Amino Acid Racemization in Heated and Alkali-Treated Proteins

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The racemization of 14 amino acids (7 nutritionally essential) has been measured in severely heated proteins, in stored and heated milk powders, and in alkali-treated casein. By means of deuterium labeling we have differentiated between the D isomers already present in the treated proteins and those formed during acid hydrolysis. In severely heated bovine plasma albumin and solvent-extracted chicken muscle (up to 27 h at 121 °C), only aspartic acid, cysteine, and serine showed any appreciable racemization. In stored and heated milk powders there was virtually no racemization except after very severe treatments (20 min at 230 °C), when aspartic acid and glutamic acid were partially racemized. In contrast, alkali treatment of casein (1 h at 80 °C in 0.2 or 1 M NaOH) caused extensive racemization of all amino acids. It was concluded that amino acid racemization has little influence on the nutritive value of severely heated or Maillardized proteins but that it is partly responsible for the nutritional losses induced by alkali treatment.

Proteins are the most reactive of the major food components, and during food processing, they can react with sugars, fats and their oxidation products, other proteins, polyphenols, and various food additives (Hurrell, 1980). These reactions influence both the organoleptic properties of the food and the nutritional quality of the proteins. Racemization of amino acids during processing is another phenomenon which can influence protein quality. This occurs most readily after alkaline treatments (Tannenbaum et al., 1970; Provansal et al., 1975; Masters and Friedman, 1979) but can occur to a lesser extent in acid conditions (Ikawa, 1964; Manning, 1970; Jacobsen et al., 1974) and during roasting of proteins (Hayase et al., 1973, 1975a), particularly in the presence of lipids or reducing sugars (Hayase et al., 1979).

Studies of amino acid racemization in food proteins in the past have been limited by the number of D-amino acids which could be detected. Masters and Friedman (1979) detected only seven and Hayase et al. (1973, 1975a, 1979) eight. Most of the essential amino acids were not included. In addition, racemization is measured after acid hydrolysis, yet, with few exceptions, the analytical data have not been corrected for the additional racemization induced by this step. This latter quantity depends on the nature of both the amino acid and the protein (Manning, 1970) but may be as high as 9% after 24-h hydrolysis in 6 M HCl (Liardon et al., 1981).

Recently, we developed a method for the corrected determination of D-amino acids in protein samples (Liardon et al., 1981). By means of deuterium labeling, we differentiate D-isomer molecules formed during the hydrolysis of the protein from those initially present in the sample. The method involves the GC separation of amino acid enantiomers on an optically active phase and their detection by selected ion mass spectrometry. D/L ratios down to 0.2% have been accurately measured.

We now report on the use of this procedure to study the racemization of amino acids after severe heating of proteins alone, after early and advanced Maillard reactions, and after alkaline treatment of casein. The racemization of 14 amino acids has been measured, including the essential amino acids lysine, methionine, cystine, threonine, valine, isoleucine, leucine, tyrosine, and phenylalanine.

MATERIALS AND METHODS

Heat Treatments. Bovine plasma albumin (BPA) and solvent-extracted chicken muscle samples were the same materials as used by Hurrell et al. (1976). Both materials were adjusted to 15% moisture and packed into glass ampules which were flushed with nitrogen before sealing and autoclaving for different times up to 27 h at 121 °C.

Spray-dried whole milk powder was packed in aluminum cans and stored for 9 weeks at 60 °C or at 70 °C. Other 5-g portions of the same material were heated in an oven, either in sealed glass bottles for 1 h at 120 °C or on a watch glass for 20 min at 230 °C.

Alkaline Treatments. Five grams of casein (type Hammarsten supplied by E. Merck, Darmstadt, West Germany) was dissolved in 100 mL of 0.2 or 1 M NaOH. The solutions were heated for 1 h at 80 °C. After being cooled, the treated protein was precipitated at pH 4.6–4.8 by the addn. of 1 M HCl, filtered under vacuum, freeze-dried, and ground. Alternatively, alkali-treated casein solutions were freeze-dried directly after heat treatment.

Amino Acid Analysis. Amino acids were estimated in duplicate in acid hydrolysates of test materials by ion-exchange chromatography using a Beckman Multichrom amino acid analyzer (Beckman Instruments, Munich, West Germany). Methionine and cystine were determined as methionine sulfone and cysteic acid, respectively, after preliminary performic acid oxidation (Moore, 1963). Nitrogen was determined by an automatic Kjeldahl procedure using the Kjeldfoss apparatus (Foss Electric, Hillerod, Denmark).

Determination of Amino Acid Enantiomers. The levels of amino acid enantiomers in the treated proteins were determined by the procedure of Liardon et al. (1981). This method differentiates, by means of deuterium labeling, those D isomers formed during acid hydrolysis from those already present in the protein. Amino acid racemization in concentrated acid involves the replacement of the α-hydrogen atom by a proton from the bulk. When a protein is hydrolyzed in deuterated hydrochloric acid, the amino acids that racemize are deuterated on the α-carbon atom and can be distinguished by mass spectroscopy from those molecules retaining their original configuration.

Protein samples (1–2 mg) were placed in vacuum reaction tubes (Pearce Chemical Co., Rockford, IL) together with 2 mL of 6 M DCl, made up from 1 mL of concentrated DCl and 1 mL of D_2O (Fluka, Buchs, Switzerland). Each sample was degassed several times under vacuum to remove oxygen. Ethyl mercaptan (50 μL) was then added as a reducing agent for converting cystine into cysteine and protecting methionine from oxidation. The tubes were sealed under vacuum and heated for 24 h at 110 °C in a heating block. Hydrochloric acid was then removed on a rotary evaporator and replaced by distilled water. The

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Table I. Racemization[a] of Amino Acids in Heated Chicken Musele Protein and Bovine Plasma Albumin (BPA)

amino acid	chicken muscle						BPA		
	0 h[c]	2 h[c]	4 h[c]	8 h[c]	16 h[c]	27 h[c]	0 h[c]	8 h[c]	27 h[c]
Ala	0	0.2 (0.04)	0.5 (0.04)	0.9 (0.1)	2.2 (0.1)	3.3 (0.1)	0	0.8 (0.04)	4.1 (0.04)
Val	0	0	0	0	0	0.4	0	0.3 (0.03)	0.4 (0.1)
Leu	0	0	0.1 (0.05)	0.3 (0.1)	0.6 (0.1)	0.8 (0.3)	0	0	1.2 (0.1)
Ile	0	0.9 (0.2)	0.3 (0.2)	0.9		1.4 (0.7)	1.8 (0.1)	1.9 (0.3)	2.7 (0.2)
Cys	0	0	0.9 (0.5)	2.6 (0.3)	6.0 (0.5)	6 (1)	0	29.1 (0.4)	32 (1)
Met	0	0	0	0	0	<0.5	0	0	<1
Phe	0	0.2 (0.1)	0.4 (0.1)	0.3 (0.2)	0.9 (0.03)	1.5 (0.02)	0	0.6 (0.1)	2.2 (0.1)
Lys	0	0	0	0.4 (0.05)	0.6 (0.2)	1.5 (0.3)	0	0.6 (0.2)	3.0 (0.1)
Asp	2.9 (0.4)	14.9 (0.3)	22.4 (0.6)	26.3 (1.4)	31 (2)	31 (1)	0	28.6 (0.5)	40 (1)
Glu	0	0	0.7 (0.03)	1.0 (0.1)	2.1 (0.1)	2.3 (0.1)	0	1.0 (0.01)	4.7 (0.02)
Ser	0	1.2 (0.1)	2.0 (0.2)	2.8 (0.3)	5.5 (0.3)	4.4 (0.1)	0	4.2 (0.5)	15 (1)
Thr	0	0	0.6 (0.1)		1.2 (0.1)	1.4 (0.1)	0	1.1 (0.2)	3.2 (0.3)
Tyr[b]	0.9 (0.4)	1.1 (0.1)	0.9 (0.2)	0.8 (0.4)	1.7 (0.4)	1.5 (0.1)	1.2 (0.2)	1.8 (0.2)	1.9 (0.6)

[a] Expressed as 100D/(D + L); each value is the average of three independent determinations; numbers in parentheses are standard deviations of the mean. [b] Racemization data for tyrosine include hydrolysis-induced racemization. [c] Heating time at 121 °C.

samples were transferred into conical reaction vials and dried under a stream of nitrogen. The elimination of water was completed by azeotropic entrainment with methylene chloride. Samples for amino acid determination were all prepared in triplicate.

In the first step of amino acid derivatization, 250 μL of acetyl chloride–2-propanol (20:100 v/v) and 50 μL of ethyl mercaptan were added to the dry residue, and the whole was kept for 30 min at 110 °C. The excess reagent was removed by evaporation under a stream of nitrogen. Then 50 μL of trifluoroacetic anhydride (TFAA) was added to the sample which was heated again for 10 min at 110 °C. After evaporation of the excess TFAA under nitrogen, the sample was finally dissolved in 200 μL of ethyl acetate. Alternatively, a third reaction step was performed for the complete derivatization of histidine. To this effect, the sample was treated with 50 μL of isobutyl chloroformate (IBCF) and maintained for 10 min at 110 °C.

The derivatized amino acids were separated on a 20 m × 0.2 mm i.d. glass capillary column coated with Chirasil-Val (Applied Science, State College, Pa), mounted in a Hewlett-Packard 5992 combined gas chromatograph/mass spectrometer. The column temperature was programmed from 70 to 200 °C at 4 °C/min. The injection port temperature was set at 200 °C. The carrier gas was helium at 3.5 mL/min; a 10:1 inlet split ratio was used. The mass spectrometer was run in multiple ion detection mode. The whole amino acid range was generally covered by analyzing each sample twice. A third run was performed with the samples in which histidine was determined. Peak surface areas were measured by postrun integration by means of the routine provided within the HP 5992 software package. The resulting data were further processed by a Basic computer program running on Hewlett-Packard 3354 Lab Automation System to calculate the amounts of D isomer in the sample before hydrolysis and the acid hydrolysis induced racemization. Of all protein amino acids, only proline and arginine could not be determined by this method. The determination of histidine, resulting from a later development, was only performed in the alkali-treated casein samples. As tryptophan is destroyed during the acid hydrolysis, this amino acid could not be detected.

RESULTS AND DISCUSSION

Racemization in Heated Protein. D-Amino acid formation on prolonged heating of protein alone was investigated with two model proteins: defatted chicken muscle and BPA. Protein samples were heated in sealed glass ampules for time periods ranging from 2 to 27 h at 121 °C

Table II. Changes in Amino Acid Composition[a] in Heated Chicken Muscle Protein and Heated Bovine Plasma Albumin (BPA)

amino acid	chicken muscle			BPA		
	unheated	8 h[b]	27 h[b]	unheated	8 h[b]	27 h[b]
Ala	6.4	91	93	5.8	94	99
Val	5.1	93	92	4.9	110	107
Leu	8.7	92	91	11.0	103	99
Ile	5.5	97	90	2.3	111	95
Met	2.9	100	100	0.9	94	100
Cys	1.2	78	65	7.1	40	23
Phe	4.9	91	92	6.3	102	105
Lys	9.7	93	92	12.2	91	92
Asp	10.5	89	91	10.0	98	98
Glu	14.7	93	92	13.2	103	101
Ser	4.4	98	80	3.8	98	88
Thr	4.7	106	89	5.5	89	82
Tyr	4.1	95	92	4.8	103	102
His	3.8	103	84	3.4	102	101
Pro	4.6	104	105	4.9	97	100
Arg	6.7	93	89	6.0	94	92
Gly	4.7	96	91	2.0	97	103

[a] Values for the unheated control samples are expressed as g/16 g of N and those for the heated samples as percentage of the corresponding control value. [b] Heating time at 121 °C.

in the presence of 15% water. The amounts of the various D-amino acids and the changes in amino acid composition are presented in Table I and II. The D-amino acid content is expressed as D/(D + L) (%). The degree of racemization is double this value. On the basis of the data in Table I, curves were established for the formation of D-amino acids. Figure 1 shows an example of such plots.

It was observed that the initial part of all curves was linear (and that this sometimes held for the whole period of heating). This part of the plot was used for calculating inversion rate constants k corresponding to the first-order reaction mechanism

$$\text{L-amino acid} \underset{k}{\overset{k}{\rightleftharpoons}} \text{D-amino acid}$$

using linear regression analysis as described previously (Liardon et al., 1981). The resulting values are reported in Table III. For comparison, this table also contains the inversion rate constants measured for free amino acids in 6 M hydrochloric acid at 110 °C (Liardon et al., 1981).

In several studies on free and bound amino acids, the differences in racemization rates among the various amino acids could be related to the inductive strength of the R

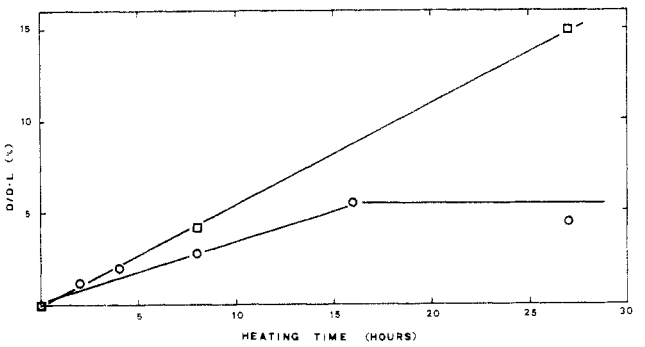

Figure 1. Formation of D-serine in chicken muscle (○) and BPA (□) heated at 121 °C.

Table III. Comparison of Amino Acid Inversion Rate Constants[a] in Heated Protein and in Acid Solution

amino acid	121 °C, 15% water: chicken muscle	121 °C, 15% water: BPA	110 °C 6 M HCl, free amino acid
Ala	36 (1.3)	45 (5)	13.2
Val	4	4	2.8
Leu	9 (0.8)	13 (4)	15.4
Ile	22 (8)	12 (2)	4.6
Cys	117 (9)	>1400	54
Met	<5	<10	17.6
Phe	15 (1.4)	24 (1)	11.8
Lys	16 (1.7)	33 (4)	13.4
Asp	1800 (210)	>1400	5.8
Glu	39 (4)	52 (5)	17.6
Ser	95 (6)	188 (11)	<1
Thr	<1	<1	<1

[a] Dimension: $10^{-8} \times s^{-1}$; numbers in parentheses are standard deviations.

substituents (Bada and Schroeder, 1975; Bada and Shou, 1980; Masters and Friedman, 1979). This conclusion was based on observed correlations between inversion rate constants and the substituent σ^* values, the best example of which has recently been presented by Friedman and Masters (1982). In all these studies, however, analytical data were only available for a limited number of amino acids. In a previous report, we presented a correlation analysis including the rate constants of 14 amino acids, as measured in strong mineral acid solution (Liardon and Jost, 1981). The results of that investigation suggested that the inductive strength of the side chain could not be considered in all cases as the main driving force in the racemization process.

A similar attempt was made here to evaluate the contribution of this factor in the racemization of heated protein. This is shown for chicken muscle in Figure 2 where the logarithm of the inversion rate constant of each amino acid (k_{AA}) normalized to that of alanine (k_{Ala}) is plotted against the inductive strength constant (σ^*) of the R substituent. The linear regression model calculated on this plot was characterized by a correlation coefficient of 0.607. On the other hand, when the linear fit computation was limited to the same six amino acids (Ala, Leu, Val, Phe, Asp, Glu) as included in the study by Friedman and Masters (1982), the correlation coefficient changed to 0.947. Similar results were obtained with the rate constants measured for BPA racemization. If it is assumed that σ^* values give a valid estimation of the R substituent inductive strength, these different observations confirmed that for several amino acids other factors have a more significant effect on the racemization rate constant.

In the case of bound acids, part of these factors may be related to the environment of each residue in the protein

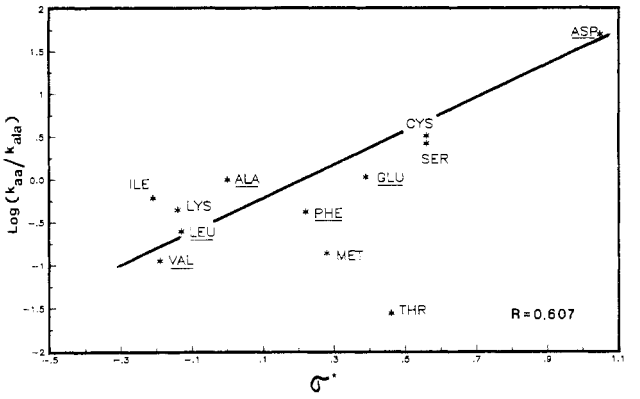

Figure 2. Relationship between the inductive strength (σ^*) of the amino acid side chain and the relative rate constant (k_{AA}/k_{Ala}) in heated chicken muscle. Values for σ^* are taken from Hansch and Leo (1979).

chain. Interaction between neighboring amino acids have been found to influence their racemization rate (Craig et al., 1954; Manning, 1970). Differences in racemization rate have already been reported for various proteins treated under the same conditions (Masters and Friedman, 1979; Liardon and Jost, 1981). The same observation can be made on the data in Table III. For half of the amino acids, the inversion rate constants in chicken muscle and BPA are significantly different. Further discrepancies can be found in the shape of the racemization curves. This is illustrated in Figure 1, where the formation of D-serine in chicken muscle is shown to level off around 5%, while in BPA it increases linearly at the double rate over the whole time range. Although both samples were protein mixtures, these different time courses confirm the importance of environmental factors in the racemization of certain amino acids.

The nonlinear kinetics of chicken muscle D-serine present some similarity with aspartic acid racemization in alkaline-treated collagen (Sol, 1978). In that case, a slope change in the racemization curve was explained by the progressive liberation of this amino acid, owing to protein partial hydrolysis, and the difference in racemization rate between free and bound aspartic acid. By analogy, serine behavior in chicken muscle might be the consequence of heat-induced modifications of the environmental factors affecting serine racemization, e.g., protein primary or secondary structure.

From the data in Table II, it appears that most amino acids do not suffer more than 10% destruction after 27 h at 121 °C. These findings and the greater decomposition rate of cysteine, threonine, and serine are consistent with the results of Bjarnason and Carpenter (1970). In contrast to a recent suggestion of Hayase et al. (1979), there does not seem to be any correlation between racemization and decomposition.

Racemization in Heated Milk Powders. The interaction of proteins and carbohydrates upon heating has long been known to proceed via Maillard reaction pathways. Because of the influence of this process on the organoleptic properties, the nutritional value, and the toxicology of food products, it has been widely studied (Hurrell, 1980). Recently, results presented by Hayase et al. (1979) have indicated that carbohydrates heated in the presence of proteins might also promote the racemization of amino acid residues. Depending on the extent of this phenomenon, it could further augment the detrimental effect on the nutritional quality of food proteins that the Maillard reaction already has (Hurrell and Carpenter, 1977a,b; Hurrell, 1980). Spray-dried milk was chosen as a model

Table IV. Racemization[a] of Amino Acids in Heated Milk Powder and Casein

amino acid	spray-dried milk 60 °C/ 9 weeks	70 °C/ 9 weeks	120 °C/ 1 h	230 °C/ 20 min	casein–glucose,[b] 230 °C/ 20 min
Ala	0	0	0	3.3 (0.3)	12
Val	0	0	0	0	6
Leu	0	0.3 (0.1)	0	1.9 (0.3)	6
Ile	0	0.2 (0.1)	0	0	4
Cys	0	0	0	0	
Met	0	0	0	0	
Phe	0	0	0	1.2 (0.2)	10
Lys	0	0	0	0	
Asp	0	3.7 (0.3)	1.2 (0.7)	31 (2)	34
Glu	0	0	0	9.2 (0.8)	20
Ser	0	0	0	0	
Thr	0	0	0	0	
Tyr	0	0	0	0	

[a] D/(D + L) (%); each value is the average of three independent determinations; numbers in parentheses are standard deviations of the mean. [b] Hayase et al. (1979); values corrected for hydrolysis-induced racemization by subtracting the level of racemization in the untreated sample.

Table V. Changes in Amino Acid Composition[a] in Heated Spray-Dried Milk

amino acid	untreated	60 °C/ 9 weeks	70 °C/ 9 weeks	120 °C/ 1 h	230 °C/ 20 min
Ala	3.6	94	106	103	93
Val	7.3	95	101	96	84
Leu	10.5	96	108	108	92
Ile	5.3	101	110	109	92
Cys	0.9	106	88	88	65
Met	3.0	93	83	113	42
Phe	5.2	95	97	103	94
Lys	8.5	83	17	21	13
Asp	8.6	98	102	96	79
Glu	22.4	108	110	99	85
Ser	5.9	104	104	102	32
Thr	4.9	100	102	98	51
Tyr	5.7	96	91	95	74
His	3.0	102	61	41	36
Pro	11.5	96	98	93	81
Arg	4.1	91	17	28	0
Gly	2.1	102	107	102	93

[a] Values for untreated control samples are expressed as g/16 g of N and those for the heated samples as precentages of the control value.

system to verify the influence of sugars on amino acid racemization. This product was subjected to various treatments, viz., 9-week storage at 60 and 70 °C, 1-h heating at 120 °C, and 20-min roasting at 230 °C.

As previously reported by Hurrell (1980), storage of this milk powder at 60 °C was an example of early Maillard damage. After 9 weeks of storage, the product still retained its natural color even though 40% of the lysine units were bound as lactulosyllysine. At 70 °C, advanced Maillard reactions took place and the product became dark brown. After 9 weeks of storage, no lactulosyllysine remained and 88% of the lysine units were not recoverable on acid hydrolysis. The powders heated for 1 h at 120 °C and roasted for 20 min at 230 °C were also examples of advanced Maillard damage. The levels of D-amino acids in these materials and their amino acid composition are shown in Tables IV and V. For comparison, Table IV also includes racemization data reported by Hayase et al. (1979) for a 1:1 mixture of casein and glucose after 20-min roasting at 230 °C.

No racemization at all was found in the proteins of spray-dried milk after storage at 60 °C (early Maillard reactions). Similarly, no significant racemization was found after the relatively severe Maillard reaction treatments of 9 weeks at 70 °C and 1 h at 120 °C. Under these conditions, there were large losses of lysine, arginine, and histidine, all three amino acids having a free functional group able to react with reducing sugars. There seemed therefore to be no correlation between the Maillard processes and racemization of amino acid residues. In roasted dried milk, only aspartic acid and glutamic acid presented a sizable racemization rate, yet in addition to the large losses of lysine, arginine, and histidine, serine, threonine, methionine, and cysteine were also considerably destroyed.

These results differ from those of Hayase et al. (1979), who used a casein–glucose system. Glucose is more reactive than lactose in Maillard reactions (Lewis and Lea, 1950). It appears that the promotion of amino acid inversion by reaction with reducing sugars is not a general rule, and further experiments will be necessary to determine how far this depends on the nature of the sugar.

Our results, however, indicate that under normal food processing conditions racemization of amino acids will have little or no influence on the nutritional quality of Maillardized proteins. This is largely determined by the loss of the essential amino acid lysine and, under more severe heating conditions, the destruction of other essential amino acids and the formation of cross-linkages within and between the protein chains which can greatly reduce protein digestibility (Hurrell and Carpenter, 1977b).

Table VI. Racemization[a] of Amino Acids in Alkali-Treated Casein[b]

amino acid	0.2 M[c] T[d]	0.2 M[c] P[d]	1.0 M[c] T	1.0 M[c] P
Ala	20 (1)	20.1 (0.1)	42 (1)	37
Val	3.7 (0.4)	3.1 (0.3)	18.0 (0.4)	12.9 (0.4)
Leu	9.0 (0.8)	9.4 (0.2)	34.3 (0.7)	27.1 (0.8)
Ile	3.7 (0.3)	5.4 (0.4)	29.9 (0.4)	20 (3)
Cys	n.d.[e]	n.d.	n.d.	n.d.
Met	30 (2)	31.2 (0.2)	43 (1)	39.8 (0.8)
Phe	31.0 (0.6)	28 (1)	44 (2)	39 (2)
Lys	13 (3)	14.2 (0.3)	41 (1)	31 (3)
Asp	36.9 (0.1)	35 (1)	51 (1)	35 (4)
Glu	28 (1)	26.9 (0.7)	42 (2)	38.1 (0.5)
Ser	43.9 (0.7)	47 (2)	54 (4)	51 (3)
Thr	28 (1)	29 (1)	34 (4)	32 (1)
Tyr	14 (2)	17 (1)	49 (1)	36 (1)
His	36 (1)	10 (1)	49 (2)	35 (1)

[a] D/(D + L) (%); each value is the average of three independent determinations; numbers in parentheses are standard deviations of the mean. [b] 1 h at 80 °C in NaOH solution. [c] Alkali concentration. [d] T, total sample; P, precipitable fraction. [e] Not detected.

Racemization in Alkali-Treated Protein. Previous studies of racemization in alkali-treated proteins have generally been concerned with the determination of a limited number of amino acids (Hill and Leach, 1964; Geschwind and Li, 1964; Pickering and Li, 1964; Pollock and Frommhagen, 1968; Tannenbaum et al., 1970; Provansal et al., 1975; Masters and Friedman, 1979). A better overview of the phenomenon is obtained by using the present analytical method. Samples of casein were heated for 1 h at 80 °C in 0.2 and 1 M NaOH. Following neutralization with HCl, the remaining protein was recovered by precipitation. Alternatively, the whole sample was lyophilized. The amounts of D enantiomers and the changes in amino acid composition are presented in Tables VI and VII.

These determinations have confirmed that alkaline solubilization is the most effective treatment for promoting amino acid racemization. Considering the freeze-dried

Table VII. Changes in Amino Acid Composition[a] of Casein after Alkaline Treatment[b]

amino acid	untreated	0.2 M[c]		1 M[c]	
		T[d]	P[d]	T	P
Ala	3.1	103	147	116	137
Val	6.3	108	130	116	124
Leu	9.9	92	134	101	144
Ile	5.3	91	108	80	82
Cys	0.5	58	29	77	38
Met	2.9	97	134	95	121
Phe	5.5	95	129	104	154
Lys	8.3	75	76	69	73
Asp	7.2	103	108	110	108
Glu	23.0	104	110	112	112
Ser	5.9	46	42	32	19
Thr	4.3	67	52	23	21
Tyr	6.0	95	183	105	202
His	2.9	97	119	76	122
Pro	10.7	106	116	114	120
Arg	3.8	76	111	38	71
Gly	1.8	139	172	192	156

[a] Values for the untreated casein are expressed as g/16 g of N and those for the treated samples as percentages of the corresponding control value. [b] 1 h at 80 °C in NaOH solution. [c] Alkali concentration. [d] T, total sample; P, precipitable fraction.

total sample (Table VI), after 1 h at 80 °C in 1 M NaOH, aspartic acid, serine, tyrosine, and histidine were completely racemized. More than 40% of alanine, methionine, phenyl alanine, lysine, and glutamic acid were also found to be present as the D enantiomer. Under our conditions, valine, isoleucine, leucine, and threonine were the least racemized. Masters and Friedman (1979) heated casein for 3 h at 65 °C in 0.1 M NaOH. They reported the following relative order of racemization: aspartic acid > phenylalanine > glutamic acid > alanine > leucine > valine. Aspartic acid racemized almost 9 times faster than valine.

Later on, the same authors (Friedman and Masters, 1982) found a close correlation between the relative inversion rates of these amino acids and the R substituent σ^* values. Applying the same approach to our data gave a very similar result: the correlation coefficient (r) for the same six amino acids was 0.966. On the other hand, the correlation was less satisfactory when all amino acids were included in the computation (r = 0.881).

Our results (Table VI) also show differences in D-amino acid contents between the total freeze-dried sample and the precipitable fraction. Racemization was generally faster in the partially degraded whole sample than in the precipitated protein. The same observation has been made with roasting-induced racemization in casein (Hayase et al., 1975).

Turning to the total amino acid analysis (Table VII), from the results for the total freeze-dried sample it would appear that serine, threonine, arginine, and cysteine are the most sensitive amino acids to modifications during alkali treatment. Considerably less of these amino acids, as well as some reductions in lysine, histidine, and isoleucine, was detected in the alkali-treated samples. The loss of isoleucine is consistent with its racemization to *allo*-isoleucine. These results are in agreement with the findings of Provansal et al. (1975). The apparent increase in glycine is probably due, at least in part, to lanthionine eluting at the same time as the glycine peak during ion-exchange chromatography (Provansal et al., 1975). Lanthionine is formed by reaction of cysteine with dehydroalanine which itself is formed by the degradation of cysteine or serine. The apparent increase in glycine, however, appeared to be greater than could be accounted for by the loss of cysteine.

Nutritional Considerations. Racemization of amino acids in proteins heated alone or during Maillard reactions appears to be of little importance nutritionally in food products. During alkali treatments, however, racemization appears to be at least partly responsible for the observed detrimental effect of these processes on the nutritive value of proteins. The loss of nutritive value during alkali treatment of proteins has been demonstrated in rats (De Groot and Slump, 1969). It is due to the combined effect of a reduction in protein digestibility, the destruction of certain essential amino acids, and the poor utilization of certain essential D-amino acids which are formed during the treatment (Hayashi and Kameda, 1980). In addition, D-serine has been reported to be nephrotoxic (Ganote et al., 1974).

The loss of protein digestibility is partly due to the formation of cross-linkages, such as lysinoalanine and lanthionine, and partly due to the loss of certain sites of enzyme attack because of amino acid destruction. But, more importantly, according to Hayashi and Kameda (1980), it is because the D-amino acids formed are not available for enzyme attack. In other words, the formation of L–D, D–L, or D–D peptides bonds would resist attack by proteolytic enzymes (Friedman et al., 1981). A reduction in digestibility of proteins severely heated alone is presumably due to similar causes (Hayase et al., 1975b).

The absorption of D-amino acids is also much slower than for the corresponding L form (Gibson and Wiseman, 1951), and even if digested and absorbed, most D isomers of essential amino acids are not utilized by man. In his review on the utilization of D-amino acids, Berg (1959) reported that, in man, D-lysine, D-threonine, D-tryptophan, D-leucine, D-isoleucine, and D-valine were not utilized. D-Phenylalanine could partially replace L-phenylalanine, and of the essential amino acids, only D-methionine was as effective as L-methionine in maintaining N balance. Kies et al. (1975), Zezulka and Calloway (1976), and Printen et al. (1979), however, have subsequently reported that D-methionine is also poorly utilized by man.

The rat, on the other hand, appears to be capable of completely utilizing D-methionine and D-tryptophan for growth and to partially utilize D-histidine, D-phenylalanine, and D-tyrosine. D-Valine and D-leucine are poorly utilized, and the D-isomers of lysine, threonine, and cystine are not used at all (Berg, 1959; Ohara et al., 1980a). The utilization of D-amino acids is explained by an oxidative deamination in the liver or kidney to the corresponding α-keto acid, followed by a L-specific reamination (Berg, 1959; Ohara et al., 1980b). Poor utilization in man is probably due to the lack of D-amino acid oxidases.

Commercial treatment of food proteins often includes heating in alkaline solution, and Friedman et al. (1981) have recently reported 9–17% D/(D + L) aspartic acid in commercial alkali-treated products containing soy protein or sodium caseinate. Although cross-link formation and amino acid destruction are also involved, racemization of amino acids in such products would be expected to contribute to some decrease in protein digestibility and to some loss in availability of most essential amino acids.

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Registry No. L-Ala, 56-41-7; L-Val, 72-18-4; L-Leu, 61-90-5; L-Ile, 73-32-5; L-Cys, 52-90-4; L-Met, 63-68-3; L-Phe, 63-91-2; L-Lys,

56-87-1; L-Asp, 56-84-8; L-Glu, 56-86-0; L-Ser, 56-45-1; L-Thr, 72-19-5; L-Tyr, 60-18-4; L-His, 71-00-1; L-Pro, 147-85-3; L-Arg, 74-79-3; Gly, 56-40-6; NaOH, 1310-73-2.

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Effect of Pre Heat Treatment on Tryptic Hydrolysis of Maillard-Reacted Ovalbumin

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Tryptic hydrolysis of a pre-heat-treated ovalbumin–glucose complex (OVG) was compared with that of pre-heat-treated ovalbumin (OV). The ovalbumin–glucose (7:3 in weight) mixture was stored at 50 °C and 65% relative humidity for a defined time (2–10 days), solubilized, and then freeze-dried. The level of hydrolysis after preheating of samples was measured with two methods, that is, determination of trichloroacetic acid precipitable protein concentration and calculation of ratio in unhydrolyzed and hydrolyzed fraction areas on a Sephadex G-75 column chromatographic pattern. Stored OVG hydrolysis was better than for OV and unstored OVG, although the number of free amino groups in stored OVG decreased with storage. Stored OVGs were irreversibly unfolded by heating (100 °C, 10 min) as indicated in the CD spectra but almost never aggregated to a high particle weight compound like the heat-treated OV. These results suggest that good tryptic hydrolysis of stored OVG depends on a heat-denatured conformation of OVG and reduced formation of aggregates.

The nutritional problems with food proteins induced by the Maillard reaction have been well investigated (Hurrell and Carpenter, 1977, 1981). The reaction is conventionally divided into early, advanced, and final stages (Hodge, 1953; Ellis, 1959; Reynolds, 1963, 1965; Mauron, 1981) which induce a different type of nutritional damage. In the stage after the early Maillard reaction, it is well-known that the formation of colored and high cross-linking protein–carbohydrate polymers having high particle weight causes a lowering of solubility, digestibility, and nutritional value of the reacted proteins (Mauron, 1970; Clark and Tannenbaum, 1974). On the other hand, different results are obtained from the nutritional estimation of protein in the early Maillard reaction. The amino–carbonyl condensation

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