Covalent Attachment of Amino Acids to Casein. 2. Bioavailability of Methionine and N-Acetylmethionine Covalently Linked to Casein

Antoine J. Puigserver, Lourminia C. Sen, Andrew J. Clifford, Robert E. Feeney, and John R. Whitaker*

Casein was used to evaluate the effect of covalent attachment, via active N-hydroxysuccinimide esters, of amino acids through isopeptide bonds on the nutritional value of the protein fed to rats. Nutritional evaluation included digestion with bovine pancreatin and rat bile–pancreatic juice, in vivo protein efficiency ratio response, and plasma amino acid patterns of rats fed isonitrogenous diets. The following isonitrogenous diets were fed: unmodified 10% casein alone or supplemented with 0.2% free L-methionine or free N-acetyl-L-methionine, and 10% protein including L-methionylcasein and N-acetyl-L-methionylcasein. In vitro digestion of the casein derivatives was significantly decreased. However, in in vivo experiments, casein modification did not significantly alter plasma amino acid patterns or protein efficiency ratio values. Thus, the isopeptide bond formed between the ε-amino group of the lysyl residues of casein and methionine or N-acetylmethionine was readily cleaved. Intestinal aminopeptidase may be responsible for in vivo hydrolysis of the isopeptide bond of ε-N-L-methionyl-L-lysine although other enzymes cannot yet be ruled out. Therefore, covalent attachment of limiting essential amino acids to proteins may be used for improving their nutritional quality.

The biological value of a food protein depends upon its ability to supply the essential amino acids (Hegsted, 1964; Meister, 1965). Proteins differ in their content of the essential amino acids, and consequently they differ in nutritional quality. Since protein and amino acid requirements of mammals, including man, have been investigated in considerable detail (Rose, 1957; Harper, 1959, 1971; Rogers and Harper, 1965) the amino acid composition of a protein may be used to predict its nutritional value.

Nutritional value of proteins from plants, single cells, and other less conventional sources is frequently limited by their low content of one or more essential amino acids. Since 1914 when Osborne and Mendel (1914) demonstrated that supplementation of wheat proteins with free lysine improved their nutritional quality for the rat, the objective of many investigations has been the improvement of the nutritional value of food proteins. Supplementation of vegetable proteins with amino acids has been extensively studied and was recently reviewed (Harper and Hegsted, 1974; Altschul, 1974).

Although fortification of foods with amino acids must be considered an easy and relatively efficient way of improving the nutritional value of food proteins, there are many disadvantages of this method. Among the disadvantages are possible losses of the added amino acid during processing and technical problems that arise when the food products must be processed or cooked. This is especially noteworthy with methionine which may undergo the Strecker reaction (Ballance, 1961) to produce methional, thereby adversely affecting the sensory properties of the finished product. In general, chemical reactions occurring during processing result in a reduction of the nutritional value. Finally, although the metabolism of amino acids which are free or covalently attached in proteins is similar, there are differences in their absorption levels (Crampton, 1972; Matthews, 1972).

There are chemical and enzymatic methods already available for the covalent attachment of amino acids to proteins in an aqueous medium. Proteolytic enzymes have been used to increase the content of essential amino acids of food proteins through the plastein reaction (Yamashita et al., 1971, 1976). Chemical methods generally involve use of either active esters of amino acids (Blumberg and Vallee, 1975; Slotboom and de Haas, 1975) or Leuchs' anhydrides (Glazer et al., 1962; St. Angelo et al., 1966). These methods have been successfully applied to food proteins (Bjarnason-Baumann et al., 1977; Puigserver et al., 1978, 1979). Therefore, the purpose of the present work was to determine the biological availability of L-methionine and N-acetyl-L-methionine covalently attached to the ε-amino group of the lysyl residues of casein through isopeptide bonds. The organ location of the isopeptide hydrolytic activity was investigated in tissue homogenates as well as cell cultures of kidneys, intestine, and liver.

MATERIALS AND METHODS

Materials. Dicyclohexylcarbodiimide, N-hydroxysuccinimide, and 2-*tert*-butyloxycarbonyloxyimino-2-phenylacetonitrile were from Aldrich Chemical Co., Milwaukee, WI. α-*tert*-Butyloxycarbonyl-L-lysine, α-*tert*-butyloxycarbonyl-L-methionine N-hydroxysuccinimide ester, N-tosyl-L-arginine methyl ester, N-acetyl-L-tyrosine ethyl ester, N-acetyl-L-alanyl-L-alanyl-L-alanyl methyl ester, alanine *p*-nitroanilide, hippuryl-L-arginine, and hippuryl-β-phenyl-L-lactic acid were from Bachem Fine Chemicals, Torrance, CA. L-[^{14}C]Methionine was from ICN Pharmaceuticals, Inc., Irvine, CA. Anhydrous trifluoroacetic acid, diethyl ether, dimethylformamide, hydroxylamine, 2-propanol, and trichloroacetic acid were from Mallinckrodt, Inc., St. Louis, MO. Vitamin-free casein was from Nutritional Biochemicals Corp., Cleveland, OH. N-Acetyl-L-methionine, L-methionine, and bovine pancreatin were from Sigma Chemical Co., St. Louis, MO. Carboxymethyl Sephadex C-25 was from Pharmacia, Uppsala, Sweden. Aquasol-2 was from New England Nuclear, Boston, MA. Isopropylcasein was a gift of H. S. Lee, Department of Food Science and Technology, University of California, Davis.

Porcine pancreatic juice was from CNRZ, Jouy-en-Josas, France. Trypsin, from Worthington Biochemical Corp., Freehold, NJ, was freed from most of the contaminating chymotrypsin activity by the procedure of Maroux et al. (1962). Rat bile–pancreatic juice was a gift of B. Schneeman, Department of Nutrition, University of California, Davis. Pure aminopeptidase was a gift of H. Ferracci, CBM-CNRS, Marseille, France. Closed membrane vesicles

Department of Food Science and Technology (L.C.S., R.E.F., J.R.W.) and the Department of Nutrition (A.J.C.), University of California, Davis, California 95616, and the Centre National de la Recherche Scientifique, Centre de Biochemie et de Biologie Molecular, Marseille, France (A.J.P.).

0021-8561/79/1427-1286$01.00/0 © 1979 American Chemical Society

was a gift of D. Gratecos, CBM-CNRS, Marseille, France. The canine kidney cell line was a gift of M. Saier, Department of Biology, University of California–San Diego, La Jolla, CA.

Methods. *Esterification of N-Acetyl-L-methionine.* Synthesis of *N*-hydroxysuccinimide ester of the N-protected amino acid was done at 4 °C according to previously described methods (Anderson et al., 1964; Blumberg and Vallee, 1975). Solid dicyclohexylcarbodiimide (16.2 g) was added with stirring to a mixture of *N*-acetyl-L-methionine (15 g) and *N*-hydroxysuccinimide (9.1 g) dissolved in dimethylformamide (150 mL). The mixture was incubated overnight at 4 °C, the reaction byproduct, dicyclohexylurea, was removed by filtration under vacuum through glass fiber paper and the resulting solution (140 mL) was evaporated to dryness at room temperature. The residue was twice crystallized from 2-propanol. *N*-Acetyl-L-methionine *N*-hydroxysuccinimide ester (16.5 g) was recovered in 75% yield.

Synthesis of ϵ-N-L-[methyl-^{14}C]Methionyl-L-lysine. A mixture of cold L-methionine (2.1 g) and L-[^{14}C]methionine (0.36 mg, 100 μCi) was converted to the *tert*-butyloxycarbonyl derivative with 2-*tert*-butyloxycarbonyloxyimino-2-phenylacetonitrile (Itoh et al., 1975). Then, the derivative (pure by thin-layer chromatography on silica gel thin-layer plates using chloroform–methanol–acetic acid (85:10:5; v/v) as solvent) was esterified as described above (Anderson et al., 1964). The resulting *tert*-butyloxycarbonyl-L-methionine *N*-hydroxysuccinimide ester, crystallized from 2-propanol, was pure (3.7 g, 79% yield). The active *N*-hydroxysuccinimide ester of *tert*-butyloxycarbonylmethionine was then reacted with α-*tert*-butyloxycarbonyllysine as described by Stewart and Young (1969) to give the amino group protected isodipeptide, ϵ-*N*-L-methionyl-L-lysine. The *tert*-butyloxycarbonyl groups were removed by HCl treatment to give a white solid (2.9 g; 60% yield; mp 126 °C) with a specific activity of 7 μCi/mmol.

Preparation of Casein Derivatives. Casein (100 g) was dissolved in 1 L of 0.1 M sodium borate, pH 9.0, with warming at 45 °C for 1 h. The solution was cooled to room temperature and 25 g of *tert*-butyloxycarbonylmethionine *N*-hydroxysuccinimide ester, dissolved in 100 mL of dimethylformamide, was added with stirring over a period of 3 h while the pH was kept constant at 9.0 by addition of 10 N NaOH. After the solution was treated for 2 h with hydroxylamine (0.5 M final concentration) at pH 8.0 in order to remove reagent covalently linked to the hydroxyl groups of tyrosyl residues (Puigserver et al., 1978), the reaction mixture was dialyzed against deionized water at 4 °C for 3 days and lyophilized. Removal of amino protecting group was achieved by treatment of the casein derivative with anhydrous trifluoroacetic acid under conditions where peptide and isopeptide bonds are stable (Puigserver et al., 1979). A 60-fold molar excess of acid over the protecting group (250 mg of protein/mL of acid) was generally used for complete removal of the amino group protector. Protein was then slowly dispersed with stirring in 0.5 M Tris-HCl buffer (pH 8.0–9.0) with continuous adjustment of pH with 10 N NaOH. Precipitation always occurred when the protein was changed from the trifluoroacetic acid solution to an aqueous medium, but on further stirring, it dissolved. Following dialysis against water, the modified protein solution was precipitated by adjusting the pH to 4.5 with 3 N HCl. The precipitate was freeze-dried and ground into a powder for the feeding studies and analyses. The overall yield by this procedure was about 85%.

The same conditions were also used for the preparation of glycylcasein and *N*-acetyl-L-methionylcasein except the trifluoroacetic acid step was omitted in the preparation of *N*-acetyl-L-methionylcasein. A 1.2-fold molar excess of active ester over amino group was used for complete modification on lysyl residues. Reagent control casein was prepared under the same experimental conditions except the *N*-hydroxysuccinimide ester of the protected amino acid was omitted.

Amino Acid Analysis and Amino Group Determination. The amino acid composition of reagent control casein and modified casein samples was determined with a Technicon Autoanalyzer equipped with an Autolab-Beckman computer (system AA) following hydrolysis of the proteins with distilled 5.6 N HCl in sealed tubes at 110 °C for 24 h (Spackman et al., 1958). Methionine was determined as methionine sulfone after performic acid oxidation of the protein (Hirs, 1956; Moore, 1963). Tryptophan content was determined by the method of Edelhoch (1967). The lithium citrate buffers for physiological fluid analysis were used for plasma samples (Durrum manual, 1973). Free amino groups of proteins were quantitated with 2,4,6-trinitrobenzenesulfonic acid (TNBS; Fields, 1972).

Activation of Pancreatic Juice and Determination of Enzymatic Activities. Porcine pancreatic juice (75 mg corresponding to about 11 mg of protein) in 0.05 M sodium phosphate, pH 8.0 (8 mL) was activated by trypsin (1:10; w/w) at 0 °C for 3 h and the resulting enzymatic activities were determined. Tryptic, chymotryptic, and elastolytic activities were determined by titrimetry (recording Radiometer pH Stat) at 25 °C and pH 7.9 using 2 mM *N*-benzoyl-L-arginine ethyl ester or 25 mM *N*-tosyl-L-arginine methyl ester, 10 mM *N*-acetyl-L-tyrosine ethyl ester in 3% methanol, and 5 mM *N*-acetyl-L-alanyl-L-alanyl-L-alanyl methyl ester as substrates, respectively. Carboxypeptidase A activity was measured by titrimetry at pH 7.5 using 10 mM hippuryl-β-phenyl-L-lactic acid. Carboxypeptidase B activity was measured at 25 °C and 254 nm with a Beckman Acta III spectrophotometer using 1 mM hippuryl-L-arginine.

Aminopeptidase activity was measured at 37 °C by the method of Roncari and Zuber (1969) with 1.5 mM alanine *p*-nitroanilide in 0.05 M phosphate buffer, pH 7.0. *p*-Nitroaniline was determined spectrophotometrically at 410 nm using ϵ_m of 8800 M^{-1} cm^{-1}. One unit of aminopeptidase activity is defined as that amount of enzyme which hydrolyzes 1 nmol of substrate/min.

In Vitro Enzyme Digestion. Liberated amino groups, determined by TNBS analysis (Fields, 1972), were used to measure the extent of enzymatic digestion after 48 h. Reaction mixtures containing 0.1% protein in 0.1 M phosphate buffer, pH 7.0, were incubated at 38 °C with 1:2 (w/w; enzyme to protein) bovine pancreatin and 1:100 (v/v) rat bile–pancreatic juice.

Digestion of Methionylcasein by Trypsin. Digestion of methionylcasein by trypsin was done at pH 8.0 and 37 °C for 1.5 h (Grosclaude et al., 1970). The casein derivative (200 mg) dissolved in 0.05 M sodium phosphate buffer (15 mL) was incubated with tosyl-L-phenylalanine chloromethyl ketone treated trypsin (4 mg) for 1.5 h. The trypsin was then denatured by incubating the mixture in boiling water for 15 min at pH 10.0 (Walsh, 1970).

Nutritional Studies with Rats. All feeding studies were done with weanling Sprague-Dawley male rats housed individually in stainless steel metabolic cages. The rats were fed a stock diet for 5 days, then divided into groups of five or nine rats of approximately equal mean initial weights (about 80 g) and fed an experimental diet (Lee et al., 1978).

Table I. Extent of Modification of Casein Derivatives

protein	amino acid content[a]				free amino groups	
	Lys	Met	Gly	modification,[b] %	no.	modification, %
commercial casein	11.3	4.6	5.6	0	10.1	0
reagent control casein	10.7	4.2	5.0	0	10.4	0
glycylcasein + BOC[c]				87	1.0	90
– BOC	11.3	4.4	15.4		10.3	
methionylcasein + BOC				91	0.7	93
– BOC	10.5	14.9	5.5		10.5	
acetylmethionylcasein	11.0	10.6	5.2	55	4.2	60
acetylmethionylcasein	10.7	15.5	6.3	96	0.1	99

[a] Number of residues/mol of protein. Average of calculated composition before and after performic acid oxidation (four de determinations). [b] Calculation based on 11 lysyl, four methionyl, and five glycyl residues and one amino terminal group per mole control casein (MW 23 000). [c] Before and after removal of *tert*-butyloxycarbonyl group is indicated by + BOC and – BOC, respectively.

Body weight and ad libitum food intake were recorded twice weekly throughout the test period. The nutritive value (protein efficiency ratio, PER) of the derivatized caseins was calculated as the weight gained per unit weight of protein consumed.

Plasma Sampling. The experiments were terminated by decapitating the rats at 11:30 a.m. on the last day of each test feeding period. Blood samples were collected at room temperature into centrifuge tubes containing 1.2% EDTA in 0.9% NaCl and centrifuged in the cold for 20 min. Plasma samples were deproteinized with equal volumes of 6% sulfosalicylic acid and centrifuged, and the supernatant liquid was kept frozen until analyzed.

Preparation of Homogenates from Kidneys, Liver, and Intestine. An adult male Wistar rat (~400 g) previously fasted for 18 h was killed and the kidneys, liver, and intestine (jejunum and ileum) were quickly excised. Kidneys and liver were weighed, minced, and homogenized in 4 volumes of cold 0.05 M sodium phosphate buffer, pH 7.0, in a Sorvall Omni-Mixer at top speed. The small intestine was first washed with cold phosphate-buffered saline (10 mM phosphate buffer/0.15 M NaCl, pH 7.4); then the mucosa was prepared and homogenized as described above. All operations were done in a cold room at 4 °C. One-half of each homogenate was centrifuged for 2 h at 18 000 rpm in a Sorvall RC-2B centrifuge. The soluble and resuspended particulate fractions were used separately to study distribution of the isopeptide bond-hydrolyzing enzyme(s).

Digestion of Methionylcasein and Trypsin-Treated Methionylcasein by Tissue Homogenates. Concentration of methionylcasein solutions before or after tryptic digestion was about 10–12 mg of protein/mL in 0.05 M sodium phosphate buffer, pH 7.0. Since the casein derivative contained an average of 10–11 methionyl residues covalently linked to the ε-amino group of lysyl residues, the concentration of ε-*N*-methionyllysine was about 5 mM. Samples were digested as follows: 0.5 mL of protein solution containing 0.1 mL of tissue homogenate was incubated overnight at 37 °C. Protein was then removed by precipitation with trichloroacetic acid (final concentration of 10%). Excess trichloroacetic acid was extracted with diethyl ether and the resulting solution was analyzed for free methionine with a Beckman Model 120C autoanalyzer equipped with an expanded scale.

Hydrolysis of ε-N-L-[methyl-^{14}C]Methionyl-L-lysine by Tissue Homogenates and Aminopeptidase. Extent of hydrolysis of the radiolabeled isodipeptide was followed by separating free radiolabeled methionine from the remaining isodipeptide and counting the resulting radioactivity in a Packard Tri-Carb liquid scintillation spectrometer, Model 2450. The isodipeptide (0.5 mL of a 5 mM solution) was incubated with tissue homogenates or pure aminopeptidase under the same experimental conditions as described for methionylcasein. After trichloroacetic acid treatment and diethyl ether extraction, free methionine was separated by column chromatography (1 × 6 cm) on carboxymethyl Sephadex C-25 equilibrated with 0.01 M sodium phosphate, pH 7.0. While free methionine eluted with buffer alone, a higher ionic strength (1.0 M NaCl) was required to elute the isodipeptide.

Pure aminopeptidase from the brush border membrane of porcine and rabbit jejunum and ileum was prepared according to Maroux et al. (1973) and Ferracci (1977). Closed membrane vesicles from rat and hog duodenal and jejunal brush border were obtained by the procedure of Louvard et al. (1973).

Preparation of Cell Suspensions. Structurally intact hepatic parenchymal cells were prepared according to the procedure of Howard and Pesch (1968). The liver of an adult (400 g) male rat (Sprague-Dawley strain) was perfused with Krebs-Ringer phosphate solution (Dawson et al., 1969) as described by Berry and Friend (1969). Intestinal cells were isolated as described by Halsted et al. (1976). The canine kidney cell line (MDCK) was also used.

Cell Culture. Liver and intestinal cells were cultured in Eagle's basal medium (BME) with Hanks' salts (Hanks and Wallace, 1949; Eagle, 1955) supplemented with 10% dialyzed fetal calf serum and antibiotics under a humid atmosphere of 90% air/10% CO_2 at 37 °C. The same experimental conditions were used for cultivation of canine kidney cells except that Eagle's minimum essential medium (MEM) was used instead of BME (Eagle, 1959). Cells were plated onto glass coverslips at about 2 × 10^6 cells per 60-mm dish and allowed to grow for 24–48 h up to subconfluency in the presence of the radiolabeled isodipeptide. An equal volume of 20% trichloroacetic acid was then added to the cell suspension and the precipitate washed several times until there was no significant count present in the washings. The precipitate was then suspended in 10 mL of Aquasol-2 and ^{14}C radioactivity counted.

RESULTS

Analysis of Casein Derivatives. The extent of amino acid incorporation into glycylcasein, L-methionylcasein, and *N*-acetyl-L-methionylcasein, determined by amino acid analysis and amino group determination, is summarized in Table I. Amino group analysis was done before and after removal of the *tert*-butyloxycarbonyl protecting group from glycyl- and methionylcasein. Under conditions used for reaction, the extent of modification of glycyl- and methionylcasein was 87 and 91%, respectively, by amino acid analysis and 90 and 93% respectively, by amino group determinations. No significant changes in other amino acids were observed in the modified casein samples. The

Table II. Free Amino Acid Patterns of Plasma from Rats Fed 10% Protein Diets[a]

amino acids, μmol/100 mL of plasma	commercial casein	control casein	methionyl-casein	glycyl-casein	isopropyl-casein
taurine	8	5	20	6	17
aspartic acid	5	4	4	3	4
hydroxyproline	2	2	3	1	1
threonine	18	19	17	19	46
serine	29	34	33	34	44
asparagine	5	6	5	4	6
glutamic acid	39	37	36	34	39
glutamine	90	72	86	36	76
proline	41	57	36	31	33
glycine	27	32	27	30	40
alanine	105	72	71	64	116
citrulline	10	9	10	6	9
α-aminobutyric acid	1	1	5	1	2
valine	18	19	15	14	20
half-cystine	2	1	6	1	6
methionine	4	5	39	8	6
isoleucine	7	7	7	6	9
leucine	11	14	11	10	15
tyrosine	12	17	10	9	10
phenylalanine	5	6	5	5	7
tryptophan	2	2	1	1	3
ornithine	6	5	5	3	6
lysine	93	101	96	72	17
histidine	9	7	9	15	11
arginine	6	7	8	5	17
urea	1	1	1	1	1
α-aminoadipic acid	2	2	2	3	3
hydroxylysine	1	1	1	3	1

[a] Pooled sample of five blood samples collected after a 3-day test period by decapitation. Weanling Sprague-Dawley male rats with approximately equal mean initial weights (63 ± 4 g) were used.

amino acid compositions of both the reagent control casein and modified proteins were in good agreement with published data (Brunner, 1977).

The level of unmodified lysine of the casein derivatives in a 10% protein diet was below the lysine requirement of growing rats (1%). Thus, lysine would be a limiting amino acid if the isopeptide bond formed between the ε-amino group of lysyl residues and the carboxyl group of the added amino acid were not hydrolyzed in vivo by the animal.

Plasma Amino Acid Patterns. Levels of free amino acids in plasma from rats fed casein highly modified by covalent attachment of glycine and methionine were measured in an effort to determine whether the isopeptide bond was cleaved in vivo. Plasma amino acid patterns of rats fed a 10% protein diet containing either unmodified casein, glycylcasein, methionylcasein, or isopropylcasein are summarized in Table II. The isopropylcasein was selected as a control since it has already been established that ε-*N*-isopropyllysine is unavailable as a lysine source for rats but appears in the plasma (Lee et al., 1978). The plasma amino acid concentration of rats fed glycyl- or methionylcasein were normal compared to the pattern of those fed isopropylcasein. The plasma amino acid pattern with isopropylcasein is typical of lysine deficiency (Gray et al., 1960; Morrison et al., 1961; Muramatsu et al., 1973).

The high level of methionine in the plasma of rats fed methionylcasein may be due to efficient cleavage of the corresponding isopeptide bond and subsequent normal absorption through the intestinal wall (Canolty and Nasset, 1975). By contrast, the level of glycine in the plasma of rats fed glycylcasein was not changed, suggesting that hydrolysis of the ε-*N*-glycyllysine bond was less efficient than in the methionyl derivative. As shown in Table III, removal of the protecting *tert*-butyloxycarbonyl group prior to feeding was essential. The isopeptide bond was not cleaved in the presence of the amino protecting group and the protected amino acid was not transported (Christensen, 1963). In contrast, hydrolysis of the isopeptide bond of ε-*N*-acetylmethionyllysine occurred readily.

Table III. Plasma Concentration of Some Free Amino Acids in Rats Fed 10% Protein Diets[a]

proteins	μmol/100 mL of plasma		
	Thr	Met	Lys
commercial casein	29 ± 4	4 ± 0	60 ± 1
BOC-methionylcasein	49 ± 12	5 ± 2	6 ± 1
acetylmethionylcasein[b]	41 ± 4	4 ± 2	34 ± 14
methionylcasein	17	39	96

[a] Determined after a 3-day test period. Mean initial weights of rats were 75 ± 6 g. Data are averages of three sets of three rats each. [b] The highly modified casein derivative (>96%; Table I) was used in this experiment.

PER Response of Rats Fed Casein Derivatives. Another way to examine efficiency of hydrolysis of the isopeptide bond is to compare PER response of rats fed diets containing the same amount of an individual amino acid, either free or covalently linked. Since casein is slightly deficient in methionine and since methionine is one of the more limiting essential amino acids in several plant proteins (Henry and Ford, 1965; Milade et al., 1972), we chose to use methionylcasein for this study. Moreover, as the nutritional similarities of methionine and *N*-acetyl-L-methionine have been demonstrated (Boggs et al., 1975; Rotruck and Boggs, 1975), it is important to know whether this similarity exists when they are covalently attached to the ε-amino groups of a protein. If so, use of acetylated amino acids in derivatizing proteins would eliminate the need to deacylate prior to feeding.

The protein composition of the isonitrogenous diets used in this feeding trial, as well as the nutritive value (PER) of the casein derivatives, are listed in Table IV. The nutritive value (PER) of methionylcasein (D_3) was comparable to that of the corresponding control (D_4) but was a little lower than commercial casein supplemented with free methionine (D_2). This was probably a result of treating

Table IV. Nutritive Values of Methionyl- and *N*-Acetylmethionylcaseins for Rats[a]

diet description	diet no.	methionine in the diet, % by w[b]	weight gain, g	protein intake, g	PER[c]
10% commercial casein	D_1	0.26	72.6 ± 10.5	29.7 ± 3.6	2.46 ± 0.29
10% commercial casein + 0.2% free L-methionine	D_2	0.26 0.20	106.8 ± 9.7	33.7 ± 2.9	3.15 ± 0.23
5% commercial casein + 5% L-methionylcasein	D_3	0.13 0.34	60.5 ± 8.6	21.2 ± 1.5	2.92 ± 0.22
5% commercial casein + 5% control casein[d] + 0.2% free L-methionine	D_4	0.13 0.13 0.20	66.7 ± 10.6	22.2 ± 3.4	2.97 ± 0.30
3% commercial casein + 7% *N*-acetyl-L-methionylcasein	D_5	0.08 0.39	89.8 ± 10.9	30.5 ± 4.0	2.95 ± 0.28
3% commercial casein + 7% control casein + 0.26% *N*-acetyl-L-methionine	D_6	0.08 0.18 0.21	107.0 ± 9.9	36.3 ± 3.9	3.18 ± 0.29

[a] Methionylcasein and *N*-acetylmethionylcasein used in these studies were 59 and 42% modified, respectively. The remainder of the diet was of standard composition (Lee et al., 1978). Groups of nine rats each with about the same mean initial weights were used. All values are mean ± SEM. [b] Calculated by amino acid analysis as methionine and methionine sulfone after performic acid oxidation. [c] Protein efficiency ratio calculated as the weight gained per unit weight of protein consumed. [d] Control casein was carried through all the steps used in preparing modified casein but without the addition of the methionine derivatives.

Table V. Plasma Concentrations of Free Amino Acids in Rats Fed L-Methionyl- and *N*-Acetyl-L-methionylcasein Diets[a]

amino acids, μmol/100 mL of plasma	D_1[b]	D_2	D_3	D_4	D_5	D_6
Tau[c]	6	10	20	8	6	11
Asp	3	3	3	3	3	3
Thr	20	14	16	12	27	20
Ser	44	30	41	33	47	35
Asn	8	6	6	8	10	10
Glu	11	12	17	17	15	16
Gln	99	84	86	91	165	96
Pro	59	42	29	24	35	28
Gly	22	22	32	24	22	23
Ala	150	127	35	58	140	105
Cit[c]	8	8	8	9	8	8
α-ABA[c]	0	1	2	1	0	2
Val	23	15	12	19	23	20
1/2-Cys	0	1	1	1	0	2
Met	5	6	3	7	6	8
Ile	9	6	7	8	9	9
Leu	17	9	12	13	16	13
Tyr	15	10	6	13	16	12
Phe	6	4	4	4	6	5
Orn[c]	5	4	5	5	5	5
Lys	63	46	57	87	35	56
His	8	5	6	8	9	7
Arg	19	9	16	12	9	6
urea	54	24	41	43	46	28
α-AAA[c]	2	3	3	2	2	2

[a] Values represent single determination on samples pooled from groups of nine rats. Blood samples were taken at the end of the feeding trial (see Table IV). [b] See Table IV for composition of diets. [c] The abbreviations are for taurine (Tau), citrulline (Cit), α-aminobutyric acid (α-ABA), ornithine (Orn) and α-aminoadipic acid (α-AAA), respectively.

the protein with trifluoroacetic acid. Therefore, covalently bound methionine appears to be as available as the free amino acid. Covalently bound *N*-acetyl-L-methionine (D_5) was somewhat less available than when added in free form (D_6) but the differences may not be significant. The plasma amino acid patterns of rats fed derivatized casein were normal and comparable to those of the corresponding controls (Table V). The plasma amino acid levels were also comparable to those reported earlier (Boomgaardt and McDonald, 1969), indicating that the bioavailability of L-methionine and *N*-acetyl-L-methionine covalently linked to casein was high and that these did not produce any clinical symptoms of digestive or metabolic disturbances.

Table VI. In Vitro Digestion Studies of Modified Caseins

protein	relative extent of digestion, %: modification,[a] %	rat bile pancreatic juice	bovine pancreatin
control casein	0	100	100
glycylcasein	90	80	81
BOC-methionylcasein[b]	59	54	67
methionylcasein	59	95	90
methionylcasein	90	71	78
N-acetylmethionylcasein	57	71	84

[a] Determined by amino group estimation and amino acid analysis as described under Materials and Methods. [b] BOC = *tert*-butyloxycarbonyl.

In Vitro Enzyme Digestion. The relative extents of digestion of modified caseins by rat bile–pancreatic juice and bovine pancreatin are summarized in Table VI. Both pancreatic mixtures digested the casein derivatives to about the same extent; however, digestibility was lower than that of unmodified casein. When the amino protecting group was still present, a marked decrease in digestibility was noted. Highly modified methionylcasein (90%) and 50% modified *N*-acetyl-L-methionylcasein were digested to a similar extent.

Since the extents of digestion were determined by amino group estimation with TNBS (Fields, 1972), hydrolysis of both peptide and isopeptide bonds would be measured. However, as shown in Table VII neither porcine pepsin nor activated pancreatic juice was able to hydrolyze the isopeptide bond of ε-*N*-methionyllysine or to release methionine from methionylcasein at a significant rate. Pancreatic juice is devoid of aminopeptidase and dipeptidase activities which could hydrolyze the isopeptide bond.

Hydrolysis of the Isopeptide Bond of ε-*N*-Methionyllysine. Methionine covalently bound to the ε-amino group of lysyl residues of casein appears to be as readily available as the free amino acid. Therefore, the organ location of the enzymatic activity responsible for this quite efficient hydrolysis of the isopeptide bond of ε-*N*-methionyllysine in vivo is important.

Isolated rat liver and intestine cells were cultured in the presence of radiolabeled ε-*N*-methionyllysine in order to

Table VII. Isopeptide Bond Hydrolysis by the Proteolytic Enzymes of the Upper Alimentary Tract

enzymes[a]	substrate	nanomoles of substrate hydrolyzed/ assay	extent of hydrolysis, %[b]	E/S (w/w)[c]
pepsin	ε-*N*-methionyllysine	85	3.4	1:0.5
	ε-*N*-methionyllysine	138	5.5	1:6.0
pancreatic juice	methionylcasein	161	6.4	1:6.0
	alanylglycine[d]	53	2.1	1:3.0
	alanine *p*-nitroanilide	6	0.8	1:3.0

[a] Porcine enzymes were used. [b] Determined after incubation for 15 h at 37 °C except for the last two where the incubation period was 10 min. Under these experimental conditions, tissue homogenates of liver, intestine, and kidney gave about 90% hydrolysis with a ratio of aminopeptidase/substrate of 1:300 (w/w). [c] Concentration of pepsin determined by weighing whereas concentrations of pancreatic juice and aminopeptidase solution were derived from an extinction coefficient value ($E^{1\%}_{1cm}$) of 19.0 at 280 nm and a specific activity of 30 000 alanine *p*-nitroanilide units/mg of protein, respectively. [d] Concentration was 5 mM in 0.05 M phosphate buffer, pH 7.0. For the other substrate concentrations see Materials and Methods.

Table VIII. Isodipeptide Uptake by Cell Cultures

medium	CPM/mL of cell suspension[a] liver	intestine
BME,[b] 5 mM isodipeptide	487	398
BME,[c] 0.2 mM isodipeptide	7	5
BME,[c] 5 mM isodipeptide	228	145

[a] Intracellular ^{14}C radioactivity was counted after cells were allowed to grow for 48 h (see Materials and Methods). One milliliter of cell culture contained about 1 mg of protein. [b] Eagle's basal medium. [c] Eagle's basal medium minus free lysine and free methionine.

establish whether they could hydrolyze the isopeptide bond and utilize the free methionine. As shown in Table VIII, the isodipeptide was not toxic for liver and intestinal cells as they accumulated this compound and grew. Isodipeptide uptake by these cells occurred in the absence of free lysine and methionine, indicating that these essential amino acids, necessary for cell growth, could be supplied by hydrolysis of the isodipeptide. With canine kidney cells it was established, using conventional polyacrylamide gel electrophoresis, that the [^{14}C]methionine was incorporated into proteins. Experiments performed with free [^{35}S]-methionine instead of ε-*N*-[^{14}C]methionyllysine gave similar incorporation of the radiolabeled amino acid into proteins.

The presence of an enzymatic activity hydrolyzing the isopeptide bond was confirmed by digestion studies performed with homogenates of kidneys, intestine, and liver (Table IX). The enzyme(s) able to release methionine from methionylcasein was apparently present in higher quantity in the intestine than in the two other organs and may be membrane-bound, at least in the intestine as indicated by the method of preparation. Digestion of methionylcasein by trypsin prior to incubation with tissue homogenates gave similar results.

It was of interest to determine whether aminopeptidase could hydrolyze the isopeptide bond effectively. The results (Figure 1) demonstrated that aminopeptidase, soluble or membrane-bound as occurring in the intestinal brush border, was efficient in hydrolyzing the isopeptide bond of ε-*N*-methionyllysine. There were no substantial differences in effectiveness between porcine and rabbit aminopeptidases or the soluble and particulate form of the enzyme since the number of nanomoles of substrate hydrolyzed per aminopeptidase unit was essentially constant.

DISCUSSION

Casein was chosen as a model for a food protein because it is highly digestible, it has a limiting amount of methionine and it is commercially available at low cost. Moreover,

Table IX. Hydrolysis of the Isopeptide Bond by Tissue Homogenates[a]

tissue	methionylcasein	ε-*N*-[^{14}C]-methionyllysine
kidney homogenate	1560	1340
liver homogenate	2820	1920
intestine homogenate	5290	5980
supernatant	1240	560
pellet	3640	5160

[a] Extent of hydrolysis (micromoles of methionine) was determined after incubation for 15 h at 37 °C of substrate with tissue homogenate aliquots containing about 20 aminopeptidase units/assay as described in the test. The results are expressed on basis of the whole organ.

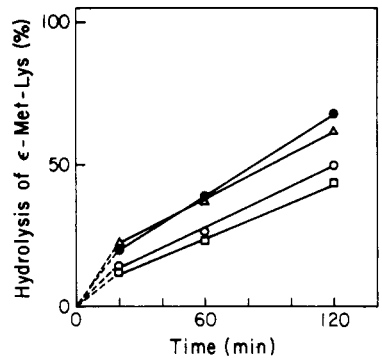

Figure 1. Rate of hydrolysis of ε-*N*-L-[*methyl*-^{14}C]methionyl-L-lysine by intestinal aminopeptidase. Solubilized enzymes from hog (44 units/assay; ●) or rabbit (34 units/assay; △) small intestine, and membrane-bound aminopeptidase from hog (32 units/assay; ○) and rat brush border (41 units/assay; □) were used.

since this protein has been widely used in feeding experiments with rats, it was considered ideal for studying the biological availability of amino acids covalently bound to the lysyl residues via isopeptide bonds. It must be stressed that the beneficial effect of adding a limiting essential amino acid would be greater with a protein even more deficient in this amino acid, such as a plant protein.

Quite efficient hydrolysis of the isopeptide bond, formed between the ε-amino groups of lysyl residues of casein and the carboxyl group of either methionine or glycine, was readily achieved in vivo. This observation suggests that essential amino acids covalently attached to the ε-amino group of lysyl residues of a food protein will be released from the protein in vivo and therefore are available for an animal.

The levels of free amino acids in plasma from rats fed casein modified by covalent attachment of glycine and methionine were normal, except for the increased level of methionine in the methionylcasein diet, in contrast to the

amino acid pattern of rats fed isopropylcasein. The unchanged level of glycine in the plasma of rats fed highly modified glycylcasein suggested that hydrolysis of the ϵ-*N*-glycyllysine bond was less efficient than with the methionyl derivative. This difference might be related to the diarrhea observed for the group of animals fed glycylcasein. The diarrhea, which was also observed when rats were fed isopropylcasein (Lee et al., 1978), may be a result of digestibility problems since ϵ-*N*-glycyllysine was able to almost completely replace lysine (Mauron, 1970). However, results obtained with the dipeptide may be completely different from those obtained with a protein because it is now well known that there are close relationships between peptide transport and hydrolysis (Ugolev, 1972; Matthews, 1975; Silk, 1977; Kim, 1977). The quite different properties of this casein derivative as compared to unmodified or methionylcasein may also be part of the explanation of the difference. The less efficient hydrolysis of the isopeptide bond of ϵ-*N*-glycyllysine as compared wiith ϵ-*N*-methionyllysine might also be related to the specificity of the enzyme responsible for the hydrolysis, as discussed later.

It is noteworthy that covalently bound methionine was about as readily available as the free amino acid. Therefore, covalent attachment of limiting essential amino acids to proteins appears to be an attractive approach for improving the nutritional value of food proteins. Such chemical modification of proteins would have a number of advantages. In addition to the biological availability of the added amino acid, it cannot be lost during processing. Moreover, the physical and functional properties of food proteins may be improved. The odor associated with addition of free methionine to foods, a result of methional formation (Ballance, 1961), is essentially eliminated when the amino acid is covalently bound to casein.

The nutritional similarities of L-methionine and *N*-acetyl-L-methionine (Boggs et al., 1975) were preserved when they were covalently attached to the lysyl residues of casein. The slight difference in the nutritive values of free and bound methionine could be the result of treatment of the protein with trifluoroacetic acid. Bound *N*-acetyl-L-methionine is also slightly less available than when added in the free form. This might be explained as above but in addition, some racemization may occur during activation of the carboxyl group of *N*-acetyl-L-methionine because of oxazolinone formation (Katsoyannis and Ginos, 1969) permitted by the acetyl group. The resulting *N*-acetyl-D-methionine is not available as a source of dietary methionine (Boggs et al., 1975). Although deteriorative reactions involving lysyl residues of proteins (Carpenter and Booth, 1973; Feeney et al., 1975; Feeney, 1977; Cheftel, 1977) may be essentially eliminated during processing and storage by covalent attachment of amino acids with protected amino groups, the presence of the protecting groups generally result in a decrease of the nutritional value of the protein.

The organ location of the enzymatic activity responsible for the hydrolysis of the isopeptide bond is of interest. Proteolytic enzymes of either gastric or pancreatic secretion were not able to hydrolyze the isopeptide bond. In contrast, isolated cells of kidneys, liver, and intestine as well as tissue homogenates, were quite efficient in hydrolyzing ϵ-*N*-methionyllysine. Since tissue homogenates released free methionine from methionylcasein before and after tryptic digestion it is concluded that an aminopeptidase rather than a dipeptidase (isodipeptidase) is involved in the hydrolysis.

More than 85% of the free methionine found in the plasma during in vivo digestion studies probably came from methionine covalently attached to the ϵ-amino group of lysyl residues. The relative concentration of intrinsic and added methionine was 1:6. Based on the primary structure of casein (Brunner, 1977), it would be difficult to explain how intrinsic methionine could be released without also releasing several other amino acids, namely glutamic acid, leucine, and proline. Yet, the level of these amino acids in the plasma was almost identical for casein and methionylcasein. Since most of the lysyl residues of casein were protected by methionyl groups, trypsin in the gut could cleave only arginyl bonds. The six–seven peptides formed by trypsin hydrolysis would favor further hydrolysis of peptide bonds. The major evidence that the isopeptide bonds are hydrolyzed is that lysine of the derivatized caseins was readily available to the rat.

Since the enzymatic activity hydrolyzing the isopeptide bond was particulate, at least as shown in this work for intestine, brush border aminopeptidase (Maroux et al., 1973) may be the enzyme involved in release of added methionine. Intestinal aminopeptidase is very abundant in the intestinal brush border (8% of the total proteins of the corresponding vesicles (Louvard et al., 1973) and accounts for practically all the peptidase activity of the absorptive membrane (Maroux et al., 1973). Aminopeptidase may also be involved in hydrolysis of the isopeptide bond of ϵ-*N*-acetylmethionyllysine after removal of the acetyl group by a specific enzyme (Birnbaum et al., 1952), although the organ location of this enzyme has not been clearly established (Rotruck and Boggs, 1975).

It is of interest that membrane-bound aminopeptidase appeared to be the single enzymatic activity of the brush border of porcine and rat jejunum and ileum able to hydrolyze the isopeptide bond effectively. Aminopeptidase, known to exist in other tissues, especially kidney and liver (Maroux et al., 1977; Garner and Behal, 1975), might be responsible for most of the observed isopeptidase activity. However, more detailed kinetic studies are necessary to preclude the existence of another type of enzyme, such as an isodipeptidase, able to catalyze the hydrolysis of an isopeptide bond more efficiently and with more specificity.

Finally, it is important to emphasize that although lysine is found bound primarily through its α-amino group in biological systems, its ϵ-amino group is also known to be involved in the formation of a few natural products (Schröder and Lübke, 1965). As a constitutive and widely distributed enzyme, aminopeptidase of the intestinal brush border appears to hydrolyze isopeptide bonds. Therefore, covalent attachment of amino acids to proteins through an ϵ-lysyl isopeptide bond may be seriously considered as a way to produce new products with higher nutritive value than the starting material.

ACKNOWLEDGMENT

The authors wish to thank D. Watts and B. Heyer for amino acid analysis, D. Sherman for the synthesis of ϵ-*N*-L-[*methyl*-^{14}C]methionyl-L-lysine, R. T. Crane and P. Ternahan for preparation of structurally intact hepatic parenchymal cells, D. Louvard for the conduct of the experiments with kidney cells, D. Filippi for hydrolysis studies of the isodipeptide, and H. S. Lee for helpful advice with the animal experiments. We are also grateful to Clara Robison for typing of the manuscript and Vicky Crampton for assistance in checking the references.

LITERATURE CITED

Altschul, A. M., *Nature (London)* **248**, 643 (1974).
Anderson, G. W., Zimmerman, J. E., Callahan, F. M., *J. Am. Chem. Soc.* **86**, 1839 (1964).
Ballance, P. E., *J. Sci. Food Agric.* **12**, 532 (1961).
Berry, M. N., Friend, D. S., *J. Cell Biol.* **43**, 506 (1969).

Birnbaum, S. M., Levinton, L., Kingsley, R. B., Greenstein, J. P., *J. Biol. Chem.* **194**, 455 (1952).
Bjarnason-Baumann, B., Pfaender, P., Siebert, G., *Nutr. Metab.* **21** (Suppl. 1), 170 (1977).
Blumberg, S., Vallee, B. L., *Biochemistry* **14**, 2410 (1975).
Boggs, R. W., Rotruck, J. T., Damico, R. A., *J. Nutr.* **105**, 326 (1975).
Boomgaardt, J., McDonald, B. E., *Can. J. Physiol. Pharmacol.* **47**, 392 (1969).
Brunner, J. R., in "Food Proteins", Whitaker, J. R., Tannenbaum, S. R., Eds., Avi, Westport, CT, 1977, p 175.
Canolty, N. L., Nasset, E. S., *J. Nutr.* **105**, 867 (1975).
Carpenter, K. J., Booth, V. H., *Nutr. Abstr. Rev.* **43**, 424 (1973).
Cheftel, J. C., in "Food Proteins", Whitaker, J. R., Tannenbaum, S. R., Eds., Avi, Westport, CT, 1977, p 401.
Christensen, H. N., *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **22**, 1110 (1963).
Crampton, R. F., in "Peptide Transport in Bacteria and Mammalian Gut", Associated Scientific Publishers, Amsterdam, 1972, p 1.
Dawson, R. M. C., Elliott, D. C., Elliott, W. H., Jones, K. M., "Data for Biochemical Research", 2nd ed, Oxford University Press, 1969.
Durrum Pico Buffer System IV Instruction Manual, Durrum Chem. Corp., Palo Alto, CA, 1973, p 1.
Eagle, H., *Science* **122**, 501 (1955).
Eagle, H., *Science* **130**, 432 (1959).
Edelhoch, H., *Biochemistry* **6**, 1948 (1967).
Feeney, R. E., in "Evaluation of Proteins for Humans", Bodwell, C. E., Ed., Avi, Westport, CT, 1977, p 233.
Feeney, R. E., Blankenhorn, G., Dixon, H. B. F., *Adv. Protein Chem.* **29**, 135 (1975).
Ferracci, H., "L'aminopeptidase intestinale de lapin", Thèse de Spécialité, Université de Provence, Marseille, 1977.
Fields, R., *Method Enzymol.* **25B**, 464 (1972).
Garner, C. W., Behal, F. J., *Biochemistry* **14**, 3208 (1975).
Glazer, A. N., Bar-Eli, A., Katchalski, E., *J. Biol. Chem.* **237**, 1832 (1962).
Gray, J. A., Olsen, E. M., Hill, D. C., Branion, H. D., *Can. J. Biochem.* **38**, 435 (1960).
Grosclaude, F., Mercier, J. C., Ribadeau-Dumas, B., *Eur. J. Biochem.* **14**, 98 (1970).
Halsted, C. H., Reisenauer, A., Back, C., Gotterer, G. S., *J. Nutr.* **106**, 485 (1976).
Hanks, J. H., Wallace, R. E., *Proc. Soc. Exp. Biol. Med.* **71**, 196 (1949).
Harper, A. E., *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **18** (Suppl. 3), 104 (1959).
Harper, A. E., in "Metabolic Adaptation and Nutrition", Scientific Publication No. 222, Pan American Health Organization, Washington, DC, 1971, p 8.
Harper, A. E., Hegsted, D. M., in "Improvement of Protein Nutriture", NAS–NRC, Washington, DC, 1974, p 184.
Hegsted, D. M., in "Nutrition", Vol. 1, Beaton, G. H., McHenry, E. W., Eds., Academic Press, Inc., New York, 1964, p 115.
Henry, K. M., Ford, J. E., *J. Sci. Food Agric.* **16**, 425 (1965).
Hirs, C. H. W., *J. Biol. Chem.* **219**, 611 (1956).
Howard, R. B., Pesch, L. A., *J. Biol. Chem.* **243**, 3105 (1968).
Itoh, M., Hagiwara, D., Kamiya, T., *Tetrahedron Lett.* **49**, 4393 (1975).
Katsoyannis, P. G., Ginos, J. Z., *Ann. Rev. Biochem.* **38**, 881 (1969).
Kim, Y. S., *Ciba Found. Symp.* **50**, 151 (1977).
Lee, H. S., Sen, L. C., Clifford, A. J., Whitaker, J. R., Feeney, R. E., *J. Nutr.* **108**, 687 (1978).
Louvard, D., Maroux, S., Baratti, J., Desnuelle, P., Mutaftschiev, S., *Biochim. Biophys. Acta* **291**, 747 (1973).
Maroux, S., Rovery, M., Desnuelle, P., *Biochim. Biophys. Acta* **56**, 202 (1962).
Maroux, S., Louvard, D., Baratti, J., *Biochim. Biophys. Acta* **321**, 282 (1973).
Maroux, S., Louvard, D., Vannier, C., Semeriva, M., *Biochem. Soc. Trans.* **5**, 523 (1977).
Matthews, D. M., in "Peptide Transport in Bacteria and Mammalian Gut", Associated Scientific Publishers, Amsterdam, 1972, p 71.
Matthews, D. M., *Physiol. Rev.* **55**, 537 (1975).
Mauron, S., *Int. Z. Vitaminforsch.* **40**, 209 (1970).
Meister, A., in "Biochemistry of the Amino Acids", Vol. 1, Academic Press, Inc., New York, 1965, p 201.
Milade, S., Hegsted, D. M., Saunders, R. M., Kohler, G. O., *Cereal Chem.* **49**, 119 (1972).
Moore, S., *J. Biol. Chem.* **238**, 235 (1963).
Morrison, A. B., Middleton, E. J., McLaughlan, J. M., *Can. J. Biochem.* **39**, 1675 (1961).
Muramatsu, K., Takeuchi, H., Sakurai, K., *J. Nutr. Sci. Vitaminol.* **19**, 277 (1973).
Osborne, T. B., Mendel, L. B., *J. Biol. Chem.* **17**, 325 (1914).
Puigserver, A. J., Sen, L. C., Clifford, A. J., Feeney, R. E., Whitaker, J. R., *Adv. Exp. Med. Biol.* **105**, 587 (1978).
Puigserver, A. J., Sen, L. C., Gonzales-Flores, E., Feeney, R. E., Whitaker, J. R., *J. Agric. Food Chem.*, in press (1979).
Rogers, Q. R., Harper, A. E., *J. Nutr.* **87**, 267 (1965).
Roncari, G., Zuber, H., *Int. J. Protein Res.* **1**, 45 (1969).
Rose, W. C., *Nutr. Abstr. Rev.* **27**, 631 (1957).
Rotruck, J. T., Boggs, R. W., *J. Nutr.* **105**, 331 (1975).
Schröder, E., Lübke, K., in "The Peptides", Vol. 1, Academic Press, New York, 1965, p 137.
Silk, D. B. A., *Ciba Found. Symp.* **50**, 15 (1977).
Slotboom, A. J., de Haas, G. H., *Biochemistry* **14**, 5394 (1975).
Spackman, D. H., Stein, W. H., Moore, S., *Anal. Chem.* **30**, 1190 (1958).
St. Angelo, A. J., Conkerton, E. J., Dechary, J. M., Altschul, A. M., *Biochim. Biophys. Acta* **121**, 181 (1966).
Stewart, J. M., Young, J. D., "Solid Phase Peptide Synthesis", W. H. Freeman, San Francisco, CA, 1969.
Ugolev, A. M., in "Peptide Transport in Bacteria and Mammalian Gut", Associated Scientific Publishers, Amsterdam, 1972, p 123.
Walsh, K. A., *Method Enzymol.* **19**, 41 (1970).
Yamashita, M., Arai, S., Tsai, S. J., Fujimaki, M., *J. Agric. Food Chem.* **19**, 1151 (1971).
Yamashita, M., Arai, S., Fujimaki, M., *J. Food Sci.* **41**, 1029 (1976).

Received for review April 30, 1979. Accepted August 14, 1979. A preliminary report of some of the work was presented at the 62nd Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, NJ, 1978 [*Fed. Proc., Fed. Am. Soc. Exp. Biol.* **37**, 839 (1978); Abstr. 3273]. Financial support was received from Grant FD 00568 from the Food and Drug Administration, from the University of California, Davis (Hatch H-2850), and from the Centre National de la Recherche Scientifique, France.