ing a glutamic acid enriched plastein in a high yield. (2) Such a plastein, though nondialyzable, was of a low molecular nature compared with an ordinary plastein. (3) Both the high glutamic acid content and the low molecular nature of this plastein seemed to cause its water solubility and stability to heating. (4) It was demonstrated that the enriched glutamic acid residue had a significant contribution to several physicochemical properties of the enzymatically synthesized polypeptides. (5) A possibility is expected that the plastein reaction can be applied to create some new functional properties from proteins.

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An Investigation of N-Substituted Methionine Derivatives for Food Supplementation

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N-Substituted methionine derivatives were prepared and evaluated as possible nutritional supplements for vegetable protein based foods. Ten of the C_1–C_{18} *N*-acyl derivatives of methionine, *N*-carbamoylmethionine (methionine urea), methionine hydantoin (cyclic urea), and *N*-carbethoxymethionine were prepared. All of the derivatives, except the carbethoxy- and methionine hydantoin, were stable to hydrolysis at pH 7, 100°. The remaining compounds were tested for reaction with reducing sugar models (Strecker degradation) and found to be stable relative to methionine at pH 5.5, 100°, for 1 hr. Isolated enzymes were used to predict the biological availability of stable derivatives. *N*-Acetyl-L-methionine was hydrolyzed faster than the other *N*-acyl-L-methionine compounds by hog kidney acylase. *N*-Acetyl-L-methionine was stable to racemization in the presence of active acetylating agents. L-Methionine and *N*-acetyl-L-methionine were incorporated into foods and evaluated for organoleptic acceptability. At levels of 0.15–0.50% of the product, *N*-acetyl-L-methionine was acceptable. L-Methionine inclusion at a much lower level (0.05%) rendered the food unpalatable.

The potential use of vegetable proteins to replace animal protein in food products has been well documented (*Chem. Eng. News*, 1971; Meyer, 1971; Hammonds and Call, 1970). It has been suggested that formulated foods, when replacing foods that make significant nutrient contribution, should at least equal the nutrient value of the food replaced (Council on Foods and Nutrition, 1968; Johnson, 1972). Vegetable proteins are deficient in sulfur amino acids, methionine and cystine. Addition of methionine to relieve this deficiency frequently makes the foods unpalatable for human consumption (Kies and Fox, 1971; FAO, WHO, UNICEF, Protein Advisory Group, 1970; Beigler, 1969). These flavor effects are caused by Maillard and Strecker degradation reactions, which yield volatile sulfides (Hodge, 1967). Methional has been identified as a major product from the Strecker degradation of methionine (Ballance, 1961). This reaction involves the amino group of methionine and proceeds *via* formation of an addition compound (hydroxyamine), followed by loss of water to yield an imine (Hodge, 1967). We have synthesized a series of methionine derivatives in which the amino group has been substituted with electrophilic groups so that the compound cannot undergo Strecker degradation. The chemical stabilities and enzyme stabilities of these compounds have been studied to evaluate them as replacements for methionine in food systems. The most promising derivative, *N*-acetyl-L-methionine, was tested for organoleptic acceptability.

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EXPERIMENTAL SECTION

Synthesis. All compounds prepared gave correct C, H, N elemental analyses and infrared and nmr spectra. Chemical purity was further established by a single spot for each compound on silica gel thin-layer chromatography plates. Solvent A (BuOH–H_2O–HOAc, 80:20:20) and solvent B (benzene–THF–HOAc, 70:30:1) were used for elution and iodine was used for development of the plates.

Acylmethionine compounds were prepared by three modifications of acylation reactions.

Method A: Reaction of Methionine with an Acid Anhydride. *Example: N-Acetyl-L-methionine.* L-Methionine (300 g, 2.0 mol) in 2 l. of water was adjusted to pH 10 with sodium hydroxide and cooled to 5°. Acetic anhydride (212 g, 2.1 mol) was added dropwise while pH 10 was maintained by addition of sodium hydroxide. After addition (~2 hr), the solution was stirred for 2 hr at 5–10° and allowed to reach room temperature. The solution was concentrated to 1 l. by vacuum evaporation, acidified to pH 1.0 with 6 *N* hydrochloric acid, and extracted with ethyl acetate. The organic layer was dried with sodium sulfate and evaporated under vacuum to yield a white solid. The crude product was recrystallized from 2 l. of acetone and 4

l. of ether to yield 265 g (70% yield) of *N*-acetyl-L-methionine, mp 102–103°. A tlc of this product (solvent A) showed one spot (R_f 0.63; methionine, R_f 0.33). Optical rotation measurements indicated greater than 99% optical purity compared to an *N*-acetyl-L-methionine standard (Tanable Seiyaku Co., Osaka, Japan).

Method B: Reaction of Methionine with an Acid Chloride. *Example: N-Decanoyl-L-methionine.* L-Methionine (5.0 g) was placed in 50 ml of water, and 50% aqueous NaOH was added to adjust the pH to 10. The solution was cooled to 5°; then 0.034 mol of decanoyl chloride was added slowly dropwise. During the addition pH 9–10 was maintained with NaOH. The mixture was stirred at 5° for 2 hr at room temperature for 4 hr. The mixture was acidified to pH 2 with HCl and extracted with ethyl acetate. The organic layer was separated, water washed, and evaporated to afford 6.37 g of crude product. This was stirred in hexane, and the insolubles were filtered off. Two recrystallizations from petroleum ether–ethyl acetate gave 4.7 g (46% yield) of white solid: mp 87–90°; $[\alpha]_D$ −29.2°, (*c* 4, EtOH). The compound showed one spot by tlc (solvent B, R_f 0.81).

Method C: Reaction of Methionine Ethyl Ester with an Acid Chloride. *Example: N-Stearoyl-L-methionine.* Methionine ethyl ester hydrochloride was prepared by refluxing an ethanolic solution of methionine that had been saturated with hydrogen chloride. The hydrochloride (15.0 g, 0.07 mol) was dissolved in 50 ml of water and adjusted to pH 10 with potassium carbonate solution. To this solution stearoyl chloride (20 g, 0.067 mol) in 100 ml of chloroform was added dropwise at room temperature. The chloroform layer was separated, dried, and evaporated under vacuum. The residue was dissolved in 75 ml of 1 *N* NaOH and 100 ml of dioxane, refluxed for 1 hr, cooled, and adjusted to pH 2.0 with 6 *N* hydrochloric acid. The precipitate was filtered and washed with water. The residue (26 g) was dissolved in 150 ml of hexane–ether (1:1) and crystallized at 5° to provide 13 g (47% yield) of *N*-stearoyl-L-methionine: mp 80–82°; $[\alpha]_D$ −18.8° (*c* 4, HOAc).

***N*-Carbethoxy, *N*-Carbamoyl, and Hydantoin Derivatives of Methionine.** α-Isocyanatomethionine ethyl ester was prepared by reaction of methionine ethyl ester hydrochloride with phosgene according to the method of Humphlett and Wilson (1961). From this intermediate the carbethoxy and carbamoyl derivatives were prepared by reaction with ethanol and ammonia, respectively. The cyclic urea (hydantoin) was prepared by the reaction of methional, ammonium hydroxide, ammonium bicarbonate, and sodium cyanide (Brzozowski, 1959).

Strecker Degradation Determination. Methionine or a methionine derivative (0.01–5 mmol) was mixed with diacetyl or ninhydrin (1–10 mmol) in water or 80% diglyme [bis(2-methoxyethyl) ether]–20% water at 100° for 1 hr. Volatile products were collected in cold traps or were trapped in sodium bisulfite solution. The products were analyzed by gas–liquid chromatography (glc) of the condensates or by titration of the bisulfite solutions (Hunter and Potter, 1958). To obtain a more sensitive analysis, L-[^{35}S]methionine, *N*-[^{35}S]acetyl-L-methionine, and *N*-[^{35}S]formyl-L-methionine were prepared and allowed to react with ninhydrin in a Na_2HPO_4–NaH_2PO_4 buffer (0.1 *M*, pH 7.0) at 100° for 1 hr. Volatile products were swept with nitrogen into three successive mercuric chloride (2%) traps whose contents were later counted for radioactivity.

Organoleptic Threshold Values Determination. A dispersion of 0.4 g of either *N*-acetyl-L-methionine or L-methionine, 20 g of vegetable protein, and 4 g of fat in 70 ml of water was heated at 70° for 1 hr to heat-set the protein. After cooling, each sample was evaluated and compared to a similar sample which did not contain the test compound.

Enzymatic Hydrolysis with Acylase. *N*-Acyl-L-methionine (5 mmol) in 10 ml of Na_2HPO_4–NaH_2PO_4 buffer (0.1 *M*, pH 7.0) was incubated at 37° with 0.2 mg of acylase enzyme (hog kidney, Catalog No. 05700-2143, Mann Research Laboratories, New York, N. Y.). Hydrolysis was followed by analysis for α-amino acids (methionine) using the ninhydrin colorimetric method (Moore and Stein, 1948). Long-chain derivatives were emulsified by addition of equimolar amounts of sodium taurocholate. Sodium taurocholate did not affect the rate of hydrolysis of *N*-acetyl-L-methionine in control experiments.

Enzymatic Hydrolysis with Urease. *N*-Carbamoylmethionine or the hydantoin of methionine (5 mmol) in 100 ml of Na_2HPO_4 buffer (0.1 *M*, pH 7.0) was incubated at 37° with 0.1 mg of urease powder (jack bean meal, Catalog No. 05300-2388, Mann Research Laboratories). Hydrolysis was followed by the ninhydrin colorimetric method (Moore and Stein, 1948).

Hydrolysis. *N*-Acylmethionines (10 mmol) in 100 ml of Na_2HPO_4 buffer (0.1 *M*, pH 7.0) were heated under the conditions specified in Table II. Hydrolysis was followed by the ninhydrin procedure (Moore and Stein, 1948). *N*-Formyl-, acetyl-, and propionyl-L-methionine were hydrolyzed in excess 0.1 *N* hydrochloric acid at 60°.

Optical Rotation and Racemization Studies. Optical rotations were measured on a JASCO Model ORD/UV-5 spectropolarimeter. Optical rotatory dispersion curves for *N*-acetyl-L-methionine and *N*-acetyl-D-methionine were determined. For the racemization studies specific rotations were routinely measured at 300 nm since they are approximately two and one-half times as large as the specific rotations at 589 nm (Table I).

Racemization studies were made in aqueous solution initially adjusted to pH 2, 6, 8, or 10 with hydrochloric acid or sodium hydroxide. Acetic anhydride was added, and the solution was stirred at 37° for 1 hr. Optical rotations were measured either directly on the reaction solution or on the *N*-acetylmethionine recovered by extraction with ethyl acetate. No significant optical rotation changes were noted. If the initial pH was adjusted to 12 and acetic anhydride was added, complete racemization was observed within 1 hr (DuVigneaud and Meyer, 1932).

Racemization was also studied at pH 7 in Na_2HPO_4–NaH_2PO_4 buffer with the addition of catalytic amounts of acetyl CoA and acetyl phosphate. No change in optical rotation was observed. Addition of equimolar acetylimidazole gave complete racemization within 1 hr.

Oxidation. A 100-g sample of *N*-acetyl-D,L-methionine was dissolved in 500 ml of water and air was bubbled through the solution for 24 hr. The effluent stream was passed through a Porapak collection column and then into a glc–mass spectrometry system to analyze for sulfur-containing volatiles. Dimethyl disulfide, mol wt 94, was identified as a major product. Another peak was identified as methional.

RESULTS AND DISCUSSION

Ten of the C_1–C_{18} *N*-acylmethionine derivatives, *N*-carbamoylmethionine, methionine hydantoin, and *N*-carbethoxymethionine were prepared (Table I). These compounds were chosen because they were expected to be stable under cooking conditions. Their susceptibilities to hydrolysis and to Strecker degradation were determined. Each compound was also tested to see whether it could be hydrolyzed by enzymes present in mammalian systems. The most promising derivative of methionine was tested for organoleptic acceptability in food analog products.

Three methods were used to prepare *N*-acylmethionine derivatives. Method A involved the reaction of methionine with an equivalent amount of an acid anhydride in aqueous alkali at 5–10°. This method gave good yields (65–75%) of optically pure products but was useful only with water-soluble anhydrides. Method B involved the same aqueous alkali system except an acid chloride was used as the acylating agent. This system was useful for making

Table I. Synthesis and Physical Properties of *N*-Substituted Methionine Derivatives

N-Acylmethionine	Preparative method	Yield, %	Mp, °C	$[\alpha]_D$
DL-Formyl-	A[d]	60	99–100	
L-Formyl-	A[d]	56	99–100	−9.75[a]
DL-Acetyl-	A	77	111–112	
L-Acetyl-	A	70	102–103	−21.4[a,e]
D-Acetyl-	A	65	100–102	+19.9[a]
L-Propionyl-	A	66	Oil	−21.1[a]
L-Butyryl-	C	51	Oil	+6.0[b]
DL-Citraconyl-	A	42	125	
L-Decanoyl-	B	46	87–90	−29.2[c]
DL-Palmitoyl-	B	37	81–82	
L-Palmitoyl-	B	42	75–77	N.D.
DL-Oleoyl-	B	33	Oil	
L-Oleoyl-	B	35	Oil	N.D.
L-Stearoyl-	C	47	80–82	−18.8[b]
L-Linoleoyl-	C	48	Oil	N.D.
Methionine urea and carbethoxy derivatives				
N-Carbethoxy-DL-methionine		92	Oil	
N-Carbamoyl-DL-methionine		21	94–96	
DL-5-(β-Methylmercaptoethyl)-hydantoin		43	104	

[a] Rotations were measured in water at a concentration of 4 and pH 2. [b] Rotation measured in acetic acid, *c* 4. [c] Rotation measured in ethanol, *c* 4, pH 2. [d] These reactions were conducted in 95% formic acid using the mixed anhydride of formic and acetic acid. [e] A rotation of −55.4° was measured for *N*-acetyl-L-methionine at 300 nm (*c* 4, H_2O).

Table II. Enzymatic and Chemical Hydrolysis of *N*-Acylmethionine Derivatives

N-Acylmethionines	% chem hydrol.[a]	% enzym. hydrol.[b]
Acetyl-	0.4	95
Decanoyl-		55
Palmitoyl-	0.1	4
Oleoyl-		3
Stearoyl-		
Carbamoyl-	0.3	0

[a] Chemical hydrolysis conducted at 100°, pH 7 for 1 hr on *N*-acyl-DL-methionine derivatives. [b] Enzymatic hydrolysis at 37°, 1 hr with 0.2 mg of acylase enzyme on *N*-acyl-L-methionine derivatives. If the enzyme quantity is increased to 0.6 mg, significant hydrolysis (~60%) of palmitoyl-, oleoyl-, and stearoyl-L-methionine can be achieved.

Table III. Strecker Degradation of Methionine and *N*-Acylmethionine Compounds[a]

Compounds	% Methional	% Radioact.
Methionine	70–100	24
Formylmethionine		0.4
Acetylmethionine	0.5	0.1
Palmitoylmethionine	0.1	

[a] *N*-Acyl-D,L-methionines were used in experiments to trap methional. *N*-[^{35}S]Acyl-L-methionines used for radioactivity experiments (see Experimental Section for details).

medium (C_3) to long-chain (C_{18}) *N*-acylmethionine in moderate yields (30–60%). Optically pure products were readily isolated by this procedure. Method C involved the reaction of an acid chloride with methionine ethyl ester in an aqueous or mixed aqueous–organic solvent. The resulting *N*-acylmethionine ethyl ester was saponified to the desired *N*-acylmethionine. This method was used when the carboxylic acid impurity formed in method B could not be removed from the desired product by simple extraction or recrystallization. Some racemization occurred in this procedure, and the products had to be purified by recrystallization or column chromatography. Moderate yields of 40–50% were realized by this method.

The extent of hydrolysis of the methionine derivatives was determined at pH 7 (Table II). All *N*-acylmethionines were relatively stable to hydrolysis even when heated to 100° for 1 hr. The relative rate constants for hydrolysis of *N*-formyl-, *N*-acetyl-, and *N*-propionylmethionine were measured to be 57:1:0.6 at pH 1, 60°. These compounds followed pseudo-first-order kinetics under these conditions. The fast hydrolysis of the *N*-formyl derivative compared to the *N*-acetyl or *N*-propionyl derivatives may indicate stability problems under usage conditions. The rates of hydrolysis of *N*-acetyl- and *N*-propionylmethionine are similar and are much less than the rate of formylmethionine. *N*-Acetyl-, *N*-propionyl-, and longer-chain *N*-acylmethionines should be stable to chemical hydrolysis under use conditions. There is no large advantage in hydrolytic stability in using an *N*-acylmethionine of chain length greater than acetyl.

N-Carbamoylmethionine (urea) was stable to hydrolysis, but methionine hydantoin and the carbethoxy derivative were partially converted to methionine and other derivatives under the hydrolysis conditions. Further study of these derivatives was not warranted.

The *N*-acylmethionines were tested in a model Strecker degradation reaction such as might occur under cooking conditions. The results from some of these experiments are presented in Table III. Acylation of methionine greatly inhibited the formation of methional, even under the experimental conditions which favor methional formation. Trace amounts of methional were formed from *N*-acetyl-, *N*-formyl-, and *N*-palmitoylmethionine, probably because small amounts of methionine became available by hydrolysis of the derivative.

If a compound is to replace methionine as a nutritional supplement in foods, it should provide methionine to support animal growth and development. Kidney acylase is known to hydrolyze *N*-acetyl-L-methionine to methionine and acetate (Birnbaum *et al.*, 1952). Several acyl-L-methionines were tested *in vitro* using kidney acylase (Table II). Under the conditions of our hydrolysis, *N*-acetyl-L-

methionine gave the fastest rate of hydrolysis, with essentially quantitative conversion to methionine in 0.5–1 hr. Activity dropped off dramatically with increasing chain length; *N*-stearoylmethionine showed no hydrolysis after 1 hr with low concentrations of acylase. The long-chain derivatives can be hydrolyzed at reasonable rates at higher enzyme concentrations. From these results we predict that *N*-acetyl-L-methionine will be cleaved by acylase *in vivo* at a rate fast enough to give complete biological availability. The long-chain derivatives, although cleaved at a slower rate, may also be of biological value.

The carbamoyl and carbethoxy analogs of methionine completely resisted hydrolysis.

The optical stability of *N*-acetyl-L- and *N*-acetyl-D-methionine was investigated. Optically active *N*-acylamino acids racemize *via* an azalactone intermediate (Bergmann and Zervas, 1928). Racemization is accelerated by acetylating agents such as acetic anhydride. Simply exposing either *N*-acetyl-L- or *N*-acetyl-D-methionine to a range of pH from 2 to 12 at 25–100° for 1 hr gave no detectable racemization. Addition of 1 equiv of acetic anhydride at 37° to *N*-acetyl-L-methionine at pH values of 2–10 did not cause racemization, but at pH 12 *N*-acetyl-L-methionine racemized completely within 30 min after addition of acetic anhydride.

From nutritional studies in rats it appears that racemization *in vivo* does not occur to a significant extent, since *N*-acetyl-L-methionine was utilized as well as L-methionine and *N*-acetyl-D-methionine was not utilized at all (Boggs *et al.*, 1974). Nevertheless, we checked the effect of "natural" acetylating agents, acetyl CoA, acetyl phosphate, and acetylimidazole (to imitate acetylhistidine). Only acetylimidazole caused racemization at 37° for 1 hr.

The final stability examination of acetylmethionine involved air oxidation. It was noticed that "aged" samples of acetylmethionine developed a slight sulfide odor. The volatile oxidation products from acetylmethionine were collected and analyzed by glc–mass spectrometry and found to contain a small amount of dimethyl disulfide. In preliminary tests this oxidation was inhibited by adding 0.1% of butylated hydroxyanisole or *tert*-butylhydroquinone.

All of the results indicate that *N*-acylmethionines, and especially *N*-acetyl-L-methionine, are practical substitutes for methionine in food products. *N*-Acetyl-L-methionine was compared to L-methionine in a vegetable protein based food system (Table IV). Organoleptic threshold results showed an approximate tenfold advantage for *N*-acetyl-L-methionine. L-Methionine imparted a strong cabbage note with objectionable aftertaste, while *N*-acetyl-L-methionine imparted a slight sulfur note.

Table IV. Organoleptic Evaluation of L-Methionine and *N*-Acetyl-L-methionine

Compound	Level of incorp., % product	Organoleptic acceptability
L-Methionine	0.02	6/8 unacceptable
L-Methionine	0.05	8/8 very unacceptable
N-Acetyl-L-methionine	0.15	Not detected
N-Acetyl-L-methionine	0.50	1/6 slightly unacceptable

Calculations based on adding L-methionine or *N*-acetyl-L-methionine to vegetable protein show that approximately 1.5% of L-methionine or *N*-acetyl-L-methionine relative to the weight per cent of the protein should be added. For a food product containing 10% protein, 0.15% of L-methionine should be added for nutritional supplementation. Since L-methionine is easily detected at one-third this level it is clearly unacceptable. It appears that *N*-acetyl-L-methionine can be used at 0.15% of the product without sensory detection.

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