(XIII) picrate as yellow needles (1.68 g.) which were recrystallized from ethanol; m.p. 140–141°, solidifying at 142° and remelting at 173–175°.

Anal. Calcd. for $C_7H_{11}N_2O_2 \cdot C_6H_3N_3O_7$: C, 42.17; H, 3.81; N, 15.13. Found: C, 42.38; H, 3.86; N, 15.13.

The picrate (500 mg.) was dissolved in 3 *N* hydrochloric acid and extracted 5 times with benzene. The colorless aqueous layer was evaporated *in vacuo* and the residue (250 mg.) was thoroughly dried and recrystallized from methanol–acetone to give the hygroscopic hydrochloride of 3,4-dehydro-DL-stachydrine, m.p. 180–182° dec. Its n.m.r. spectrum in D_2O shows a peak for two olefinic protons and two singlets for the non-equivalent N-methyl groups.

Conversion of 3,4-Dehydrostachydrine (XIII) into 2,3-Dehydrostachydrine (XII).—A tube containing 3,4-dehydrostachydrine (XIII) picrate (20 mg.) was placed in an oil-bath at 150°. When the compound had melted and resolidified, it was cooled and crystallized from ethanol yielding yellow plates of 2,3-dehydrostachydrine (XII) picrate, m.p. 173–175°, having an infrared spectrum identical with that of the authentic sample. The infrared spectra of the isomeric picrates show noticeable differences, as do those of the two hydrochlorides in KBr.

[CONTRIBUTION FROM THE NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, NATIONAL INSTITUTES OF HEALTH, BETHESDA 14, MD.]

Alkylation and Cleavage of Methionine Peptides

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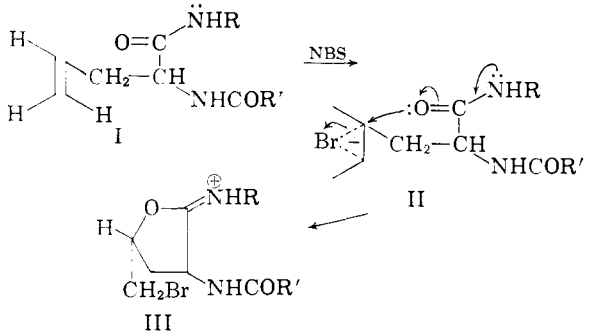
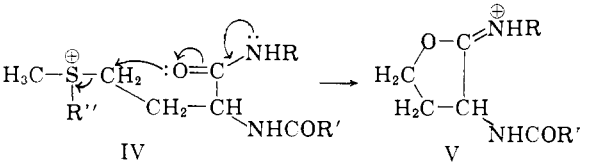
RECEIVED NOVEMBER 15, 1961

Intramolecular participation of the peptide carbonyl in the displacement of the sulfur function in sulfonium salts of methionine peptides has been utilized for peptide cleavage. Alkylation and decomposition of methionine peptides has been studied with regard to optimal conditions and the effect of the nature of the alkylating agent on the yields in the cleavage reaction.

The oxidative cleavage of peptides of tryptophan,[2] tyrosine,[3] histidine[4] and allylglycine[5] appears to proceed through participation of the C-peptide bond in the ring opening of a labile bromonium intermediate (I–III).[6]



In a peptide of a methioninesulfonium derivative IV, participation of the C–peptide group is easily possible by 1,5-interaction, concerted elimination of the sulfur function in the γ-position and formation of a homoserine (imino)lactone (V).[7]



The literature records numerous instances of such a breakdown of methioninesulfonium salts leading to homoserine or its lactone. However, the intramolecular assistance from the methionine carboxyl group in these elimination reactions has not always been properly recognized.

The decomposition of N-formylmethioninemethylsulfonium acetate to α-formamido-γ-butyrolactone on evaporation of an aqueous solution to dryness *in vacuo* implies an internal displacement of methyl sulfide by the carboxylate ion.[8] The sulfonium salt, derived from bis-(2-chloroethyl) sulfide and methionine, when heated in aqueous solution at 100° for several hours, yielded largely methionine and a small amount of homoserine.[9]

A heat-labile principle of cabbage juice was found to be a methioninemethylsulfonium salt giving rise to homoserine when autoclaved in water.[10] Homoserine was obtained from the methioninemethylsulfonium salt isolated from asparagus when boiled with alkali.[11] In the decomposition of methioninemethylsulfonium bromide in acid solutions methionine was regenerated, whereas in hot neutral and alkaline solutions homoserine and dimethyl sulfide were formed.[12]

The presence of homoserine, homoserine lactone and S-carboxymethylhomocysteine in addition to methionine, among the products of acid hydrolysis of ribonuclease, 50% inactivated by iodoacetate at *p*H 2.8, indicated elimination of the sulfur function of methionine.[13] The same prod-

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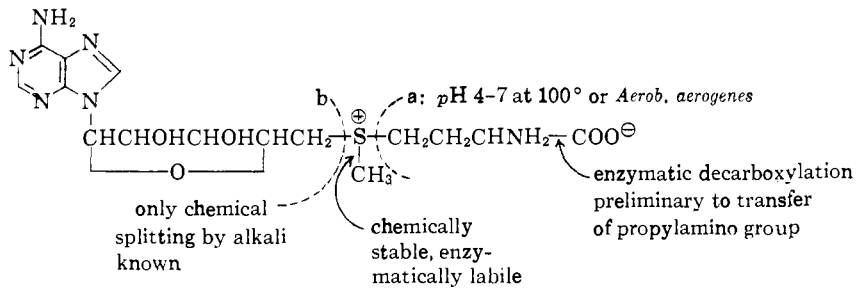

VI

ucts in varying proportions were observed in the decomposition of methioninecarboxymethylsulfonium iodide at 100° as a function of *p*H.[14] It was suggested in these instances that the homoserine lactone arose from homoserine and not directly by an internal displacement reaction.

The instability of S-adenosylmethionine (VI) and its modes of reaction have been studied extensively.[15–17]

Heating a slightly acid or neutral aqueous solution for 30 minutes gives nearly quantitative yields of 5′-methylthioadenosine and homoserine. Homoserine lactone occurs among the products of similar hydrolyses. The lactone must have been produced directly by an intramolecular displacement since it is not formed from homoserine under these conditions.[18] These products arise by the rupture of bond a. Enzymatic degradation of S-adenosylmethionine by cell-free extracts of *Aerobacter aerogenes* led to 5′-methylthioadenosine and homoserine lactone,[19] while the S-adenosylmethionine cleaving enzyme of bakers' yeast produces homoserine lactone directly by an intramolecular displacement and not *via* 2-amino-3-butenoic acid.[20]

This intramolecular elimination *via* methioninesulfonium salts has now been developed into a method for the specific cleavage of methionine peptides (VII–X). In the first step a methionine peptide reacts with a suitable alkylating agent to form a sulfonium salt which is degraded by heating in aqueous solution. The yields of glycine ethyl ester obtained from N-acetyl-DL-methionylglycine ethyl ester under the same conditions of alkylation and cleavage with various halides are summarized in Table I. Alkylating agents containing electron-withdrawing groups, such as CO-OEt and $CONH_2$, facilitate the intramolecular displacement. Iodoacetamide, which gave the best yield of liberated amino acid, was used in subsequent experiments.

The degree of alkylation was measured by argentometric titration according to the method of Toennies and Kolb.[22] In order to determine the time required for alkylation of L-methionine, aqueous solutions, 0.05 *M* in amino acid and 0.16 *M* in iodoacetamide, were kept at 0°, 25° and 37.5°. The results of titrations of aliquots at various times shown in Table II indicate that, after 48 hours, alkylation had occurred to the extent of 48% at 0° and 93% at 25°. At 37.5° alkylation was complete after 21 hours.

TABLE I

INFLUENCE OF ALKYL GROUP ON THE CLEAVAGE OF SULFONIUM SALTS DERIVED FROM N-ACETYL-DL-METHIONYLGLYCINE ETHYL ESTER

Alkylating agent	Concn. of peptide in reacn. mixt.,[a] *M*	Equiv. of alkylating agents	% of peptide cleavage[b]
Iodoacetic acid[c]	0.005	3	6.0
Methyl iodide[c,d]	.01	4	3.6
Ethyl bromoacetate[c,d]	.01	4	43
Iodoacetamide[e]	.01	3	53
2,5-Dinitrofluorobenzene[e]	.01	4	0
Diethyl bromomalonate[e]	.01	3	5

[a] Reactions were allowed to proceed at room temperature for 24 hr. [b] After removal of excess of alkylating agent by ether extraction the reaction mixture was heated for 1 hour at 100°. The liberated amino acid was determined by ninhydrin assay.[21] [c] Reaction medium was 0.1 *M* *p*H 3 citrate buffer. [d] Alkylation was conducted in a sealed tube. [e] Reaction medium was 1:1 mixture of EtOH and 0.1 *M* *p*H 3 citrate buffer.

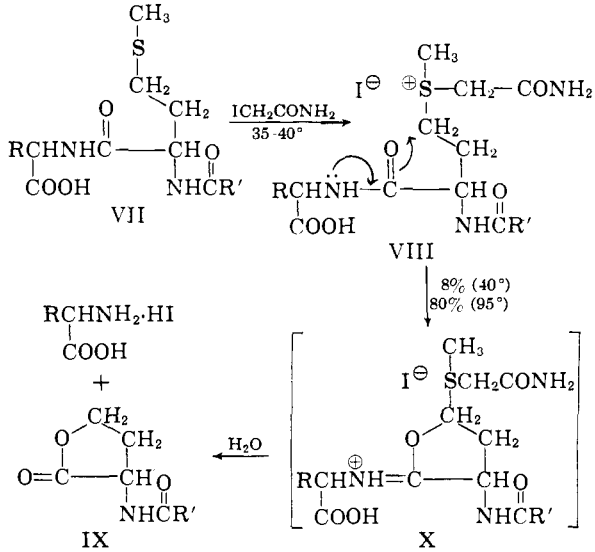

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TABLE II

FORMATION OF SULFONIUM SALT IN THE REACTION OF L-METHIONINE WITH IODOACETAMIDE AS A FUNCTION OF TIME AND TEMPERATURE

Reacn. temp., °C.	% of alkylation after 4 hr.	8 hr.	21 hr.	24 hr.	48 hr.
0	7.6	9.4	14	16	35
25	34	54	79	83	93
37.5	59	85	100	..	..

In a reaction mixture of L-methionine (0.1 *M*) and iodoacetamide (0.3 *M*) which had been kept at 38° for 31 hours, homoserine, sulfonium salt (decomposed during drying at 80°) and homoserine lactone were present as shown by paper electrophoresis and by chromatography in three different solvent systems. Homoserine does not lactonize under these conditions or on electrophoresis. Therefore, homoserine lactone must have been formed by an intramolecular displacement reaction.

The cleavage procedure was applied to model dipeptides, and the course of alkylation followed by argentometric titration. With over 90% formation of the carbamylmethylsulfonium salts at 40°, peptide cleavage occurred to the extent of 8% during alkylation (Table III). Analysis of such mixtures by the ninhydrin method[21] indicated 54–85% cleavage of the peptide bond (Table III). Brief heating prior to analysis increased slightly the yield of liberated amino acid.

The sulfonium salt of N-benzoylmethionylglycine ethyl ester was heated at 95° for one hour and then subjected to amino acid analysis according to Spackman, Stein and Moore.[23] The results are included in Table III.

Toennies and Kolb[8] had noted spontaneous decomposition of N-formylmethioninemethylsulfonium acetate on concentration of an aqueous solution with formation of α-formamido-γ-butyrolactone. A similar ease of decomposition was not observed with the halide salts. To determine whether anions such as acetate or nitrate facilitate intramolecular decomposition of sulfonium salts, such salts of methionine peptides were prepared and cleaved concurrently with the corresponding iodides. The iodides, acetates and nitrates with and without heating before analysis gave comparable cleavage yields (Table IV).

Experimental

Melting points are corrected. Electrophoresis: Schleicher and Schuell paper #598; 1000–1200 v. at *p*H 1.9 (acetic acid–formic acid–water, 150:50:800) and *p*H 6.5 (acetic acid–pyridine–water, 10:100:890). Paper chromatography: ascending technique; Schleicher and Schuell paper #598; solvent systems: A, ethanol–water, 75:25; B, *t*-butyl alcohol–formic acid–water, 70:15:15; C, 1-butanol–acetic acid–water, 4:1:1.

Acetyl-DL-methionylglycine Ethyl Ester.—To a cooled solution (−15°) of 955 mg. (0.005 mole) of acetyl-DL-methionine and 505 mg. (0.005 mole) of triethylamine in 50 cc. of absolute tetrahydrofuran was added 540 mg. (0.005 mole) of ethyl chlorocarbonate. After 15 min., 698 mg. (0.005 mole) of glycine ethyl ester hydrochloride and 505 mg. of triethylamine (0.005 mole) dissolved in 5 cc. water was added with agitation. Tetrahydrofuran was evaporated *in vacuo* and the residue taken up in 50 cc. of 1-butanol. Unchanged peptide components were extracted with three 5-cc. portions of 5% ammonium carbonate and 5% acetic acid, respectively. The solution was washed with water and the butanol evaporated *in vacuo*. The residue was taken up in water and lyophilized, leaving 852 mg. (62% of theory) of acetyl dipeptide ester, m.p. 97°. Recrystallization from ethyl acetate–ether (1:5) gave colorless needles, m.p. 114°.

Anal. Calcd. for $C_{11}H_{20}N_2O_4S$: C, 47.82; H, 7.30; N, 10.14; S, 11.61. Found: C, 47.78; H, 7.20; N, 10.16; S, 11.38.

Benzoyl-DL-methionylglycine Ethyl Ester.—To a mixture of 2.8 g. (0.02 mole) of glycine ethyl ester hydrochloride, 2.8 ml. of triethylamine and 5.06 g. (0.02 mole) of benzoyl-DL-methionine[24] in 75 ml. of dichloromethane, a solution of 4.2 g. (0.02 mole) of dicyclohexylcarbodiimide in 50 ml. of dichloromethane was added and the mixture was stirred

TABLE III

ALKYLATION AND CLEAVAGE OF METHIONYL PEPTIDES

	Time of alkylation, hr.	Degree of alkylation by titration, %	% of peptide cleavage
Carbobenzyloxy-L-methionyl-L-glutamic acid[a,b]	68[c]	92	8.1[d]
	135[e]	91	80[f]
	135	91	84[g]
	135	91	85[h]
	135	91	81[i]
Benzoyl-DL-methionylglycine[a,b]	68[c]	99	8.6[d]
	72[j]	97	49[k]
Benzoyl-DL-methionylglycine ethyl ester[a,b]	135[e]	93	54[f]
	135	93	63[g]
	135	93	65[h]
	135	93	62[i]
Carbobenzyloxy-L-methionyl-L-tyrosine[a,b]	68[c]	92	7.9[d]
	65[c]	84	84[h]

[a] Concentration in ethanol–water (50% v./v.) was 2 × 10^{-2} *M*. [b] Three equivalents of alkylating agent was used. [c] Temperature of alkylation was 40°. [d] Reaction mixture was fractionated on columns of Amberlite IR-120 and the cleaved amino acid determined with automatic recording equipment.[23] [e] Temperature of alkylation was 35–40°. [f] Reaction mixture was analyzed directly by ninhydrin method.[21] [g] Reaction mixture was heated for 15 minutes at 95° before analysis.[21] [h] Reaction mixture was heated for 1 hr. at 95° before analysis.[21] [i] Reaction mixture was extracted with ether, heated for 1 hr. at 95° and analyzed.[21] [j] Temperature of alkylation was 38°. [k] Reaction mixture was heated for 1 hr. at 95° before fractionation on columns of Amberlite IR-120 and analysis with automatic recording equipment.[23]

TABLE IV

Peptide	% of peptide cleavage[a] — Iodide: Analyzed directly	Iodide: Heated[b] before analysis	Acetate: Analyzed directly	Acetate: Heated[b] before analysis	Nitrate: Analyzed directly	Nitrate: Heated[b] before analysis
Carbobenzyloxy-L-methionyl-L-glutamic acid[c]	79	87	82	81	81	86
Benzoyl-DL-methionylglycine ethyl ester[c]	40	63	46	66	47	68
Carbobenzyloxy-L-methionyl-L-tyrosine[c]	74	92	77	85	77	85

[a] Determined by ninhydrin assay of the released amino acid.[21] [b] One hour at 95°. [c] Alkylation mixture was ethanol–water (1:1 by vol.) solution which was 0.02 *M* in peptide and 0.06 *M* in iodoacetamide. Alkylation temperature was 40°. Alkylations were 90–100% complete by titration.

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overnight. After filtering the dicyclohexylurea from the mixture, the solution was extracted with 100 ml. of 1 *N* hydrochloric acid and 100 ml. of 1 *N* potassium bicarbonate, dried over sodium sulfate and evaporated to dryness. Crystallization of the residue from benzene–cyclohexane yielded 5.5 g. of benzoyl-DL-methionylglycine ethyl ester, m.p. 113–116°; after recrystallization from 95% ethanol, m.p. 118–120°.

Anal. Calcd. for $C_{16}H_{22}N_2SO_4$: C, 56.78; H, 6.55; N, 8.28. Found: C, 56.35; H, 6.59; N, 8.25.

Benzoyl-DL-methionylglycine.—A mixture of 1.0 g. (0.003 mole) of benzoyl-DL-methionylglycine ethyl ester, 4 ml. of 1 *N* sodium hydroxide and 10 ml. of water was stirred for 2 hours at room temperature and then allowed to stand overnight. The slightly turbid mixture was filtered and the filtrate acidified with 6 *N* hydrochloric acid. The colorless crystals were separated by centrifugation, washed with cold water and dried *in vacuo;* 0.76 g., m.p. 175–177°. Recrystallization from ethyl acetate–petroleum ether (b.p. 60–70°) gave 0.51 g. of benzoyl-DL-methionylglycine, m.p. 177–179°.

Anal. Calcd. for $C_{14}H_{18}N_2O_4S$: C, 54.17; H, 5.84; N, 9.03; S, 10.33. Found: C, 54.43; H, 5.83; N, 8.84; S, 10.25.

Carbobenzyloxy-L-methionyl-L-tyrosine.—Seven grams (0.025 mole) of carbobenzyloxy-L-methionine[25,26] and 2.5 g. (0.025 mole) of triethylamine were dissolved in 20 ml. of dry tetrahydrofuran. The solution was cooled to −5° and treated with 3.4 g. (0.025 mole) of isobutyl chloroformate in 10 ml. of tetrahydrofuran.

The mixture was maintained at −5° for 5 minutes and then added to a mixture of 5.8 g. (0.025 mole) of L-tyrosine methyl ester hydrochloride[27] and 2.5 g. (0.025 mole) of triethylamine in 20 ml. of tetrahydrofuran. Concentration at reduced pressure yielded a viscous residue which was taken up with water and ethyl acetate. The ethyl acetate extract was washed with 3% sodium bicarbonate solution, water, 0.1 *N* hydrochloric acid and water and dried over sodium sulfate. The ethyl acetate was evaporated. Crystallization of the residue from ethyl acetate–petroleum ether (b.p. 60–70°) yielded 6.7 g. of carbobenzyloxy-L-methionyl-L-tyrosine methyl ester, m.p. 138–140°, $[\alpha]^{20}D$ +11.6° (*c* 2 in pyridine). Twenty ml. of 1 *N* sodium hydroxide was added to 2.65 g. (0.006 mole) of carbobenzyloxy-L-methionyl-L-tyrosine methyl ester. After standing at room temperature for 40 min. with occasional shaking the mixture was extracted with ethyl acetate, adjusted to *p*H 4 with 2 *N* hydrochloric acid and again extracted with ethyl acetate. The second extract was washed with water and concentrated at reduced pressure to an oil which was dissolved in boiling water and decolorized with charcoal. On standing, 1.85 g. of colorless needles separated from the solution; m.p. 141.5–143.5° (lit.[28] m.p. 137–138.5°).

Anal. Calcd. for $C_{22}H_{26}N_2O_6S$: C, 59.18; H, 5.87; N, 6.28; S, 7.17. Found: C, 58.90; H, 6.00; N, 6.25; S, 7.29.

Carbobenzyloxy-L-methionyl-L-glutamic Acid.—A solution of 3.0 g. (0.022 mole) of isobutyl chloroformate[29] in 10 ml. of dry tetrahydrofuran was added to a solution of 6.2 g. (0.022 mole) of carbobenzyloxy-L-methionine and 2.2 g. (0.022 mole) of triethylamine in 20 ml. of dry tetrahydrofuran cooled to −5°. After 15 min. of vigorous stirring 4.4 g. (0.022 mole) of L-glutamic acid diethyl ester[30] in 10 ml. of dry tetrahydrofuran was added to the mixture. Stirring was continued for 1 hour at −5°. The solvent was removed *in vacuo*. The residue was taken up with water and ethyl acetate. The aqueous phase was extracted twice with ethyl acetate. The combined extracts were washed with 1 *N* hydrochloric acid, 3% sodium bicarbonate solution and water, dried over sodium sulfate and concentrated *in vacuo*. Crystallization of the residue from ethyl acetate–petroleum ether (b.p. 60–70°) gave 3.6 g. of colorless crystals of the peptide diester, which was dissolved in 25 ml. of methanol and saponified with 16 ml. of 1.0 *N* sodium hydroxide. Acidification of the solution with hydrochloric acid yielded an oil which was extracted with ethyl acetate. The extract was washed with water, dried over sodium sulfate and concentrated *in vacuo*. Rubbing of the semi-solid residue with several changes of petroleum ether (b.p. 60–70°) produced crystals which on recrystallization from ethyl acetate–petroleum ether (b.p. 60–70°) gave 1.4 g. of carbobenzyloxy-L-methionyl-L-glutamic acid, m.p. 139–141° (lit.[26] m.p. 138–140°).

Anal. Calcd. for $C_{18}H_{24}N_2O_7S$: C, 52.42; H, 5.87; N, 6.79; S, 7.77. Found: C, 52.44; H, 6.17; N, 6.57; S, 7.62.

Alkylation of Methionine with Iodoacetamide.—In order to determine the time required for alkylation of methionine by iodoacetamide, three solutions were prepared each containing 0.075 g. (0.5×10^{-3} *M*) of L-methionine, 0.301 g. (1.6×10^{-3} *M*) of iodoacetamide per 10 ml. Three different temperatures of alkylation were investigated, namely, 37.5°, 25° and 0°. One-ml. aliquots were withdrawn from each flask periodically and titrated with silver nitrate.[22] One drop of 1 *N* nitric acid and 2 drops of 0.1% erythrosin in ethanol were added to the sample and the mixture titrated with 0.01 *N* silver nitrate solution. The results obtained are given in Table I.

Cleavage of Model Peptides with Iodoacetamide.—Alkylations were carried out at 35–40° in ethanol–water (1:1 by volume) with 2×10^{-2} *M* peptide and 6×10^{-2} *M* iodoacetamide solutions. The course of alkylation was followed by titration with silver nitrate as described. With 90–100% of alkylation completed the *p*H of the reaction mixture was between 3 and 4. In cases where ether extractions were carried out before heating or analysis the aliquot of the reaction mixture was extracted three times with ether saturated with water. One-ml. aliquots of the alkylation mixtures, which had been diluted to a peptide concentration of 2×10^{-4} *M* with ethanol–water (1:1 by volume), were mixed with 1 ml. of ninhydrin reagent. The resulting mixtures were heated for 15 minutes in a boiling water-bath and assayed in the usual manner.[20] Aliquots of the reaction mixture which were heated before analysis were diluted with an equal volume of water before heating. Results are given in Table III. Paper chromatography and paper electrophoresis (*p*H 1.9) using appropriate reference compounds confirmed the identity of the liberated amino acid.

Cleavage of N-Carbobenzyloxymethioninecarbamylmethylsulfonium Nitrates and Acetates.—In a typical case 2 drops of 0.1% erythrosin solution and 1 drop of 1 *N* nitric acid were added to 1 ml. of a mixture of carbobenzyloxy-L-methionyl-L-glutamic acid (2×10^{-2} *M*) and iodoacetamide (6×10^{-2} *M*). When titration with 0.01 *N* silver nitrate indicated 98% alkylation the mixture was centrifuged and the silver iodide and adsorbed indicator washed twice with water. The combined supernatants were adjusted to *p*H 3 with sodium hydroxide solution and brought to 5 ml. with water.

To prepare the acetates, 2 drops of glacial acetic acid and 2 drops of 0.1% erythrosin solution were added to 1 ml. of a mixture of carbobenzyloxy-L-methionyl-L-glutamic acid (2×10^{-2} *M*) and iodoacetamide (6×10^{-2} *M*). The mixture was titrated with 0.01 *N* silver acetate solution and the same procedure followed as for the nitrates. Aliquots of the supernatants were used in subsequent operations. Samples which were heated prior to analysis were diluted with an equal volume of water before heating. Results are given in Table IV.

Acknowledgment.—The authors wish to thank Dr. Leon Levintow for the analyses performed with the automatic amino acid analyzer.

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