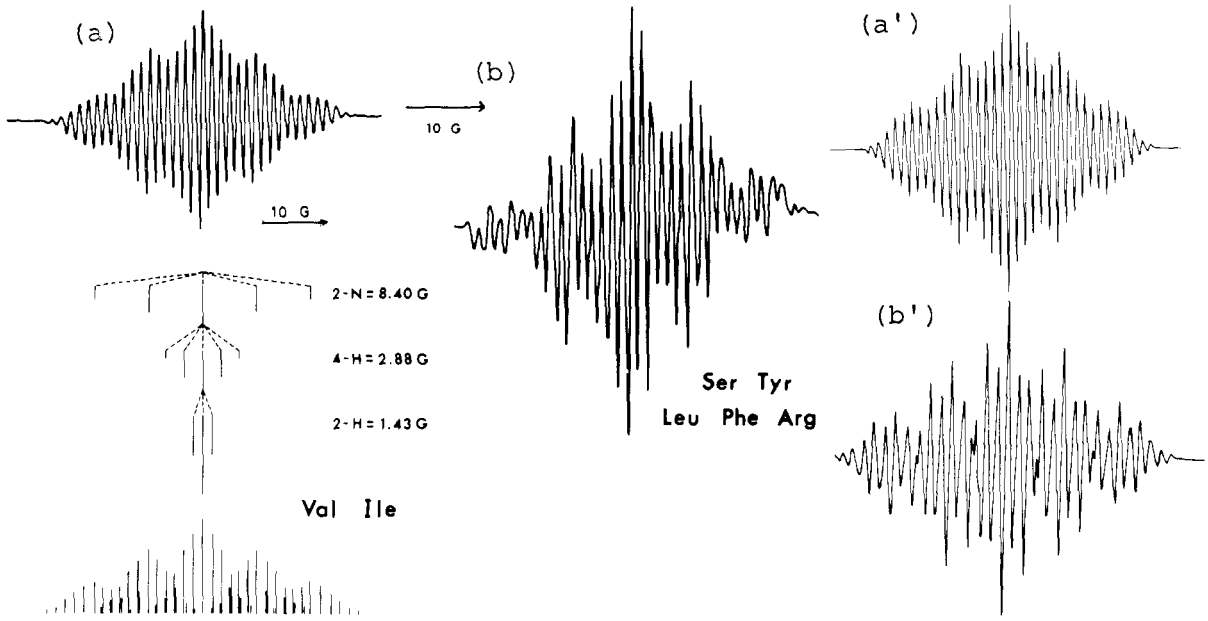


Figure 3. ESR spectra of the reaction mixtures of D-arabinose with various amino acids and the synthesized spectra based on their splitting constant: (a) L-valine, L-isoleucine; (b) L-phenylalanine, L-serine, L-tyrosine, L-arginine, L-leucine; a′ and b′ synthesized spectra for a and b, respectively.

TABLE III. Analyses of the Hyperfine Structures of ESR Spectra[a]

Amino acid or Amine	Splitting constants, G			
	α-H		2-N	4-H
Glycine	4.69	(4H)	8.15	3.04
L-α-Alanine	2.93	(2H)	8.41	2.86
L-β-Alanine	5.36	(4H)	8.15	2.84
L-Valine	1.43	(2H)	8.40	2.88
L-Phenylalanine	1.31	(2H)	7.99	3.03
Other amino acids[b]	1.5 ± 0.1	(2H)	8.3 ± 0.15	2.9 ± 0.1
tert-Butylamine			8.69	2.82
Methylamine	7.98	(6H)	8.35	2.83
Methyl-d_3-amine	1.25	(6D)	8.38	2.86
Ethylamine	5.37	(4H)	8.35	2.85
1,4-Diethylpyradine cation radical (synthesized)	5.33	(4H)	8.37	2.82

[a] Sugars: D-glucose, D-arabinose, D-xylose, or glycolaldehyde. [b] Other amino acids; L-serine, L-methionine, L-leucine, L-isoleucine, L-tyrosine, L-arginine, etc.

As has been mentioned, glycine gave very complicated hyperfine structural ESR spectrum as shown in Figure 4 with glycolaldehyde, and it could also be analyzed in the same way.

The analyzed data of the hyperfine structures of ESR spectra observed in various amino acids or amines with arabinose or glucose are summarized in Table III.

ESR Spectra of Three-Carbon Aldehydes with Amino Compounds. As has been mentioned in Table I, the three-carbon carbonyl compounds glyceraldehyde and dihydroxyacetone, gave exceptionally the ESR spectra of a different type from other carbonyl compounds in the reactions with amino compounds.

As can be seen in the representative spectra shown in Figure 5, glyceraldehyde and dihydroxyacetone with a given amino acid gave quite similar ESR spectra in their hyperfine splitting, and both spectra have an overlapped broad singlet even in the early stage of the reaction. The fact that dihydroxyacetone showed behavior similar to glyceraldehyde in the radical formation might be due to its isomeric conversion to glyceraldehyde through endiol structure, as the scheme shows in Table II.

Analysis of the hyperfine structure of these spectra seemed rather difficult, so the structure of radical product formed from these three-carbon carbonyl compounds remained obscure. However, in connection with the assumption that the radical products formed by the reaction of glycolaldehyde with amines are 1,4-disubstituted pyrazine cation radicals, the radical product from three-carbon aldehyde is expected to be 2,5-disubstituted, probably a CH_2OH or CH_3 group, derivative of the above pyrazine radical. Then, 2,5-dimethyl-1,4-diethylpyrazine cation radical was prepared in a similar way as above from 2,5-dimethylpyrazine, and the ESR spectrum is shown in Figure 5e. In comparison of this spectrum with those observed in the cases of three-carbon aldehydes, we can observe some corresponding signals between them, but the spectrum of synthetic radical has more complicated hyperfine splitting, so assignment of the radical product from glyceraldehyde with ethylamine to the 2,5-dimethyl derivative of pyrazine radical seemed unsuccessful and the structure remains to be identified.

Here an interesting fact is that the reactions of these three-carbon carbonyl compounds with *tert*-butylamine gave the ESR spectra apparently different from those observed in the cases with other amino compounds in the splitting and whole pattern of the hyperfine structure, and rather it well resembles the spectrum observed in the reaction of glycolaldehyde with *tert*-butylamine (see Figure 1).

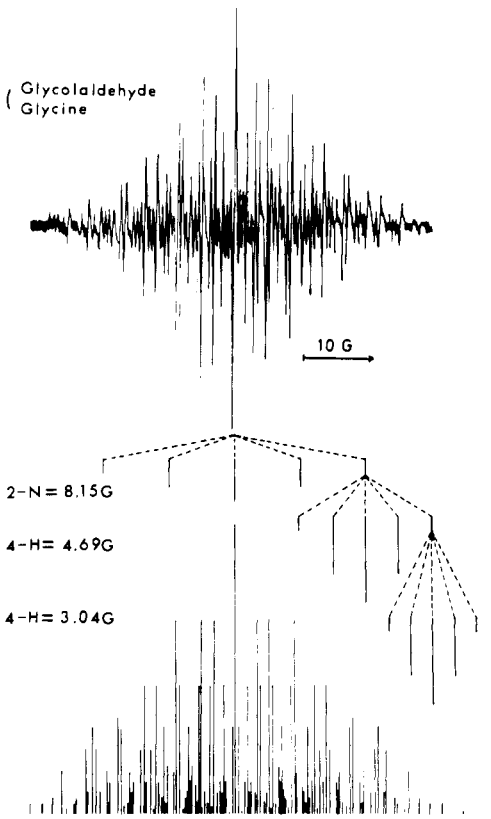
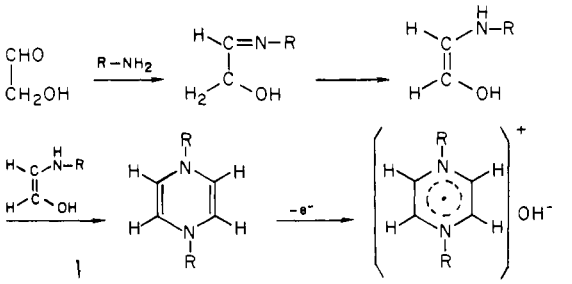


Figure 4. ESR spectrum of the reaction mixture of glycine with glycolaldehyde and analysis of its hyperfine structure.

The reasons why only glyceraldehyde gave a different type of ESR spectrum among the C-2 to C-6 sugar analogues and, moreover, why it provides the common sugar-type spectrum with *tert*-butylamine have not yet been made clear.

Mechanism of Free Radical Formation in the Reaction of Sugar with Amino Acid. From the above spectral analyses, it seems adequate to say that the free radicals which are formed at the initial stage of the reaction of sugar with amino acid and showed characteristic hyperfine structures are probably 1,4-disubstituted pyrazine

Scheme I



radical cations. The mechanism of formation of the pyrazine radicals from glycolaldehyde, which is the simplest sugar analogue, may be considered as shown in Scheme I.

Each of the individual steps in this scheme seems probable from a chemical viewpoint, and it would be supported by the fact that glycolaldehyde is the most effective one among carbonyl compounds in giving the characteristic ESR spectrum and its reaction with ethylamine or others develops intense ESR signals far more rapidly than any other sugar–amino acid system.

However, there are many problems to be investigated on the mechanism of free radical formation in the reactions of sugars with amino acids. Firstly, if one postulates that the radical products could only be from glycolaldehyde as an essential material, an adequate amount of glycolaldehyde must be produced at an early stage of the reaction. Formation of such small molecular carbonyl compounds as glycolaldehyde, glyceraldehyde, and others in the sugar–amino acid reaction systems has been reported (Hodge, 1953), but it seems not fully substantiated experimentally. If the formation of these aldehydes occurred in the reaction processes, they should be formed by C–C scission of the sugar molecule, probably not of the material sugar molecule but of some reaction intermediate products such as glucosones. Therefore, further investigation is necessary to elucidate whether enough amount of glycolaldehyde is produced at the initial stage of the reaction of sugar with amino acid, though quantitative determination in such reaction systems seems difficult, because of its high reactivity for amino compound.

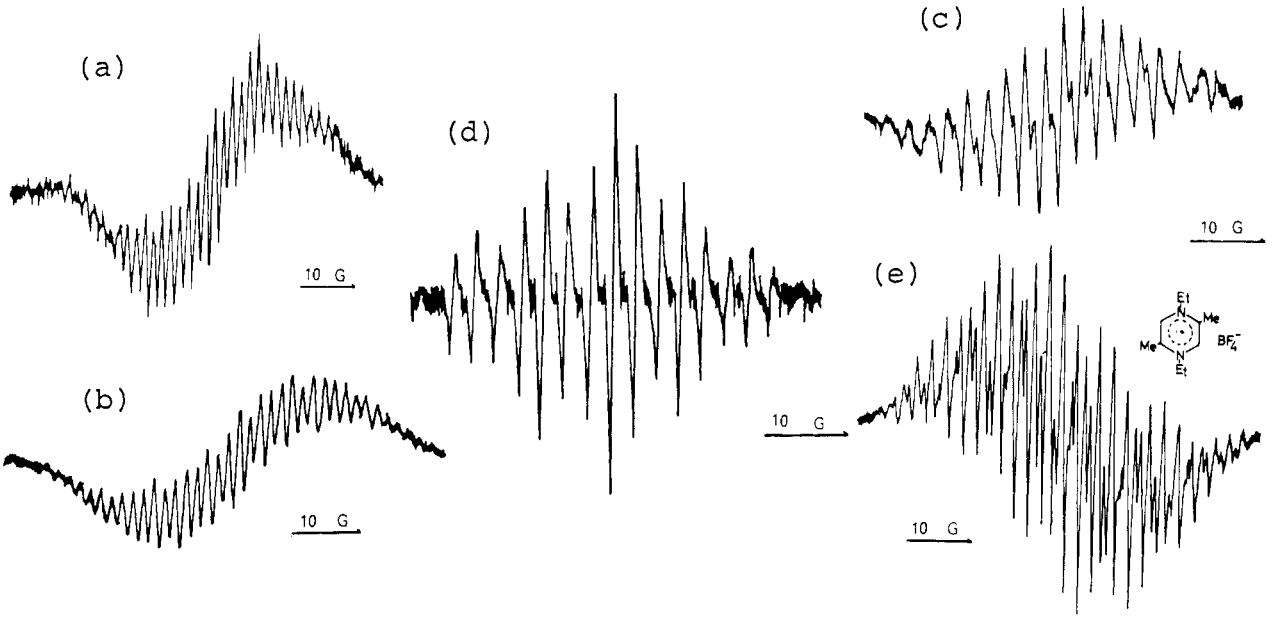


Figure 5. ESR spectra of the reacton mixtures of three-carbon carbonyl compounds with amino compounds: (a) glyceraldehyde with β-alanine, (b) dihydroxyacetone with β-alanine, (c) dihydroxyacetone with α-alanine, (d) glyceraldehyde with *tert*-butylamine, (e) 1,4-diethyl-2,5-dimethylpyrazinum fluoroborate cation radical.

On the other hand, if one considers the formation pathway of the radicals from the various compounds which have been presented as the intermediate products in an early stage of the sugar–amino acid reaction system, the following pathway seems probable; the pyrazine derivative possessing 1,4-diamino acid residues and 2,5-disugar residues would be formed by condensation of two molecules of the enaminol product, e.g., that of N-substituted 1-amino-1-deoxyketose in glucose–amino acid system, and subsequent elimination of the subsitutents of sugar residues by C–C scission will give the proposed pyrazine radical products. However, there are many problems with this postulation, since the presence of such 1,4-disubstituted pyrazine drivatives as the reaction product is hardly reported and, moreover, possibility of the following C–C scission process remains obscure.

To see the possibility of this mechanism, the following experiment was carried out as a preliminary investigation. 1-Alanino-1-deoxy-D-fructose, prepared according to Anet and Reynolds (1957), was heated in alkaline solution, with D-glucose in distilled water, or with β-alanine in distilled water, at 90 °C for 15 to 30 min. In either case no detectable ESR signal with characteristic hyperfine structure was observed.

In any event, it is not at present possible to decide whether the free radical products formed necessarily from glycolaldehyde or from other unknown pathways as presented above. Further investigation is necessary to explain fully this interesting observation.

LITERATURE CITED

Anet, E. F. L. J., Reynolds, T. M., *Aust. J. Chem.* **10**, 182–197 (1957).
Curphey, T. J., *J. Am. Chem. Soc.* **87**, 2063 (1965).
Hodge, J. E., *J. Agric. Food Chem.* **1**, 928–943 (1953).
Namiki, M., Hayashi, T., *J. Agric. Food Chem.* **23**, 487–491 (1975).
Namiki, M., Hayashi, T., Kawakishi, S., *Agric. Biol. Chem.* **37**, 2935–2936 (1973).

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Reconstitution of Petroleum Ether Soluble Wheat Lipopurothionin by Binding of Digalactosyl Diglyceride to the Chloroform-Soluble Form

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Lipopurothionins, which are lipid–protein complexes extracted with petroleum ether from the endosperms of wheat and other Gramineae, are converted to a petroleum ether insoluble, chloroform-soluble form by extraction with acetone. It has been found that digalactosyl diglyceride (DGDG) is the only component of the acetone extract that is able to restore petroleum ether solubility when added back to the chloroform-soluble form.

Purothionins are high sulfur, basic polypeptides from wheat that inhibit papain (Balls et al., 1942b), have antimicrobial (Stuart and Harris, 1942; Fernandez de Caleya et al., 1972; Hernandez-Lucas et al., 1974) and uterus contracting properties (Coulson et al., 1942), and are toxic to small animals when injected intravenously or intraperitoneally (Coulson et al., 1942). They were first discovered as lipid–protein complexes (lipopurothionins) in petroleum ether extracts from the endosperm of hexaploid wheat, *Triticum aestivum* L. (Balls et al., 1942a). Analogous complexes were later found in barley (Redman and Fisher, 1969) and in 22 species of the *Aegilops–Triticum* group (Carbonero and García-Olmedo, 1969).

Although the protein moieties of these complexes have been well characterized (Redman and Fisher, 1969; Nimmo et al., 1968; Fisher et al., 1968; García-Olmedo et al., 1968; Nimmo et al., 1974; Fernandez de Caleya et al., 1976) and even sequenced in some cases (Othani et al., 1975; Mak and Jones, 1976), the characterization of the lipid components has been only partially successful because it has not been possible to obtain petroleum ether soluble complexes free of other lipid–protein associations (Balls et al., 1942a; Redman and Fisher, 1968; Hoseney et al., 1970; Fisher, 1976). Here, we present evidence that digalactosyl diglyceride [DGDG; 2,3-di-*O*-acyl-1-*O*-β-(6-*O*-α-D-galactopyranosyl-D-galactopyranosyl)-D-glycerol] is a component of the complex which is essential for its solubility in petroleum ether.

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MATERIALS AND METHODS

Kernels from *Triticum aestivum* L. cv "Aragon 03" and from *T. durum* Desf. cv "Senatore Capelli" were milled without preconditioning in a Brabender Quadrumat mill, and 60–65% yields of milled endosperm (flour) were obtained.

Extractions of flour were carried out with five volumes of the appropriate solvent in a glass column (2 × 15 cm). The solvent was evaporated in vacuo and the extract redissolved in a small volume of the same solvent.

For quantitation purposes, purothionins were precipitated from the extracts with three volumes of ethanolic 1 N HCl, separated by centrifugation (3,000 *g*; 15 min), and redissolved in a known volume of 0.015 M aluminum lactate–lactic acid buffer, pH 3.2, 3 M urea (100 μL/10 g of original flour). Quantitation was carried out after electrophoresis as previously described (Fernandez de Caleya et al., 1976).

Acetone extracts were fractionated by preparative thin-layer chromatography in silica gel GF_{254} (Merck) using chloroform–methanol–water (65:25:4 v/v/v) as solvent system (system I). The recovered DGDG was further purified by a second chromatographic run using chloroform–methanol–acetic acid–water (170:25:25:4 v/v) as solvent system (system II). Fractions were visualized under