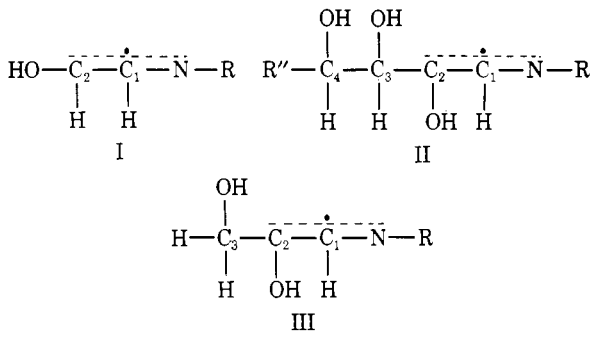
formula of the radical products, the structures of the radical products from glycolaldehyde, aldoses, and glyceraldehyde are shown as I, II, and III, respectively.



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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Table I. Degree of Racemization (%) of Each Amino Acid Residue in Casein on Roasting[a]

Amino acid[a]	Unheated	At 230° under air	At 250° under air	At 250° under N_2
Ala	3.0	24 (85)	66 (30)	66
Val	0	8.8 (82)	28 (31)	26
Ile	0	7.6 (66)	32 (17)	24
Leu	3.8	14 (76)	32 (24)	26
Pro	0	2.0 (76)	15 (25)	0
Asp	6.2	62 (70)	100 (18)	88
Phe	0	0 (76)	38 (28)	28
Glu	0	32 (72)	72 (19)	64

[a] Attempts to analyze quantitatively basic amino acids, hydroxy amino acids, tyrosine, and sulfur-containing amino acids were unsuccessful. The number in parentheses is the remaining ratio (%).

Table II. Degree of Racemization (%) of Each Amino Acid Residue in Lysozyme on Roasting[a]

Amino acid	Unheated	At 180° under air	At 230° under air	At 250° under N_2
Ala	4.0	6.8 (92)	24 (89)	48
Val	2.8	2.6 (92)	5.0 (86)	26
Ile	0	2.8 (95)	7.0 (80)	20
Leu	3.6	7.2 (92)	15 (73)	46
Pro	0	0 (90)	0 (64)	0
Asp	4.3	17 (91)	64 (70)	96
Phe	0	0 (98)	13 (66)	*b*
Glu	0	0 (90)	36 (69)	50

[a] Number in parentheses is the remaining ratio (%). [b] Neither D nor L was detected.

Table III. Degree of Racemization of Each Amino Acid Residue which Occurred by Roasting Poly(L-α-amino acids)

Poly-(L-amino acid)	Degree, % Unheated	Degree, % Heated	Remaining ratio, %
Poly-L-α-alanine			
At 250° under air	4.0	8.0	82
At 300° under air	4.0	14	47
Poly(L-glutamic acid)			
At 180° under air	0	20	*a*
At 210° under air	0	48	*a*
At 250° under air	0	100	48
At 250° under N_2	0	86	*a*
Poly-L-lysine			
At 230° under air	2.0	88	*a*

[a] Not analyzed.

The present study is focused on racemization of amino acid residues in proteins roasted at 180–300°. As protein samples, casein and lysozyme were used; lysozyme was selected on account of a pure polypeptide of which the structure had been clarified. Furthermore, as simpler models, some poly(L-amino acids) were also used. D- and L-amino acids in the acid hydrolysates of roasted samples were analyzed conveniently by gas chromatography on a capillary column according to the method reported by Nakaparksin *et al.* (1970).

MATERIALS AND METHODS

Proteins and Poly(L-amino acids). Proteins used were Hammarsten casein (Merck; moisture, 1.2%) and lysozyme (Seikagaku Kogyo Co. Ltd., 6× crystallized; moisture, 7.4%), and poly(L-amino acids) used were poly-L-α-alanine (Miles, mol wt 2675), poly(L-glutamic acid) (Miles, mol wt 7100) and poly-L-lysine HBr (Miles, mol wt 15,000). Poly-L-lysine HBr was treated with paper electrophoresis to obtain free poly-L-lysine.

Roasting Procedure. Proteins and poly(L-amino acids) (10–200 mg) were uniformly placed on the glass boat (3.5 × 2 cm), which had been cleaned with $K_2Cr_2O_7$–H_2SO_4 solution, and roasted at 180–300° for 20 min under air or nitrogen with the apparatus described in the previous paper (Fujimaki *et al.*, 1972), an electric roaster made by coiling nichrome wire around a quartz glass tube of 54 × 2.5 cm dimensions. The inner space of the roaster was heated at the rate of 60°/min to reach the intended temperature; air or nitrogen was blown at the rate of *ca.* 55 ml/min. Nitrogen was purified by 4% pyrogallol solution and reduced iron powder heated at 300° in order to remove oxygen.

Preparation of Volatile Derivatives of Amino Acids. Authentic D,L-amino acids were treated with 3 *N* HCl containing dried isopropyl alcohol and trifluoroacetic anhydride to prepare *N*-trifluoroacetyl-D,L-amino acid isopropyl esters (Nakaparksin *et al.*, 1970). Proteins and poly(L-amino acids) before and after roasting were hydrolyzed by 6 *N* HCl at 110° for 20 hr. These hydrolysates were concentrated, completely dried on P_2O_5, and then isopropylated and trifluoroacetylated in the similar manner as the preparation of authentic amino acid derivatives. These mixtures of *N*-trifluoroacetylamino acid isopropyl esters were analyzed in comparison with the retention time of authentic *N*-trifluoroacetyl-D- or -L-amino acid isopropyl ester by gas chromatography.

Gas Chromatography. Following are the gas chromatographic conditions applied: equipment, Shimadzu GC-5A; detector, hydrogen flame ionization; column, stainless steel capillary column (45 m × 0.5 mm) coated with trifluoroacetyl-L-valyl-L-valine cyclohexyl ester; column temperature, 110°; carrier gas, nitrogen at a flow rate of 20 ml/min.

Degree of Racemization. The quantity of each D- or L-amino acid derivative was calculated from peak area on the gas chromatogram. The degree of racemization was expressed as follows: racemization (%) = $2D/(D + L) \times 100$ (%), where D is the peak area of the D-amino acid derivative and L is the peak area of the L-amino acid derivative.

The degree of racemization of the isoleucine residue was calculated from the amount of D-alloisoleucine, because neither D-isoleucine nor L-alloisoleucine was detected.

Racemization of the lysine residue was determined by specific rotation at 589 mμ in 6 *N* HCl solution after acid hydrolysis of roasted poly-L-lysine.

Amino Acid Analyses. Proteins and poly-L-amino acids were hydrolyzed with 6 *N* HCl and the amounts of amino acids before and after roasting were determined by Hitachi Amino Acid Analyzer KLA-5. The remaining ratio of amino acid residues was shown as the per cent remaining after roasting to that present before roasting.

RESULTS

Racemization of Amino Acid Residues of Casein and Lysozyme during Roasting. Table I shows the results of analysis of the racemization and remaining ratio of each amino acid residue in casein roasted at 230 and 250° for 20 min. Since it had been reported that racemization of amino acid residues in proteins proceeded during acid hydrolysis (Neuberger, 1948), racemization of the acid hydrolysate of unheated casein was also analyzed.

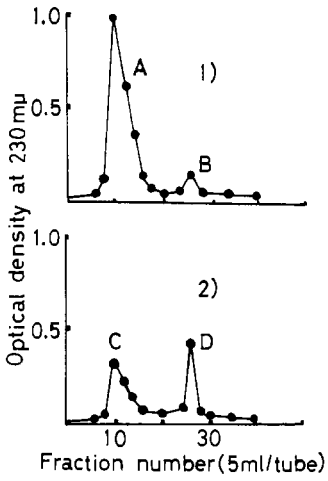

Figure 1. Gel filtration of the roasted poly(L-glutamic acid) on a Sephadex G-25 column: (1) roasted at 180°; (2) roasted at 210°; column, 2 × 50 cm; eluent, 0.02 *M* phosphate buffer solution (pH 7.0).

Table IV. Degree of Racemization (%) of Glutamic Acid and Each Fraction Obtained from Roasted Poly(L-glutamic acid) by Gel Filtration

	Roasting temp, °C	
Glutamic acid	210	68
	230	74
	250	100
Poly(L-glutamic acid)	180	20
	180 (A[a])	14
	180 (B[a])	50
	210	48
	210 (C[a])	19
	210 (D[a])	100

[a] Fraction obtained in Figure 1.

The remaining ratio of amino acid residues in casein roasted at 230° was 66–85% but racemization was observed in each amino acid residue except phenylalanine. Aspartic acid, glutamic acid, and alanine residues were especially strongly racemized. On roasting at 250° under air, racemization of each amino acid residue was more remarkable. The degree of racemization of alanine and glutamic acid reached around 70% and that of aspartic acid was 100%. Substituting the atmospheric medium by nitrogen, racemization of amino acid residues was restrained a little. The proline residue was not racemized under nitrogen.

Table II shows the racemization of amino acid residues in lysozyme roasted at 180, 230, and 250°. The aspartic acid residue was already racemized at 180°, in spite of the fact that the decomposition rate of that residue was less than 10%. On roasting lysozyme at 230°, alanine, aspartic acid, and glutamic acid residues were racemized in almost the same degree as in the case of casein. The proline residue was not racemized at three roasting temperatures.

Since isoleucine has two asymmetric carbon atoms, there are four isomers. In this experiment, however, the formation of D-alloisoleucine only was observed in roasted proteins and neither D-isoleucine nor L-alloisoleucine was detected with gas chromatography.

Rearranging the above experimental results, the order of racemization of amino acid residues in roasted casein and lysozyme is as follows: Asp > Glu > Ala > Phe, Leu, Ile, Val > Pro; any significant difference is not recognized between casein and lysozyme.

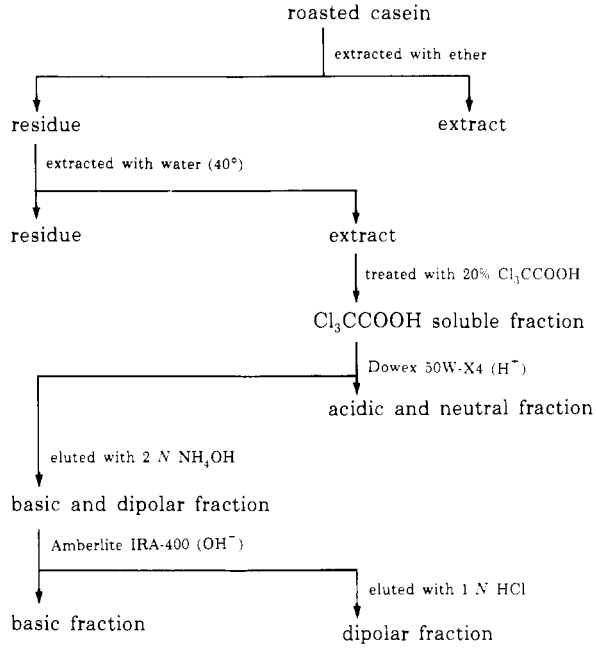

Figure 2. Fractionation of roasted casein using ion exchanger. Roasting conditions were: 20 g of casein was heated in the roasting apparatus at the rate of 60°/min and the heating was stopped when the temperature reached 200°.

Table V. Degree of Racemization (%) of Amino Acids before and after Acid Hydrolysis of the Dipolar Fraction Obtained in Figure 2

Amino acid	Free amino acid	Hydrolysate of the dipolar fraction
Ala	100	96
Val	34	28
Thr	*a*	*a*
Ile	94	68
Leu	88	78
Pro	78	84
Asp	96	100
Met	*a*	*a*
Phe	76	88
Glu	86	82

[a] Neither D nor L was detected.

Racemization of Amino Acid Residues of Poly(L-amino acids) during Roasting. Poly-L-α-alanine, poly(L-glutamic acid), and poly-L-lysine were roasted at 180–300° for 20 min under air or nitrogen. Racemization of roasted poly(L-amino acids) is shown in Table III. The remaining ratio of alanine residues after roasting at 300° was 47%, of which 14% was racemized. The remaining ratio of glutamic acid residues after roasting at 250° was also one-half, but the degree of racemization reached 100%. Glutamic acid residue was racemized more strongly at higher roasting temperatures. Racemization of this residue was slightly suppressed on roasting under nitrogen in comparison with air. The lysine residue also was remarkably racemized as well as glutamic acid.

In order to investigate the mechanism of racemization of amino acid residue on roasting proteins, the water-soluble fraction obtained from roasted poly(L-glutamic acid) was fractionated by gel permeation chromatography. Figure 1 shows the chromatogram of poly(L-glutamic acid)

roasted at 180 and 210°. Each fraction of peak was concentrated, dried up, and hydrolyzed by 6 *N* HCl. *N*-Trifluoroacetylgutamic acid isopropyl esters of these hydrolysates were prepared and analyzed. Table IV shows the racemization of A, B, C, and D fractions obtained in Figure 1 and also that of glutamic acid roasted at 210, 230, and 250°. From the results, it became clear that racemization of lower molecular peptide fractions proceeded more rapidly than that of higher molecular peptide fractions and, furthermore, roasted glutamic acid was easier to racemize than roasted poly(L-glutamic acid).

Racemization of Free Amino Acids and Oligopeptides in Roasted Casein. A dipolar fraction, which contained free amino acids and oligopeptides, was obtained from roasted casein as described in Figure 2. The fraction was converted into *N*-trifluoroacetylamino acid isopropyl esters and analyzed before and after acid hydrolysis. Table V shows the results. Each free amino acid except valine was racemized at a degree of 75–100%. Amino acid residues in oligopeptides were also found to racemize in almost the same degree. Free proline and proline residue in oligopeptides were racemized similarly to the other amino acids in contrast with the proline residue in proteins.

DISCUSSION

During roasting, proteins contained in foods, *e.g.* coffee, cookies, meat, and fish, should be chemically changeable accompanying heat denaturation. When pure proteins were roasted at 150–300°, amino acid residues, especially basic, β-hydroxy- and sulfur-containing amino acid residues, were decomposed (Fujimaki *et al.*, 1972). It has been proven in the present investigation that the amino acid residues were racemized simultaneously with their decomposition during roasting of proteins. Moreover, lower molecular peptides and free amino acids were more remarkably racemized, as shown in Tables IV and V.

The racemization will proceed by dissociation of the α-hydrogen atom attached to peptide linkage. Considering the experimental results shown in Tables I and III, atmospheric oxygen takes only a little part in the racemization. It is therefore assumed that the dissociation of α-hydrogen atom occurs mostly by heat and other factors such as the action of acid produced by the degradation of proteins. Meybeck and Windle (1969) have shown the formation of a radical by dissociation of the α-hydrogen atom in peptides irradiated with mercury vapor lamps. Moreover, Gomyo and Fujimaki (1970) have discussed the formation of a similar radical by dissociation of the α-hydrogen atom in the study of photosensitized oxidation of lysozyme. When the α-hydrogen atom is dissociated, a C=N double bond is formed in the peptide bond. The peptide bond is consequently broken and an α-keto acid is formed. The authors had observed the formation of some α-keto acids in roasted casein (Fujimaki *et al.*, 1972; Kato *et al.*, 1972). Dissociation of the α-hydrogen atom could occur also through enolization of the carbonyl group in another peptide bond, which would be promoted by the action of acid and/or base (Neuberger, 1948). Sato *et al.* (1970) and Sakieki and Mitsuno (1959) have reported that amino acids are racemized by heat in the existence of lower fatty acids. In the case of acidic amino acid or lysine residue, which has been found to racemize strongly, it is considered that the carboxyl or ε-amino group promotes the dissociation of the α-hydrogen atom. On the other hand, the formation of the oxazolone ring has been well known in the field of peptide synthesis (Goodman and McGahren, 1965). Therefore, concerning C-terminal amino acid residues formed by radical cleavage or hydrolysis, the formation of the oxazolone ring is considered as another mechanism of racemization. As already mentioned, since the L-isoleucine residue produces D-alloisoleucine only, dissociation of the β-hydrogen atom appears to be a difficult occurrence.

The findings described in this paper would be of importance in food science and dietetics, because the flavor of D-amino acids differs from that of L-amino acids (Kaneko, 1939) and the nutritive value of D- or racemized amino acids is known to be very low (Gibson and Wiseman, 1951). Consequently, we must notice the racemization as well as the decomposition of amino acid residues in proteins during heating.

The authors are now studying the racemization of proteins roasted with lipid and/or carbohydrate and also the racemization of some other foods.

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