Amino Acid Analyzer Studies of the Products of Peroxide Oxidation of Cystine, Lanthionine, and Homocystine

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Mixtures of several sulfur amino acid oxidation products were formed in the oxidation of cystine, lanthionine, or homocystine by aqueous hydrogen peroxide in the presence of hydrochloric acid. After 10 min at 100 °C in 6% H_2O_2 and 0.8 N HCl, cysteic acid was the main oxidation product of cystine; lanthionine sulfoxide, lanthionine sulfone, and unidentified products were also formed. In a similar oxidation of lanthionine, lanthionine sulfoxide was the most abundant product; cysteic acid, lanthionine sulfone, and unidentified products were also formed. An identical oxidation of homocystine afforded homolanthionine sulfoxide as the main product; homolanthionine sulfone and homocysteic acid were also formed. When homocystine was oxidized at 50 °C, homolanthionine was the principal product. However, when the HCl concentration was increased to 4.8 N, homocysteic acid and homolanthionine sulfone were the main products. Sulfoxide products were distinguished by reduction with dimethyl sulfide in the presence of HCl. Thus, they were converted into their corresponding sulfides and shifted to new chromatographic locations. These desulfurizations by hydrogen peroxide in the presence of HCl are discussed in relation to known chemical changes of amino acids, including lanthionine formation, which occur in alkali-treated foods and to the use of peroxide in food technology.

The oxidation of the sulfur amino acids of proteins by peroxide reagents is important because hydrogen peroxide is extensively used in the food industry (Guy et al., 1968; Slump and Schreuder, 1973) and the sulfur amino acids limit the protein nutritional value of many foods and feeds (Swendseid and Wang, 1970). The peroxide reagent performic acid, originally introduced into protein chemistry by Sanger (1949), is used prior to hydrolysis of proteins for amino acid analysis to oxidize these labile amino acids into acid-stable forms (more specifically, to oxidize cysteine and cystine to cysteic acid and to oxidize methionine and methionine sulfoxide to methionine sulfone). The modified performic acid procedure of Moore (1963) was designed to minimize sulfur amino acid losses due to over-oxidation by a final addition of HBr to destroy the excess performic acid. On the other hand, the presence of halides during oxidation has been avoided since peroxides convert them into halogens which promote over-oxidation (see Hirs, 1967). Since chlorides cannot be avoided during peroxide oxidation of many food and feed materials for sulfur amino acid analysis, a knowledge of the sulfur amino acid oxidation products which are produced by peroxide in the presence of HCl would contribute to an understanding of the sulfur amino acid analysis of foods, as well as to an understanding of the chemical changes produced by peroxide used in food processing.

Our current investigation of the oxidation products of cystine, lanthionine, and homocystine (see formulae in Table I) and the conditions which affect these oxidations was stimulated by the report of Clopath and McCully (1976) that homolanthionine sulfoxide and sulfone were formed by oxidation of homocystine by H_2O_2–HCl mixtures. Our interest in oxidation products of sulfur amino acids was further whetted by the reported growth hormone activity of homocysteic acid in hypophysectomized rats (Clopath et al., 1976) and by a reported experimentally induced taurine deficiency in the cat (Hayes, 1976). Although methionine and cystine have been considered to fully supply the sulfur amino acid requirements of animals, the amino sulfonic acids such as taurine and homocysteic acid deserve further nutritional study.

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EXPERIMENTAL SECTION

Materials. Amino acids and other chemicals were purchased or obtained as noted. The L-cystine (H. M. Chemical Co.), although purchased many years ago, had no detectable amino acid contaminants. The *meso*-lanthionine, prepared over 30 years ago by Dr. Millard J. Horn (Horn et al., 1941), contained about 10% of the DL isomer and traces of a few other amino acids. No appreciable impurities were detectable by amino acid analysis of the following products which were used: DL-cystine, Sigma; L-homocystine, Calbiochem; DL-homocystine, either Sigma or General Biochemicals. (Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.)

Amino Acid Analyses. A Phoenix Biolyzer, Model 3000, was used for quantitative amino acid analyses, by referenced procedures (Lipton and Bodwell, 1973), except for the changes noted. Because of its toxicity, we have discontinued use of the methyl Cellosolve ninhydrin reagent and replaced it with the dimethyl sulfoxide ninhydrin reagent of Moore (1968). Product distributions within the oxidation mixtures were calculated from manually measured peak areas of the acidic and neutral region by assuming that all amino acid components had the same peak area per mole; yields were thus only approximate. (Analyses of the oxidation mixtures for basic amino acids indicated that ammonia was the only significant detectable basic product.) Products were identified chromatographically but unambiguous structural assignments would require their individual separation.

Peroxide Oxidation. The sulfur amino acid (0.1 mmol) was transferred into the bottom of a 12-mL graduated conical centrifuge tube by use of Glassine weighing paper (Eli Lilly & Co.). A 0.20 mL volume of aqueous HCl (either 1 or 6 N) was added by pipet and the tube was warmed briefly to partly dissolve the amino acid (the insoluble portion dissolved during the oxidation). Oxidation was initiated by accurately adding 0.05 mL of 30% H_2O_2 from a serological pipet with a drawn-out tip and then immediately incubating the tube in a water bath for a 10-min reaction time. The use of peroxide and aqueous HCl in a 4:1 volume ratio resulted in a 6% H_2O_2 concentration. When 1 N HCl was used, the HCl concentration was 0.8 N; with 6 N HCl, the HCl concentration was 4.8 N.

Table I. Structures of Some Sulfur Amino Acids

Cystine	$HOOCCH(NH_2)CH_2SSCH_2CH(NH_2)COOH$
Homocystine	$HOOCCH(NH_2)CH_2CH_2SSCH_2CH_2CH(NH_2)COOH$
Lanthionine	$HOOCCH(NH_2)CH_2SCH_2CH(NH_2)COOH$
Homolanthionine	$HOOCCH(NH_2)CH_2CH_2SCH_2CH_2CH(NH_2)COOH$
Lanthionine sulfoxide	$HOOCCH(NH_2)CH_2S(O)CH_2CH(NH_2)COOH$
Lanthionine sulfone	$HOOCCH(NH_2)CH_2S(O_2)CH_2CH(NH_2)COOH$
Homolanthionine sulfoxide	$HOOCCH(NH_2)CH_2CH_2S(O)CH_2CH_2CH(NH_2)COOH$
Homolanthionine sulfone	$HOOCCH(NH_2)CH_2CH_2S(O_2)CH_2CH_2CH(NH_2)COOH$
Cysteic acid	$HOOCCH(NH_2)CH_2SO_3H$
Homocysteic acid	$HOOCCH(NH_2)CH_2CH_2SO_3H$

Reaction temperature was either 50 or 100 °C. The reactions were stopped by immersion in an ice bath and the volume was adjusted to 1.0 mL with water. By use of a Drummond microliter pipet, a 10 μL volume of the reaction mixture, equal to 1 μmol of amino acid, was then immediately placed on the 60 cm column of the amino acid analyzer for separation of the acidic and neutral amino acids. This deliberate use of four times the usual level of amino acid was intended to magnify minor products. Oxidations of L- and DL-cystine, *meso*-lanthionine, and L- and DL-homocystine were done. The usual or "standard" conditions of oxidation were for 10 min at 100 °C in 6% H_2O_2 and 0.8 N HCl. A few oxidations of L-methionine and L-cysteine were also carried out under the standard conditions.

Reduction of Sulfoxides by Dimethyl Sulfide. Dimethyl sulfide was used as a reducing agent because we had observed (Lipton and Bodwell, 1976) that this volatile sulfide reduced methionine sulfoxide to methionine. A measured volume (0.10–0.20 mL of the 1.0 mL total volume) of the peroxide-oxidized amino acid solution (see previous section) was pipetted into an ice-cooled 10-mL hydrolysis tube (Scientific Glass No. JT-7240), an equal volume of 12 N HCl was added and finally a volume of liquid dimethyl sulfide equal to the combined aqueous volume was added. The tube was quickly sealed by use of a metal clamp and an O-ring joint attached to a glass stopcock adapter. The mixture was heated for 3 min in a boiling water bath and then the stopcock was momentarily opened to allow escape of the excess dimethyl sulfide (bp 38 °C). The sample was cooled in an ice bath and immediately placed on the 60-cm resin column of the amino acid analyzer.

RESULTS

Cystine Oxidation. The ion-exchange chromatograms (Figure 1) demonstrate the complexity of the mixtures of amino acid oxidation products. As shown in Figure 1A, the oxidation of L-cystine under the standard conditions afforded cysteic acid as the main product (ca. 75% of the total; authentic cysteic acid had an identical chromatographic behavior). Lanthionine sulfoxide was the second largest peak (about 10% of the total amino acids) on the basis of the reduction with dimethyl sulfide (Figure 1B). The shift caused by reduction of the sulfoxide was in accordance with the reduction of lanthionine sulfoxide to L-lanthionine. If this product were instead a sulfoxide of cystine, its reduction would have increased the cystine peak. The peak designated as lanthionine sulfone was so assigned since it was not reduced by dimethyl sulfide. The other peaks constituted 1–5% of the total and were not identified (the peak just ahead of cystine was probably *meso*-cystine). Oxidation of DL-cystine under the standard conditions (Figure 1C) gave a similar mixture of products, but the sulfoxide appeared as a triple-headed peak which was at the same location as the L-sulfoxide. Reduction with dimethyl sulfide lowered this triple-headed peak (a mixture of two *meso*- and one DL-sulfoxide) and approximately

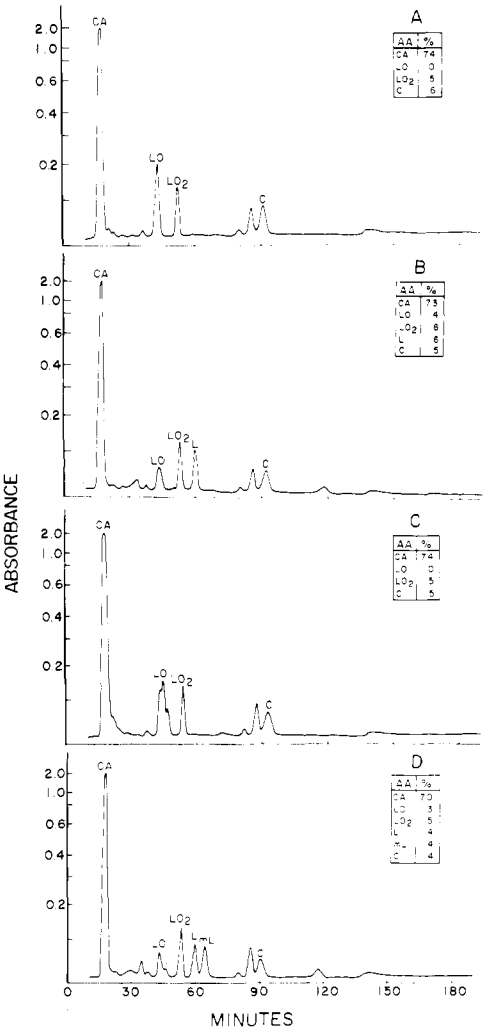

Figure 1. Amino acid analyzer chromatograms of acidic and neutral amino acids from the peroxide oxidation of L-cystine and DL-cystine: chromatogram A, oxidation of L-cystine was for 10 min at 100 °C in 6% H_2O_2 and 0.8 N HCl; chromatogram B, L-cystine was oxidized as in A and then reduced with dimethyl sulfide (see text); chromatogram C, same as A except DL-cystine was used; chromatogram D, same as B except DL-cystine was used. Abbreviations: CA, cysteic acid; L, L- or DL-lanthionine; mL, *meso*-lanthionine; LO, lanthionine sulfoxide; LO_2, lanthionine sulfone; C, cystine; AA, amino acid; %, mole percentage (see text).

equal sized peaks of DL- and *meso*-lanthionine appeared in the reduced mixture (Figure 1D).

Lanthionine Oxidation. In Figure 2, 2A shows that the untreated *meso*-lanthionine had about a 10% contaminant of DL-lanthionine. Chromatogram B illustrates the mixture of oxidation products which were obtained from *meso*-lanthionine under the standard conditions. The cysteic acid peak was accompanied by three sizable and several lesser peaks. The assignments were based on the reduction with dimethyl sulfide (Figure 2C). The two large

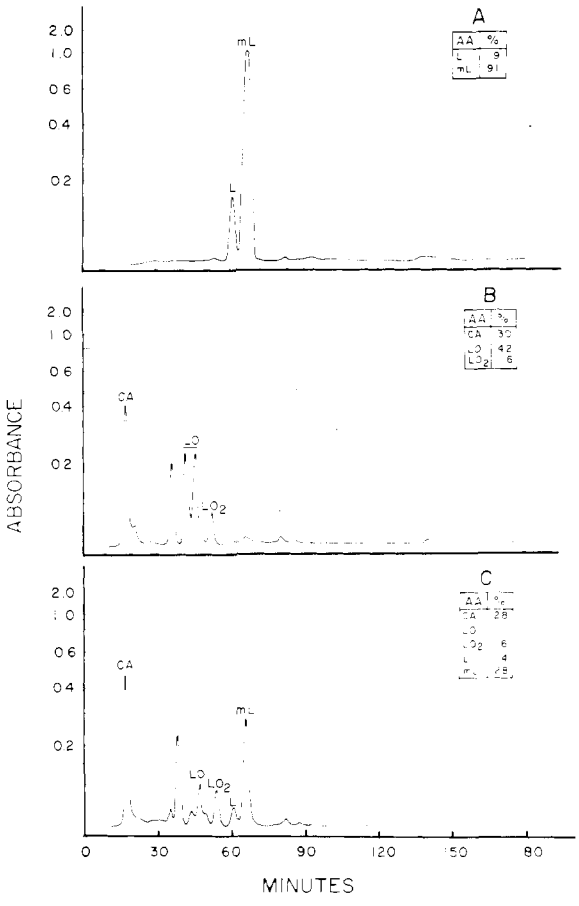

Figure 2. Amino acid analyzer chromatograms of acid and neutral amino acids which resulted from the peroxide oxidation of *meso*-lanthionine: chromatogram A, untreated *meso*-lanthionine (see text); chromatogram B, oxidation was for 10 min at 100 °C in 6% H_2O_2 and 0.8 N HCl; chromatogram C, *meso*-lanthionine was oxidized as in B and then reduced with dimethyl sulfide (see text). Abbreviations: same as in Figure 1.

sulfoxide peaks were greatly diminished by reduction and at the same time both *meso*- and DL-lanthionine peaks reappeared. The large peak (ca. 17%) just ahead of the sulfoxide peaks was not identified.

Homocystine Oxidation. Chromatogram A of Figure 3a, illustrates the mixture of products from the oxidation of L-homocystine under the standard conditions. These products included homocysteic acid (ca. 27% of the total), homolanthionine sulfoxide (ca. 36% of the total), homolanthionine sulfone, and several other amino acids. The basis of the homolanthionine sulfoxide assignment was the dimethyl sulfide reduction (Figure 3B). Disappearance of the sulfoxide and appearance of the large homolanthionine peak at the much later norleucine location verified this assignment. This confirmed the reported location of homolanthionine (Perry et al., 1966; an authentic sample of homolanthionine was not compared in our laboratory). If the sulfoxide were a disulfide derivative, reduction would have regenerated homocystine, which was eluted about 40 min after homolanthionine. Since a NaOH column regenerant was used in our autoanalyzer just after the elution of phenylalanine, homocystine ordinarily was not seen in our chromatograms.

Chromatograms C and D correspond respectively to A and B of Figure 3a, except that DL-homocystine was used instead of L-homocystine. The main difference was the resolution of the sulfoxide into three peaks (two *meso*- and one DL-sulfoxide). All three were reduced by dimethyl sulfide and shifted to the homolanthionine location (chromatogram D). Our results confirmed those of Clopath and McCully (1976). They attributed the sulfoxide structure to the three peaks on the basis of their subsequent oxidation by permanganate to homolanthionine sulfone which they crystallized and characterized.

Chromatogram E (Figure 3b) illustrates that homolanthionine formation preceded sulfoxide formation when the temperature of oxidation was the milder 50 °C. Chromatogram F demonstrates that with 4.8 N HCl the main products were homocysteic acid and homolanthionine sulfone. Chromatogram G illustrates that by omitting dimethyl sulfide, the higher HCl concentration used in the reduction procedure caused oxidation of the sulfoxide to the sulfone with no formation of free homolanthionine. The huge excess of dimethyl sulfide used in the normal reduction procedure eliminated excess peroxide.

The experiments on the oxidation of L-methionine indicated that the only product was methionine sulfoxide under the standard oxidation conditions. In the case of oxidation of L-cysteine, a mixture of products was obtained similar to those from oxidation of L-cystine. (These experiments are not illustrated by chromatograms.)

DISCUSSION

The chemistry of the sulfide amino acid, lanthionine, and the mechanism of its formation from the cystine of proteins under alkaline conditions (see citations in Friedman, 1973) have been followed with special interest in our laboratory where this amino acid was first correctly characterized and named lanthionine [Horn et al., 1941; 1942a,b; the structure was verified by synthesis (du Vigneaud and Brown, 1941); L-lanthionine derivatives were synthesized by selective desulfurization by tris(diethylamino)phosphine (Harpp and Gleason, 1971)]. Lanthionine had been previously reported (Küster and Irion, 1929) as an artifact of the alkaline treatment of wool but it was incorrectly identified. Lanthionine occurs naturally as a free amino acid in chick embryo (Sloane and Untch, 1966) and in plant pollen (Rossetti, 1966), as a constituent of insect haemolymph (Rao et al., 1967), and in the antibiotics subtilisin (Lewis and Snell, 1951) and nisin (Gross and Morell, 1970).

Our investigation confirmed the conclusions of Clopath and McCully (1976) that the sulfoxide and sulfone of homolanthionine were products of similarly oxidized homocystine. We used a 10-min instead of 1-h oxidation time with the same proportions of amino acid, H_2O_2, and HCl. Our amino acid analyses were of the crude reaction solutions instead of the ethanol-precipitated fraction. Our observed formation of homolanthione itself indicated that under mild conditions desulfurization preceded oxidation. In addition to this information on sequence of product formation, we found that increasing the HCl concentration resulted in the formation of homolanthionine sulfone instead of the sulfoxide. Our technique of reduction with dimethyl sulfide provided strong evidence for the formation of sulfoxides of sulfide amino acids.

Our primary purpose, to study the H_2O_2–HCl oxidation of cystine, provided evidence of desulfurization with a formation of lanthionine sulfoxide and lanthionine sulfone. However, these products were formed in lower yields than were obtained for the corresponding products from homocystine and instead cysteic acid was the main product. The failure to demonstrate an accumulation of lanthionine itself under milder oxidation conditions indicated that lanthionine was more susceptible to oxidation than homolanthionine.

The preparation of sulfoxide and sulfone derivatives of *meso*-lanthionine has been reported (Zahn and Osterloh,

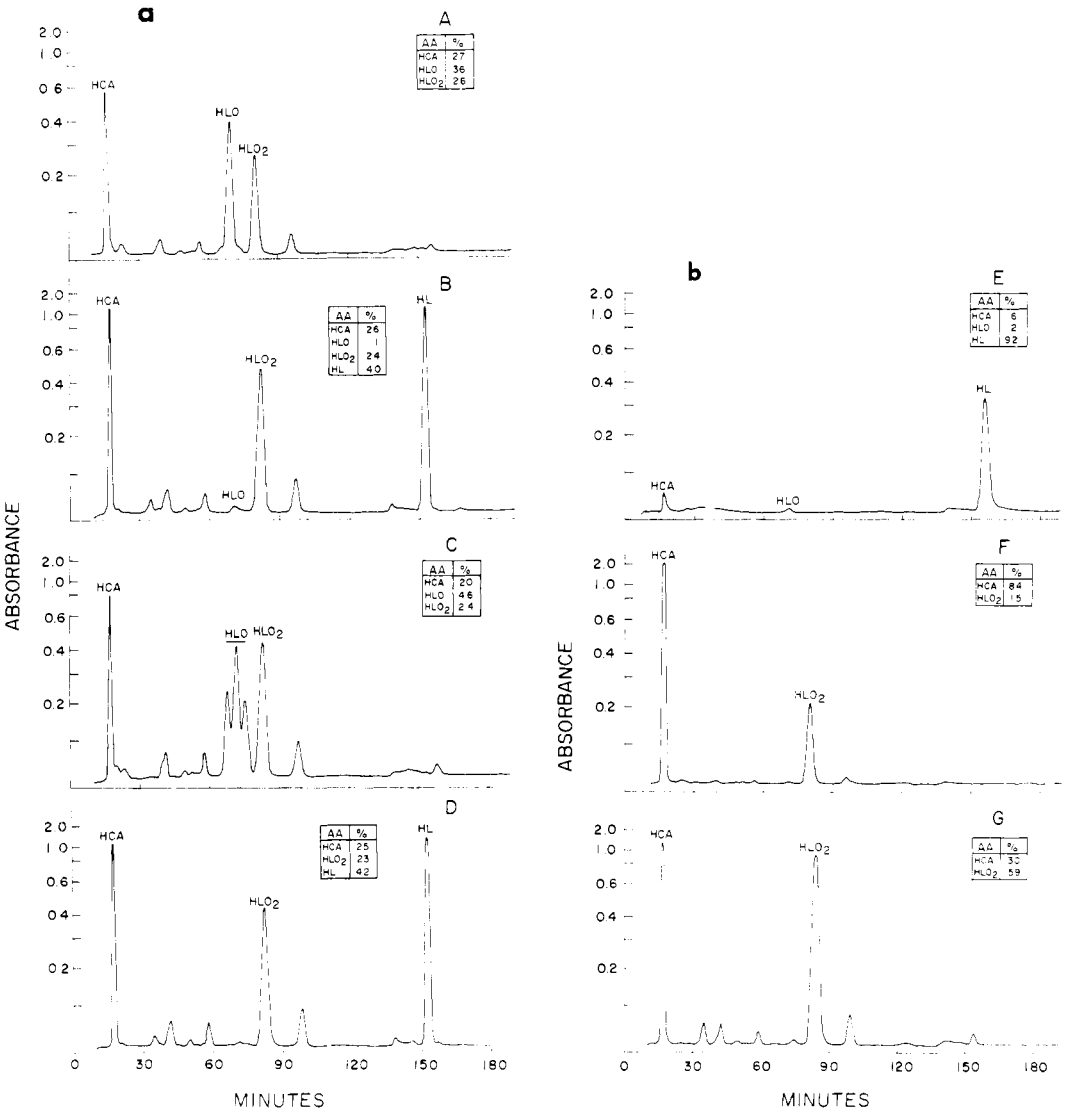

Figure 3. Amino acid analyzer chromatograms of acidic and neutral amino acids which resulted from the peroxide oxidation of L and DL isomers of homocystine: chromatogram A, oxidation of L-homocystine was for 10 min at 100 °C in 6% H_2O_2 and 0.8 N HCl; chromatogram B, L-homocystine was oxidized as in A and then reduced with dimethyl sulfide (see text); chromatogram C, oxidation of DL-homocystine was for 10 min at 100 °C in 6% H_2O_2 and 0.8 N HCl; chromatogram D, DL-homocystine was oxidized as in C and then reduced with dimethyl sulfide (see text); chromatogram E, oxidation of L-homocystine was for 10 min at 50 °C in 6% H_2O_2 and 0.8 N HCl; chromatogram F, oxidation of L-homocystine was for 10 min at 100 °C in 6% H_2O_2 and 4.8 N HCl; chromatogram G, oxidation of L-homocystine and subsequent reduction procedure were as in B except that the dimethyl sulfide was omitted. Abbreviations: HCA, homocysteic acid; HL, homolanthionine; HLO, homolanthionine sulfoxide; HLO_2, homolanthionine sulfone.

1955). Zahn (1961) indicated that when mixtures of free cystine and cysteine were heated, lanthionine was formed even under acid conditions. We have not identified all of the products of the oxidations of the sulfur amino acids and thus cannot exclude the presence of small amounts of other products such as sulfinic acids or disulfide sulfoxides. [Our chromatographic system did not resolve cysteic acid and cysteine sulfinic acid. Savige and Maclaren (1966) listed various oxidation products of disulfide amino acids but did not include lanthionine or homolanthionine sulfoxides or sulfones.]

L-Homolanthionine has been reported to occur as a free amino acid in a methionine-requiring mutant of *Escherichia coli* (Huang, 1963) and as a urinary excretion product associated with the human metabolic disease homocystinuria (Perry et al., 1966). Syntheses have been reported of the mixed DL and meso isomers (Stekol, 1948) and of the individual isomers (Weiss and Stekol, 1951). The sulfur of homolanthionine was found to be utilized by the rat for the synthesis of cystine (Stekol and Weiss, 1949). A low yield of homolanthionine was obtained from methionine sulfoxide in a sealed tube of hot 12 N HCl, but not 6 N HCl (Morihara, 1964).

Strong alkali treatments are used in food and feed technology for a variety of purposes (de Groot et al., 1976a; Feeney, 1976): the most notable of these is perhaps for the solubilization of proteins for spinning fibers (*Nutr. Rev.*, 1967). These alkali treatments may chemically alter several amino acids (Provansal et al., 1975), adversely affect nutritive values (de Groot and Slump, 1969), and produce the toxic amino acid lysinoalanine (Woodard and Short, 1973, 1975; *Nutr. Rev.*, 1976) [chemical name is N^{ϵ}-(DL-2-amino-2-carboxyethyl)-L-lysine]. Evidence has been presented (de Groot et al., 1976a,b) that lysinoalanine is toxic only in the form of the free amino acid or in an oligopeptide and is nontoxic when protein bound. It is formed in alkali-treated proteins, mainly as a protein-bound residue, by way of the intermediate, dehydroalanine (Bohak, 1964; Patchornik and Sokolovsky, 1964). The dehydroalanine arises mostly from desulfurization of a

cysteinyl residue and partly from dehydration of a serine residue. This intermediate reacts either with a lysyl residue to form lysinoalanine or with another cysteinyl residue to form lanthionine. Thus, lanthionine and lysinoalanine have been considered to be formed in parallel.

Recently, however, lysinoalanine has been reported to have wide distribution in cooked foods and in commercial products (Sternberg et al., 1975a,b) and it may be formed even under acidic conditions. Since lanthionine has no reported toxicity, its distribution in foods and feeds has not been extensively studied. We have no evidence of the mechanism of lanthionine sulfoxide formation during peroxide oxidation of cystine, but peroxides, either formed or added during food processing, might be related to the formation of both lanthionine and lysinoalanine.

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