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Isolation[1] and Biosynthesis of L-Homomethionine (L-5-Methylthionorvaline)

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Homomethionine was first isolated from nature and some of its biosynthesis was studied by using methionine-^{35}S, methionine-$^{14}CH_3$, and cystine-^{35}S.

The isolation of S-methylcysteine sulfoxide[3] and S-methyl methionine[4] from cabbage have been reported, but S-methylcysteine has not been found in Cruciferae. S-Methylcysteine sulfoxide occupies a large amount in sulfur-containing amino acids in cabbage. In cabbage sulfur-containing organic compounds except amino acids are those of volatile isothiocyanates released from the thioglucosides and thiooxazolidine derivatives[5]. The presence of allyl, 3-methylthiopropyl and 3-butenyl isothiocyanates has been indicated by Kjaer, *et al.*[6] and Clapp, *et al.*[7].

Using tracer technique for the detection of sulfur-containing amino acids, the authors observed S-methylcysteine and an unknown methionine-like amino acid on a radioautogram in cabbage. This amino acid was finally proved to be L-homomethionine (L-5-methylthionorvaline) CH_3–S–CH_2–CH_2–CH_2–CH(NH_2)–COOH. The authors believe homomethionine to be of importance as a precursor of 3-methylthiopropyl isothiocyanate.

From interest in the metabolic pathway of homomethionine comparing with S-methylcysteine sulfoxide, the authors have for some time been engaged in a study on the biosynthesis of homomethionine and S-methylcysteine sulfoxide in an excised aerial part of cabbage.

Wolff, *et al.*[8] have indicated that S-methylcysteine is enzymatically formed in an extract of yeast from methylmercaptan and L-serine.

The abbreviations used are: MC, S-methylcysteine; MCS=O, S-methylcysteine sulfoxide; MetS=O, methionine sulfoxide; SMMet, S-methylmethionine; homo, homomethionine; homoS=O, homomethionine sulfoxide.

Suzuki, *et al.*[9] also have reported studies on the biosynthesis of S-methylcysteine and its sulfoxide using methionine-^{35}S in garlic. If methylmercaptan is derived from methionine in cabbage, homomethionine may be formed from α-amino-δ-hydroxyvaleric acid[10,11] and methylmercaptan derived from methionine. Recently, Thompson, *et al.*[11] have isolated α-

1) Preliminary communication of this work: M. Sugii, Y. Suketa, and T. Suzuki, *Chem. Pharm. Bull.* (Tokyo), **12**, 115 (1964).
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3) R.L.M. Synge and J.C. Wood, *Biochem. J.*, **60**, 15 (1955).
4) R.A. McRorie, G.L. Sucherland, M.S. Lewis, A.D. Barton, M.R. Glazener, and W. Shive, *J. Am. Chem. Soc.*, **76**, 115 (1954).
5) M.R. Althamura, L. Long, Jr., and T. Hasselstrom, *J. Biol. Chem.*, **234**, 1847 (1959).
6) K.A. Jensen, J. Conti, and A. Kjaer, *Acta Chem. Scand.*, **7**, 1267 (1953).
7) R.C. Clapp, L. Long, Jr., G.P. DaTeo, F.H. Bissett, and T. Hasselstrom, *J. Am. Chem. Soc.*, **81**, 6278 (1959).
8) E.C. Wolff, S. Black, and P.F. Downey, *J. Am. Chem. Soc.*, **78**, 5958 (1956).
9) M. Sugii, S. Nagasawa, and T. Suzuki, *Chem. Pharm. Bull.* (Tokyo), **11**, 135 (1963).
10) T. Yura and H.J. Vogel, *J. Biol. Chem.*, **234**, 339 (1959).
11) J.F. Thompson, C.J. Morris, and G.E. Hunt, *J. Biol. Chem.*, **239**, 1122 (1964).

amino-δ-hydroxyvaleric acid from Jack Bean seeds.

The present paper describes the isolation of L-homomethionine, and some evidences of biosynthesis of L-homomethionine.

Experimental

Materials

DL-Methionine-$^{14}CH_3$ (2.3 μc/μ mole) was obtained from Daiichi Pure Chemicals Co. Ltd., Tokyo (Japan). L-Methionine-^{35}S (15.2 μc/μ mole) and L-cystine-^{35}S (13.0 μc/μ mole) were prepared from *Torula utilis* incubated in a culture medium containing sulfuric acid (^{35}S) according to the method described by Niklas.[12]

Paper Chromatography and Paper Electrophoresis——A part of the amino acid fraction was subjected to two dimentional ascending paper chromatography on Toyo No. 50 filter paper with (first) PhOH/H_2O (4:1, v/v) and (second) *n*-BuOH/AcOH/H_2O (4:1:1, v/v/v) as solvents, paper electrophoresis with pyridine/AcOH/H_2O (10:0.4:90, v/v/v) at pH 6.5, 30 v/cm and 10—20 mA, and the radioautogram was made.

Generally, the radioactive regions along the paper chromatogram were cut out and eluted with water. The radioactivity measurements were performed with a GM-counter with a thin mica window or a windowless gas flow counter, after radioactive compounds were evaporated as an infinite thinness on stainless steel planchettes.

Determination of the Isolated Amino Acids——Determination of amino acids were carried out by cochromatography with authentic samples and by color reaction with ninhydrin and iodoplatinate reagents, oxidation with 30% hydrogenperoxide, reduction with hydriodic acid, and desulfurization with Raney–Nickel.

Isolation of Amino Acids——At a fixed period of cultivation, the plant was homogenized and extracted with 80% methanol adjusted to pH 4.0 with glacial acetic acid at room temperature. The amino acids in the extract were separated from other substances by passing through a column of Amberlite IR-120 (H^+ form). The resin was washed with deionized water. The adsorbed amino acids were eluted with 4% ammonium hydroxide solution. The eluate was evaporated *in vacuo* at 40° and then dried in a evacuated desiccator containing sodium hydroxide and phosphorus pentoxide.

The amino acid fraction obtained by the above procedure, was adsorbed on a column of Dowex 50-X4 (200—400 mesh) equilibrated with 0.05M-ammonium formate buffer at pH 2.50 and separated by the method of Hirs, *et al.*[13] The amino acid fraction was dissolved in the buffer (0.05M ammonium formate, pH 2.50) and added to the top of the column and developed with 0.2M ammonium formate at pH 3.00 to 3.50. An elution curve was obtained by plotting the radioactivity against the tube number. Each peak showing radioactivity was pooled, lyophilized and desalted.

Results

Incorporation of ^{35}S of $H_2{}^{35}SO_4$ into Amino Acid Fraction

An excised aerial part of cabbage (275 g) was cultivated in 5 ml of water containing 1 mc of carrier free $H_2{}^{35}SO_4$. After all of the solution was absorbed, the plant was further cultivated with a SO_4^{2-} free hydroponic solution for four days. The amino acid fraction was prepared by the method as described above. The total radioactivity of amino acid fraction was 91.5 μc(9.15%). Yield 1.71 g. The radioautogram is shown in Fig. 1. The distribution of radioactivity into each spot (as shown in Fig. 1) is shown in Table I.

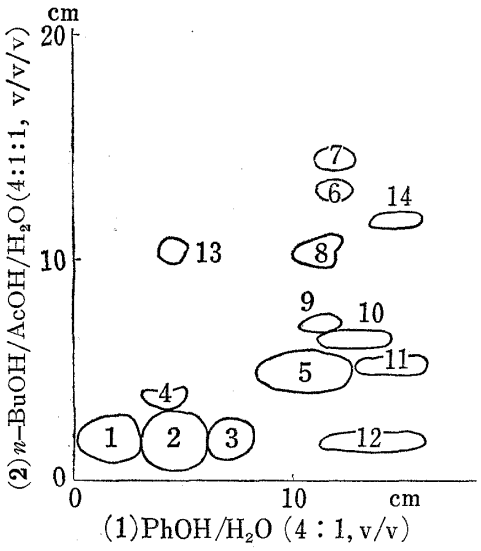

Fig. 1. Radioautogram of ^{35}S-Labeled Amino Acid in an Excised Aerial Part of Cabbage

The highest labeling spot was found as S-methylcysteine sulfoxide, which is the major sulfur-containing amino acid in cabbage. Among the ^{35}S-labeled amino acids on the radioautogram,

12) A. Niklas, *Z. Physiol. Chem.*, **301**, 194 (1955).
13) C.H.W. Hirs, S. Moore, and W.H. Stein, *J. Biol. Chem.*, **195**, 669 (1952); A.R. Thompson. *Biochem. J.*, **61**, 253 (1955).

Table I. Distribution of ^{35}S in Amino Acid Fraction

Spot No.	Radioactivity (cpm)	(%)	Deduced amino acid
1	2450	14.4	
2	3584	21.0	cysteic acid
3	826	4.9	cystine
4	228	1.2	
5	4322	25.3	S-methylcysteine sulfoxide
6	124	0.8	methionine
7	132	0.8	homomethionine
8	254	1.5	S-methylcysteine, γ-glutamyl-S-methylcysteine
9 } a)	500	3.0	
10 }			
11	74	0.5	
12	366	2.1	S-methylmethionine
13	72	0.4	
14	118	0.7	
Recovery	13152	76.6	17074 cpm (total radioactivity)

This result was obtained from the radioautogram, which is given in Fig. 1.
a) The total radioactivity of both spots No. 9 and 10 is presented in the table because the two spots overlapped with each other on the radioautogram (Fig. 1).

the authors were aware of an unknown methionine-like amino acid (as shown in Fig. 1 (spot 7)). On a Dowex 50–X4 column it emerged just after fractions of methionine and leucine. It (Rf=0.49) moved faster than methionine (Rf=0.44) by paper chromatography with *n*-BuOH/AcOH/H_2O (4:1:1, V/V/V) and moved at the same speed as methionine (Rf =0.80) with PhOH/H_2O (4:1, V/V) as given in Fig. 1. Electrophoretic mobility of this amino acid (30 V/cm) with pyridine/AcOH/H_2O (10:0.4:90, V/V/V) at pH 6.5 was identical to that of methionine.

The product which was obtained by oxidation with 30% hydrogenperoxide at room temperature for 24 hours, moved with the same speed as methionine sulfoxide in the paper chromatography in the both solvent systems. When the oxidized product was reduced with hydriodic acid, it became to the substance having original Rf values.

Isolation of Homomethionine

To isolate this unknown compound in large scales, 20 kg of fresh cabbage was treated in a manner similar as described above. Fresh cabbage (20 kg) was sliced and stirred until thoroughly homogenized in 20 liters of 80% methanol adjusted to pH 4.0 with glacial acetic acid and allowed to keep for one day at room temperature. The crude extract was obtained from the homogenate by cloth–filtration. The clear extract was prepared by the filtration of the crude extract through Celite-545. The amino acids in the filtrate were collected by passing the filtrate through a column of Amberlite IR-120 (H^+ form) at room temperature. The resin was washed with deionized water until the effluent was neutral and then eluted with 4% ammonium hydroxide solution. The eluate was concentrated to dryness under reduced pressure at below 40°C. The residue was equally divided into two portions. Each portion was separately subjected to a column chromatography as follows. The resin (Dowex 50-X4, 200—400 mesh) was prepared for chromatography according to the procedure of Hirs, *et al.*[13] and a column (6.5×65 cm) was packed. The residue containing the amino acids was dissolved in 50 ml of the 0.05M ammonium formate buffer, pH 2.50, and loaded on the top of column, and washed with 50 ml of ammonium formate buffer (pH 2.50). The development was carried out first with the 0.2M ammonium formate buffer, pH 3.00.

Each fraction (10 ml) was collected at a rate of 1 ml. per minute. After 200 fractions had been collected, the buffer was changed to one having pH 3.50 (0.2M). Fractions corresponding to each peak were combined, desalted and concentrated to dryness *in vacuo* at below 40°. The compounds in each fraction were identified by paper chromatography.

An unknown methionine-like amino acid was contained in fraction of tube No. 570—629 in the first run. A second similar run was performed, and the corresponding fraction on the runs were combined and concetrated to dryness *in vacuo*. The combined residue from the two fractions was dissolved in solvent system of *n*BuOH/AcOH/H_2O (4:1:1, V/V/V) and an unknown methionine-like amino acid was purified by a column (2.5×85 cm) of cellulose powder using the same solvent. The only methionine-like amino acid emerged in a fraction of tube No. 3—10, which were evaporated under reduced pressure and dried in an evacuated desiccator containing phosphorus pentoxide. Final purification was performed by recrystallization in aqueous ethanol.

Fine scales were obtained: Yield, 50 mg, mp 223—225° (decomp.); $[\alpha]_D^{25.5}$ +21.0 (*c*: 0.30 in 6NHCl); $C_6H_{13}O_2NS$ (Calcd: C, 44.16; H, 8.03; N, 8.58. Found: C, 44.39; H, 8.10; N, 8.74). This amino acid was positive to iodoplatinate and ninhydrin test, negative to cyanide and nitroprusside test on the filter paper, and gave norvaline by the desulfurization with Raney-Nickel. The NMR spectrum of the amino acid (refer to the previous paper[14]) showed a presence of thiomethyl group at 7.43 τ. The ORD spectrum of the amino acid is given in Fig. 2.

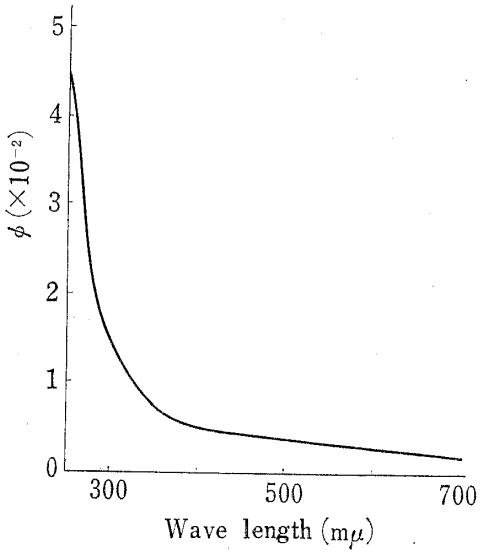

Fig. 2. The ORD Spectrum of the Isolated Amino Acid in 6N-HCl. (c=0.30)

From these results, the amino acid isolated was considered to be L-homomethionine (L-5-methylthionorvaline) CH_3–S–CH_2–CH_2–CH_2–CH(NH_2)–COOH. Final proof was performed by comparing the *Rf* values (0.85: PhOH/H_2O (4:1, V/V), 0.49: *n*BuOH/AcOH/H_2O (4:1:1, V/V/V)) and NMR spectrum with those of the authentic sample of DL-homomethionine which was synthesized by the method of Kjaer and Wagner[15]. Furthermore, the *Rf* values of oxidized product of the isolated methionine-like amino acid with hydrogenperoxide agreed with those of authentic homomethionine sulfoxide.

Biosynthesis of Homomethionine (Its Sulfoxide) and S-Methylcysteine (Its Sulfoxide)

An excised aerial part of cabbage (30 g) was cultivated in 5 ml of water containing 100 μc of L-methionine-^{35}S (15.2 μc/μ mole) or 100 μc of DL-methionine-$^{14}CH_3$ (2.3 μc/μ mole) or 298 μc of L-cystine-^{35}S (13.0 μc/μ mole). After all of the solution was absorbed into the plant, the plant was cultivated with a SO_4^{2-} free hydroponic solution for 24 hours or four days or one week. The plant was homogenized and extracted with 80% methanol adjusted to pH 4.0 with glacial acetic acid. The extract was passed through a column of Amberlite IR-120 (H^+ form) and the adsorbed amino acids were eluted with 4% ammonium hydroxide solution. The eluate was concentrated to dryness *in vacuo* at below 40° and then dried over in evacuated desiccator as the above procedure. As shown in Table II, incorporation of the above labeled amino acid into amino acid fraction was about 30% and decreased proportional to time feeded.

14) M. Sugii, Y. Suketa and T. Suzuki, *Chem. Pharm. Bull.* (Tokyo), **12**, 1115 (1964).
15) A. Kjaer and S. Wagner, *Acta Chem. Scand.*, **9**, 721 (1955).

Table II. Incorporation of L-Methionine-^{35}S, DL-Methionine-$^{14}CH_3$ and L-Cystine-^{35}S into Amino Acid Fraction

Radioactive amino acid added	Methionine-^{35}S 100 μc	100 μc	100 μc	Methionine-$^{14}CH_3$ 100 μc	Cystine-^{35}S 298 μc
Feeded time	24 hr	4 days	1 week	4 days	24 hr
Weight of cabbage	30 g	30 g	30 g	30 g	30 g
Radioactivity of extracts	63.77 μc (63.77%)	47.48 μc (47.48%)	58.48 μc (58.48%)	56.92 μc (56.92%)	161.25 μc (54.11%)
Weight of amino acid fraction	184 mg	226 mg	180 mg	201 mg	247 mg
Radioactivity of amino acid fraction	38.28 μc (38.28%)	27.14 μc (27.14%)	29.76 μc (29.76%)	26.60 μc (26.60%)	75.76 μc (25.42%)

The percentage in parenthesis indicates recovery of radioactivity.

The amino acid fraction obtained by the above procedure was adsorbed on a column (3.5×78 cm) of Dowex 50-X4 equilibrated with 0.05M ammonium formate buffer (pH 2.50) and separated by a stepwise elution[13]. Fractions of 10 ml were collected at a rate of 1 ml per minute. Elution curve was obtained by plotting the radioactivity against the tube number and is given in Fig. 3. Each peak having radioactivity was pooled, lyophilized, desalted, and examined by paper chromatography and paper electrophoresis.

In all experiments, each peak was radioactively homogeneous except a fraction which was eluted with 4% ammonium hydroxide solution. The data in Table III summarize the results of incorporation of labeled amino acid into homomethionine *etc.*

On the other hand, a part of the amino acid fraction before separation was subjected to two directional paper chromatography on Toyo No. 50 filter paper with *n*BuOH/AcOH/H_2O (4:1:1, V/V/V) and PhOH/H_2O (4:1, V/V) as solvents, and took the radioautogram. On the above paper chromatography, the separation between homomethionine sulfoxide and methionine sulfoxide was not succeeded, but it was achieved easily by the use of column chromatography. Determination of the separated radioactive amino acids was carried out as follow. Homomethionine and its sulfoxide, methionine and its sulfoxide, S-methylcysteine and its sulfoxide and cystine could be identified by cochromatography with authentic samples. Furthermore, they were confirmed by oxidation with hydrogenperoxide, reduction with hydriodic acid and desulfurization with Raney–Nickel.

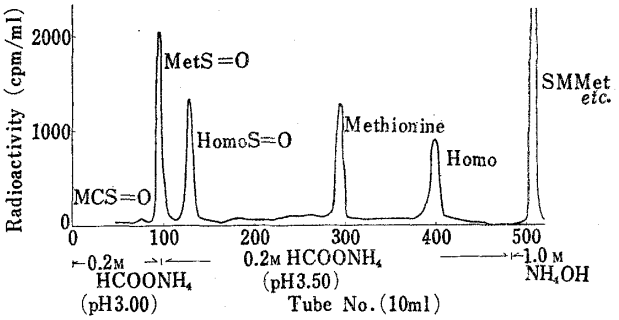

Fig. 3. The Column Charomatogram of Cultivation with Methionine-^{35}S for 24 hours

The amino acid fraction was loaded and developed with the ammonium formate as shown.

In experiments with L-methionine-^{35}S, it was found that homomethionine (involved its sulfoxide) was highly labeled as shown in Table III. ^{35}S-Incorporation into S-methylcysteine sulfoxide was slowly increased from 24 hours to one week, but the increase was lower than ^{35}S-incorporation into homomethionine. The radioactivity was obserbed in the spot of S-methylcysteine on a radioautogram, but was not obserbed as a peak of S-methylcysteine on a column chromatogram.

When DL-methionine-$^{14}CH_3$ was added to culture medium, S-methylcysteine sulfoxide was labeled higher than homomethionine as shown in Table III. This result was contrary to the cultivation with L-methionine-^{35}S.

Table III. Incorporation of L-Methionine-^{35}S, DL-Methionine-$^{14}CH_3$ and L-Cystine-^{35}S into Homomethionine(its Sulfoxide), and S-Methylcysteine (Its Sulfoxide)

Radioactive amino acid added	Methionine-^{35}S 100 μc			Methionine-$^{14}CH_3$ 100 μc	Cystine-^{35}S 298 μc
Feeded time	24 hr	4 days	1 week	4 days	24 hr
MCS=O	0.15 μc (0.39%)	1.02 μc (3.76%)	1.38 μc (4.64%)	2.11 μc (7.93%)	44.29 μc (58.46%)
MC	+[a]	++[a]	(0.59%)[a]	(0.32%)[a]	2.15 μc (2.84%)
HOMOS=O	2.08	2.78	1.94	1.01	
HOMO	1.36 3.44 μc (8.99%)	0.21 2.99 μc (11.02%)	1.18 3.12 μc (10.48%)	0.21 1.22 μc (4.59%)	—
MetS=O	2.97 μc	2.99 μc	2.50 μc	8.74 μc	9.66 μc (12.70%)
Methionine	3.73 μc	0.84 μc	0.42 μc	1.80 μc	0.78 μc (1.03%)
Cystine	—			—	0.76 μc (1.00%)
NH_4OH eluate (SMMet *etc.*)	21.60 μc	19.15 μc	21.81 μc	2.79 μc	3.56 μc
Radioactivity incorporated into amino acid fraction	38.28 μc	27.14 μc	29.76 μc	26.60 μc	75.76 μc
Recovery of chromatogram	31.89 μc 83.31%	26.99 μc 99.46%	29.23 μc 98.23%	22.24 μc 83.61%	61.20 μc 80.73%

The number in parenthesis indicates the percentage of incorporation of labeled amino acid obtained from column chromatogram.

a) The number in starred parenthesis shows incorporation of amino acid obtained from paper chromatogram.

In experiments with L-cysteine-^{35}S, a large extent of radioactivity assembled in S-methylcysteine sulfoxide (58.46%). S-Methylcysteine was labeled 2.84%. Labeled cystine used was recovered about 1%, but homomethionine was not labeled.

Discussion

The occurrence of homomethionine in cabbage was chemically and biochemically proved by these results. This report is the first proof of occurrence of homomethionine in nature. From these results using labeled amino acids or $H_2{}^{35}SO_4$, it is suggested that the mechanism of biosynthesis of homomethionine is different from that of S-methylcysteine sulfoxide: It can be mentioned that sulfur of methionine is hardly utilized for the biosynthesis of S-methylcysteine and its sulfoxide, but is easily utilized for the biosynthesis of homomethionine and its sulfoxide.

In cabbage S-methylcysteine sulfoxide may be mainly produced by transmethylation from methionine and on the other hand homomethionine may be mainly produced by reaction of α-amino-δ-hydroxyvaleric acid[10,11] with methylmercaptan derived from methionine.

From our experiments of cultivation with L-cystine-^{35}S, it is suggested thathomo methionine is not formed *via* HOOC–CH(NH_2)–CH_2–S–CH_2–CH_2–CH_2–CH(NH_2)–COOH.

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