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Development of Novel Free Radicals during the Amino-Carbonyl Reaction of Sugars with Amino Acids

Mitsuo Namiki* and Tateki Hayashi

Novel free radicals were generally found to be developed in an early stage of the amino-carbonyl reaction with sugars and amino acids by using ESR spectrometry. The radicals showed characteristic hyperfine structures and apparently differed from those existing in melanoidin formed with browning. The results of many combined reactions of various sugars and their related carbonyl compounds with various amino acids and amines indicate that the spectral patterns were mainly influenced by the structure of amino compounds as primary and secondary amino groups but not by the structures of sugars and most of their analogous carbonyl compounds. On the development of free radicals, effects of oxygen, pH, temperature, molar ratio, and some additives were investigated. These results suggest that the radicals did not locate in a highly conjugated structure such as melanoidin but they may exist at a particular position in some products formed at an early stage of the sugar-amino acid reactions and still containing the residues of either reactant.

The amino-carbonyl reaction of sugars with amino acids has been studied extensively as a principal reaction which causes important changes in food qualities such as browning along with melanoidin formation and development of various roast flavors. Concerning the reaction mechanism a proton transfer chain reaction has been proposed as an early stage of the reaction (Isbell and Frush, 1958), but little is known about the formation of free radicals except for the presence of stable free radicals in melanoidin (Mitsuda et al., 1965).

Recently, the authors have found the development of free radicals in some amino-carbonyl reaction systems (Namiki et al., 1973, 1974), and this report deals with the details of development of novel free radicals in an early stage of the browning reaction of sugars with amino acids.

EXPERIMENTAL AND RESULTS SECTION

Experimental procedures employed generally were as follows. In a Pyrex test tube was placed a solution containing equimolar amounts of sugar and amino acid prepared with distilled water and heated in a boiling water bath. Development of free radicals was checked at regular intervals of the heating time by use of a JES-ME-1X ESR spectrometer with a quartz tube for liquid samples, and development of browning was determined with the absorbancy at 420 nm. The reagents used were Guaranteed Grade and distilled water was prepared with Pyrex apparatus.

Reactions of α- and β-Alanine with Various Sugars. A mixture of D-arabinose and β-alanine was employed representatively, since a pentose-β-alanine system has been known as a remarkable one in browning with sugars and amino acids at neutral pH (Kato, 1956). When a mixture of D-arabinose (1.0 g) and β-alanine (0.6 g) in distilled water (2.0 ml) was heated in a boiling water bath, development of the ESR signal could be observed as soon as the reaction was started. As shown in Figure 1, relative intensity of the signal increased rapidly during several minutes in an initial stage of the reaction where the spectrum showed a characteristic hyperfine structure as shown in Figure 1a (g = 2.0034, 23 lines of a splitting constant of 3 G). The intensity of the signal then started to fall with further heating and decreased gradually to a constant level along with changes in the ESR spectral pattern from a to b at 10 min and finally to a broad singlet (c) after 90 min; the development of type a radical appeared likely to precede the browning reaction.

Figure 2 shows the reaction of D-arabinose with α-alanine, where the development of either free radical and browning were observed to proceed in a similar manner to the above case, although here either one proceeded with more moderation, and it was clearly demonstrated that the development of a characteristic ESR spectrum occurred prior to browning and the hyperfine structure (g = 2.0036, 17 lines of a splitting constant of 3 G) differed apparently from that presented above. Thus, the change in the relative intensity of the ESR signal shown in Figure 1 can be seen as a sum of the changes in those of spectra a and c as indicated by the broken lines A and C, respectively.

Further ESR studies were done on similar reactions of various sugars and some carbonyl compounds with α- or β-alanine, and the results are summarized in Table I with some features of their characteristic ESR spectra. As to the line number of the hyperfine structure, it is to be noted that almost all the sugars and their related carbonyl compounds gave essentially the same ESR spectra with a

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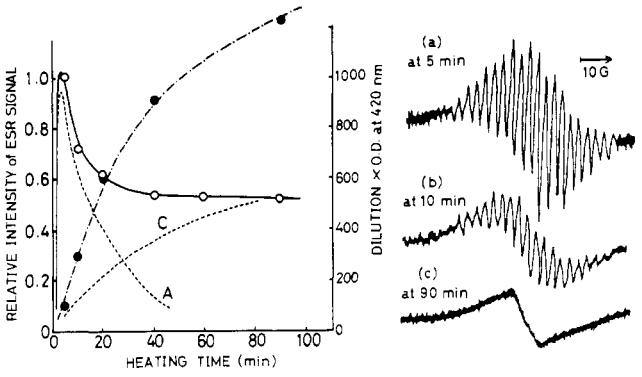


Figure 1. Formation of free radicals and browning in the reaction of D-arabinose with β-alanine (each 3 *M*), and ESR spectra of the reaction mixture heated at 98°: (–O–) ESR signal; (· - ● · -) browning; broken lines A and C, see text.

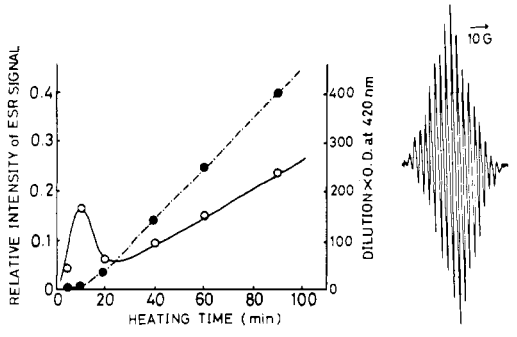


Figure 2. Formation of free radicals and browning in the reaction of D-arabinose with α-alanine (each 3 *M*), and ESR spectrum of the reaction mixture heated at 98° for 10 min: (–O–) ESR signal; (· - ● · -) browning.

Table I. ESR Spectral Data of the Free Radicals in the Amino–Carbonyl Reaction of Sugars and Other Carbonyl Compounds with α- or β-Alanine[a]

		Max. of ESR signal		
	Line no. of hfs	Time, min	Intensity	Browning
α-Alanine				
D-Glucose	16–17	20	0.05	+
D-Fructose				+
D-Arabinose	~17	~10	~0.2	++
D-Xylose	~17	~10	~0.1	++
D-Ribose	17	8	0.1	+++
Glycolaldehyde	17	3	16	+++
β-Alanine				
D-Glucose	23	15	1.0	++
D-Fructose	23	20	0.8	++
D-Arabinose	23	5	1.0	++++
D-Xylose	~23	~1	~1.0	++++
D-Ribose	~23	~1	~0.5	++++
Glyceraldehyde	31–32	0.2	5	+++++
Dihydroxyacetone	31–32	0.2	5	+++++
Glycolaldehyde	23	1	44	+++++
3-Deoxyglucosone	23	13	0.8	++++
5-Hydroxymethyl-furfural				++
Furfural				+++++
Glyoxal	23	0.2	5	+++++
Crotonaldehyde				++++
Propionaldehyde				+

[a] Aqueous solutions (each 3 *M*) were heated in a boiling water bath.

given amino acid, except for glyceraldehyde and dihydroxyacetone whose spectra resembled each other and which were indicative of a more complicated hyperfine structure. Free-radical formation was not observed with the saturated aldehyde nor with the unsaturated ones involving furfural and 5-hydroxymethylfurfural, although the latter aldehydes have been assumed as important precursors of melanoidin and showed a marked browning.

Effect of Amino Compounds on ESR Spectra. To investigate the structural effect of amino compounds on the ESR spectrum, the reactions were undertaken with 0.15 *M* solutions of each reactant at pH values around 8.0–8.5 by the use of a 0.1 *N* NaOH solution, because almost all the amino acids other than glycine and alanine were insoluble at high concentrations such as 1.0 *M* and if the reactions were done at lower concentrations in distilled water only a moderate browning with no appreciable development of free radicals was observed.

Figure 3 shows some typical ESR spectra obtained in the reactions of D-arabinose with various amino acids. In the case of glycine no significant ESR spectrum of hyperfine structure except a broad singlet was observed even in an early stage of the reaction. The spectra of leucine, phenylalanine, serine, arginine, and tyrosine were assumed to be essentially the same patterns having 35 lines of splitting constants of 8.5 (quintet or triplet), 3 (triplet), and 1.6 G (multiplet), and those of isoleucine and valine appeared to be somewhat different in the whole pattern though they also have the hyperfine structure of 35 lines of a splitting constant of 1.6 G. It seems reasonable to consider that there exist some differences in the ESR spectra due to the difference in the common structure between the former and the latter groups, $RCH_2CH(NH_2)$-COOH and R,R′-$CH(NH_2)$COOH, respectively, but further spectral work is required to distinguish them. Lysine showed a complicated spectral pattern probably due to the involvement of the spectrum of β-alanine type with the ε-amino group. The reaction of glucose with *n*-butylamine also exhibited the ESR spectrum of characteristic hyperfine structure which was in good agreement with that obtained with β-alanine.

It was therefore made clear that novel free-radical products are formed generally at an early stage of the amino-carbonyl reaction of sugars with amino acids and the characteristic hyperfine structure of ESR spectra is greatly influenced by the structural difference of amino compounds such as α- and β-amino acids but not by that of sugars.

Effects of Various Reaction Conditions on the Development of Free Radicals. In order to know some properties of the radical products as well as their formation mechanism, ESR studies were done by changing the following conditions.

Effect of Reaction Temperature. The experiments were done with a mixture of glucose and β-alanine (each 3 *M*). As shown in Figure 4, the radical development was observed remarkably at above 80° and the ESR signal intensity increased rapidly with an increase in the heating temperature. This enhancement was known to precede browning.

Effect of Molar Ratio of the Reactants. The free-radical development as well as browning were measured with the glucose–β-alanine systems of different molar ratios. The results shown in Figure 5 indicate that the radical formation occurred preferably at a molar ratio of 2:1 in amino acid and sugar, and in a similar manner for browning.

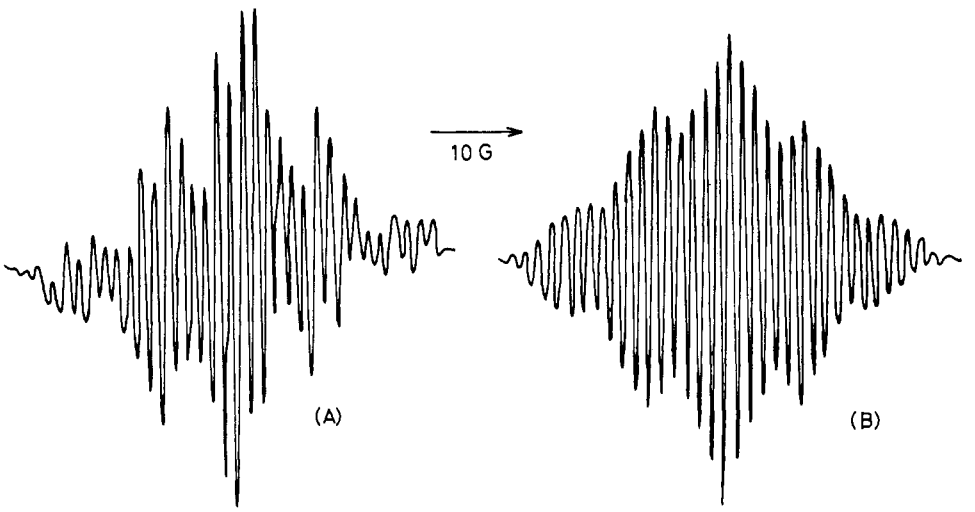


Figure 3. ESR spectra of the reaction mixtures of D-arabinose and amino acids (each 0.15 *M*) in aqueous alkaline solution (0.1 *M* NaOH), heated at 98° for 5–30 min: (A) Ser-Tyr-Phe-Leu-Arg; (B) Val-Ile.

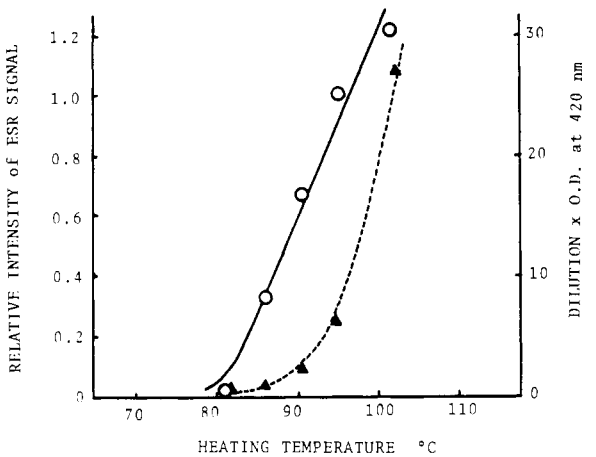


Figure 4. Effects of heating temperature on the formation of free radicals and browning in the reaction of D-glucose with β-alanine (each 3 *M*) heated for 10 min: (–O) ESR signal; (- - -▲) browning.

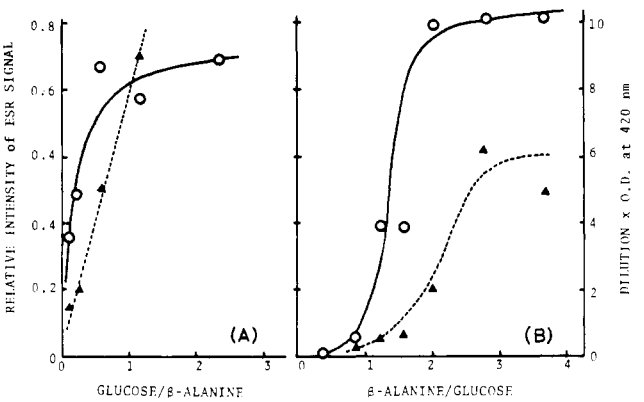


Figure 5. Effects of molar ratio on the formation of free radicals and browning in the reaction of D-glucose with β-alanine: (A) β-alanine 2.3 *M*, heated at 98° for 10 min; (B) D-glucose 1.4 *M*, heated at 98° for 6 min: (–O) ESR signal; (- - -▲) browning.

Effect of pH. The reactions presented above were undertaken at a pH of about 5–6 in distilled water, but the browning reaction of sugars and amino acids was known to proceed remarkably in alkali solution. Therefore, effects of pH on the free-radical development and browning were examined by changing the pH of a mixture of glucose–α-alanine with NaOH solution.

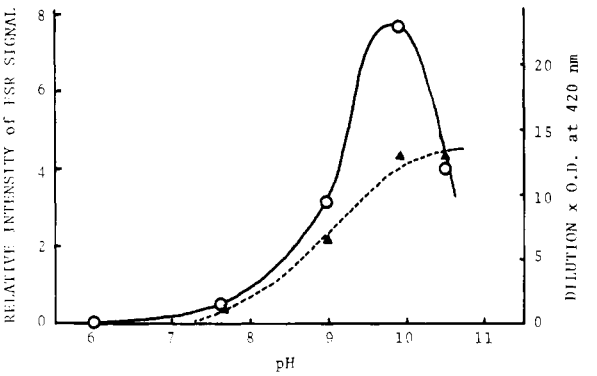


Figure 6. Effect of pH on the formation of free radicals in the reaction of D-glucose with α-alanine (each 1.5 *M*) heated at 98°. Each point indicates a maximum of ESR signal intensity during the reaction at the respective pH values: (–O) ESR signal; (- - -▲) browning.

It has been known that reducing sugars such as glucose give rise to the radical structure in a strong alkali solution (Lagercrantz, 1964). Whether the free radicals observed in the sugar–amino acid systems involve the radicals originated from sugar alone in alkali was checked out by measuring ESR spectra of the glucose solutions adjusted to high pH values. Consequently, it was verified that the free radicals developed in the system of sugar alone were negligibly small at a pH below 12 as compared with those observed in the mixed systems.

As shown in Figure 6, the free radicals liable to develop in alkali and the ESR signal intensity increased parallel to browning with increasing pH values especially at a pH above 8.0, although the intensity started to decrease at a pH above 10. The reason for these changes is not yet clear but the decrease at higher pH values might be caused by the lability of the free-radical products under such conditions as shown in the following experiment.

Effect of Various Additives on Stability of the Free Radicals. Changes in the relative intensity of the ESR signal by various additives were examined with the glucose–β-alanine system heated in boiling water for 10 min, and the results are summarized in Table II. The radicals rapidly disappeared in alkali solutions at pH values above 10, while they were unchanged in acid solutions. They were also abolished almost completely by the addition of

Table II. Changes in the Relative Intensity of ESR Signals with Various Additives[a]

Additives	Concn, *M* (final pH)	Rel intensity, %[b]
HCl	1 (0.8)	~100
NaOH	1 (10)	15
NaOH	(11)	0
Benzoquinone	0.0014	13
Hydroquinone	0.45	100
$K_3Fe(CN)_6$	0.0019	13
$K_4Fe(CN)_6$	0.12	100

[a] The additives were mixed immediately after heating for 10 min. [b] Untreated control is taken to 100%.

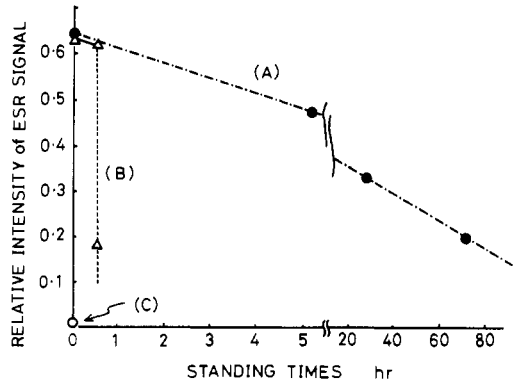


Figure 7. Effects of oxygen on the formation and stability of free radicals in the reaction of D-glucose with β-alanine (each 1.5 *M*), heated at 98° for 10 min and then kept at room temperature: (A) the reaction done in vacuo and kept intact; (B) the reaction done in an open test tube and after standing for 30 min was aerated for 2 min; (C) the reaction done under air bubbling.

weak oxidizing reagents such as benzoquinone and ferricyanide. The concentrations presented in the table are those which are minimum in effectiveness, while they are unchanged by the reducing reagents even with very high concentrations.

Effect of Oxygen. It has been well known that oxygen plays an important role in free-radical reactions and usually causes a rapid disappearance of free radicals, while in a case such as 4-NQNO a slight amount of oxygen is required to give the free-radical product (Kataoka et al., 1967).

Thus, the effect of oxygen on the development of the free radical in the sugar–amino acid system as well as on its stability was examined by comparing the signals in the glucose–β-alanine systems heated in an evacuated shield tube and in an open test tube. The results shown in Figure 7 indicate that there were no significant differences in the relative intensity of ESR signal between these two systems and also in their lifetimes when they were kept intact at room temperature for 30 min or more. However, the ESR signal disappeared instantly when the reaction mixture was exposed to air bubbling, and then no significant ESR signal could be detected when the reaction mixture was heated under aeration from the start.

Thus, it seems likely that oxygen is not required for the radical formation in an early stage of the sugar–amino acid reaction and the radicals react rapidly with oxygen to lose the ESR signal but they are considered not extremely sensitive to oxygen in the reaction system from the facts that they remained for a fairly long time at room temperature in a narrow open reaction tube and in addition the radical development was not inhibited by the oxygen dissolved initially in the reaction system.

DISCUSSION

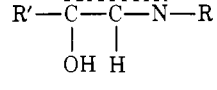
From the results presented above, it is clearly demonstrated that novel free radicals developed generally at an early stage of the amino–carbonyl reaction with reducing sugars and amino acids.

As to the early stage of the browning reaction of sugars with amino acids, the following mechanism has been proposed as the main process: with glucose and glycine, the initial reaction gives *N*-glucosylglycine which undergoes the Amadori rearrangement to provide the enaminol derivative and/or difructose glycine. The second step is oxidation and dehydration of these products to yield glucosone and, in addition, furfural derivatives, which have been assumed to be important precursors of melanoidin formation (Isbell and Frush, 1958). In accordance with this proposed mechanism, speculations on the structures of the novel radical products and their situations in the browning reaction will be attempted from the above results.

The stage of the radical formation could be estimated from the results shown in Figures 1 and 2, which showed that the radicals might be derived from some products in an early stage of the sugar–amino acid reaction prior to the formation of melanoidin. This consideration was also supported by the fact that 3-deoxyglucosone and furfural did not exhibit any superiority in the radical formation as compared with the original sugars, and moreover by the effects of temperature and pH on the radical development.

In relation to the structure of the radical products, two facts are of great importance: the ESR spectra of various sugar–amino acid reaction systems could be classified into several types in terms of the splitting line number of the hyperfine structure; their spectral patterns are mainly dependent upon the structural feature of the reactant amino acids or amines but not upon that of the reducing sugars and their analogous carbonyl compounds other than glyceraldehyde and dihydroxyacetone. These facts suggest that the radical products necessarily involve the residual structures of either reactant. Thus, they are apparently different from those observed in the reaction of amino acids with dehydroascorbic acid where the radicals appeared to be formed from the Strecker degradation products (Namiki et al., 1974).

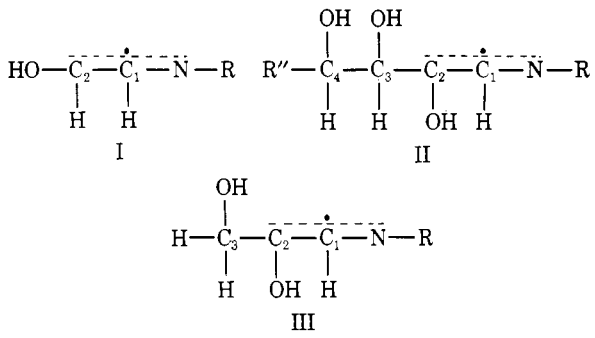
On the basis of the results of the effects of carbonyl compounds it seems likely that the structure of α-hydroxy carbonyl, potential endiol, is required to give the free radical. These considerations lead us to the following proposal that a principal structure of the radical products is that which may be derived by elimination of one proton from CH or NH in the enaminol type intermediate(s) to give the conjugated radical structure



Here, R represents the amino acid residue. Its effects on the situation of the free radical can be appreciated from the fact that the difference in the number of protons located at C-1 and C-2 from the nitrogen corresponds well to the difference in the splitting line numbers of the spectra.

On the other hand, the effect of sugar residues seems to be more simple because of the fact that almost all the sugars and their analogous carbonyl compounds gave essentially the same ESR pattern in their reactions with a given amino acid and the only exceptions were observed in the cases of glyceraldehyde and dihydroxyacetone. These results could reasonably be interpreted by the following considerations. Namely, according to the above general

formula of the radical products, the structures of the radical products from glycolaldehyde, aldoses, and glyceraldehyde are shown as I, II, and III, respectively.



Here, one attempts to postulate that the coupling of the C-2 proton (in I) to the radical is equivalent to that of C-3 proton(s) (in II and III) and that of the C-4 proton could be neglected, where such equivalence in coupling to the free radical between two protons located at different carbons would be supported by the analogous situation in the polyacrylate radical (Harris et al., 1974). According to this postulation, glycolaldehyde and sugars have one proton at C-2 and C-3, respectively, while glyceraldehyde and dihydroxyacetone have two protons at C-3. This speculation might explain well the similarity and differences observed in their ESR spectral patterns. Further speculation on the structure of radical products seems difficult at present, but from the hyperfine structures of their ESR spectra it seems unlikely that the radicals locate at such simple enaminol products as were derived from condensation of equimolar amounts of sugar and amino acid and they probably have no further conjugated structure. It has also been known that the reaction of sugars with amino acids gave various heteroaromatic products such as pyrazine and pyrimidine derivatives, and some of them were known to have sugar residues (Tsuchida et al., 1973). Thus, it seems probable that the radicals are present in some products involving such conjugated systems and still remain the residues of either reactant.

In any event, the facts that such free radicals could easily be formed at the neutral aqueous system of sugars and amino acid even in the presence of air and that they existed as a fairly stable form are of great interest in elucidation of mechanisms of some quality changes such as browning and oxidation in food and biological systems.

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Racemization of Amino Acid Residues in Proteins and Poly(L-amino acids) during Roasting

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Casein, lysozyme, poly-L-α-alanine, poly(L-glutamic acid), and poly-L-lysine were heated in an electric roaster at 180–300° for 20 min under air or nitrogen and racemization of amino acid residues in these roasted materials was investigated mainly by capillary column gas chromatography. Aspartic acid, glutamic acid, alanine, and lysine residues were remarkably racemized, and the other amino acid residues except proline were also racemized to a considerable extent at higher temperatures. A gel filtration study on poly(L-glutamic acid) revealed that, on roasting, it was decomposed into fractions with various molecular weights and racemization of the glutamic acid residue proceeded more markedly in the lower molecular fractions. Free amino acids and oligopeptides formed in roasted casein were found to be mostly or completely racemized.

Chemical changes during heat processing of proteins contained in foods are important subjects in food chemistry and dietetics. However, only minor investigation has been done, especially as to chemical changes of proteins under roasting conditions. In previous papers, the authors studied the decomposition of amino acid residues during roasting of casein and lysozyme at 150–300° (Fujimaki *et al.*, 1972) and, furthermore, identified volatile and nonvolatile products formed in roasted casein (Kato *et al.*, 1972).

Bjarnason and Carpenter (1970) studied the changes in amino acid composition during heating of bovine plasma albumin at 115 or 145° for 27 hr and showed that isoleucine residue was partially racemized to form alloisoleucine at 145°. It is reasonably considered that, during roasting, racemization of amino acid residues in proteins also occurs in addition to their decomposition.

Neuberger (1948) reviewed racemization of free amino acids by heating in the presence of acid or alkali. Recently it has been reported that free amino acids are easily racemized in the neutral solution at 150–250° (Chibata *et al.*, 1973). Racemization of amino acids has also been studied from the view of peptide synthesis (Smart *et al.*, 1960; Williams and Young, 1963) and of elucidation of the lifetime of soil and fossil (Wehmiller and Hare, 1971). Racemization which occurs by alkali treatment of proteins is well known (Neuberger, 1948).

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