BIOSYNTHESIS OF SINIGRIN. III.

Mitsuyoshi Matsuo and Mikio Yamazaki

National Institute of Radiological Sciences, Anagawa Chiba, Japan

Received August 8, 1966

Recently several papers on the biosynthesis of mustard oil glucosides in Cruciferae plants have been reported to suggest that the α-amino acids which have an intimate structural correlation with the glucosides are the direct precursors. In the previous paper (Matsuo and Yamazaki, 1964) we reported that sinigrin in Brassica juncea COSSON was derived from aspartic acid and malonic acid or acetic acid, while Chisholm and Wetter (1964) showed that methionine was incorporated into sinigrin in Armoracia lapathifolia GILIB. with high efficiency. Both results obtained above would not be incompatible each other, since aspartic acid and methionine are very closely related in biosystems. It is quite probable to assume that the direct biogenetic precursor of sinigrin should be a condensation product of methionine and malonate or acetate because of the high incorporation ratio of methionine and malonate into sinigrin. According to the recent data of the feeding experiment (Matsuo and Yamazaki, 1964) and the competitive experiment (Chisholm and Wetter, 1964) it was assumed that allylglycine would be a direct precursor of sinigrin.

The present paper describes that homomethionine is more probable as a direct precursor of sinigrin than allylglycine in horseradish (A. lapathifolia GILIB.) leaves, and homomethionine would arise by the condensation of methionine and malonic acid.

The labelled compounds were administered to horseradish leaves by immersing the cut end in the aqueous solution of the tracer. After 3 or 24 hour cultivation, mustard oil was isolated from the leaves, which was converted into allylthiourea. The comparative incorporation of tracers into sinigrin were shown in Table I.

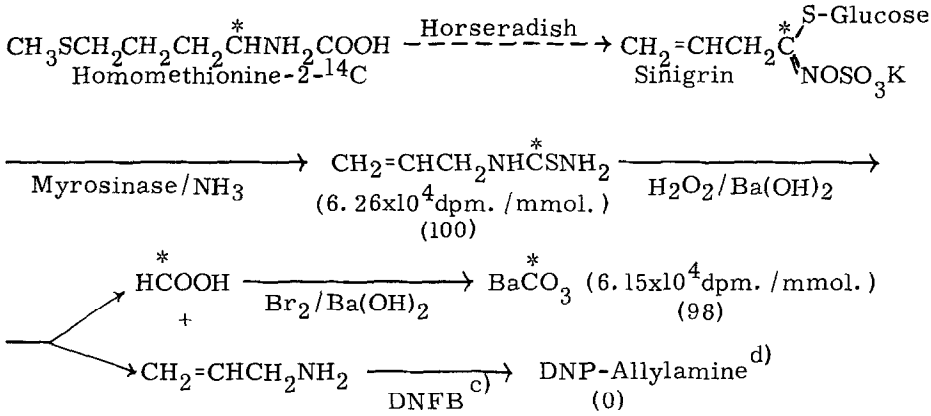
Table I. Incorporation of Labelled Compounds into Sinigrin. [a]

Compounds	Amt. fed mg.	Total act. μC.	Fresh wt. of plants g.	Sp. act. of allyl-thiourea nC./mmol.	Specific incorp.[b] %
DL-Methionine-2-^{14}C	8.9	9.4	216	713	0.45
DL-Homomethionine-2-^{14}C	10.2	2.7	212	277	0.65
DL-Homomethionine-G-^{3}H	19.1	300	216	5500	0.22
DL-Allylglycine-2-^{14}C	6.8	2.3	200	4.5	0.01
4-Methylthiobutyramide-1-^{14}C	15.6	30	209	7.4	0.003
3-Methylthiopropionamide-1-^{14}C	7.1	10	212	0.3	0.0002

a) Cultivated for 24 hours.

b) Specific incorp. = $\frac{\text{Sp. act. (μC./mmol.) of allylthiourea}}{\text{Sp. act. (μC./mmol.) of precursor}}$ X 100.

The allylthiourea obtained in the homomethionine-2-^{14}C feeding experiment was diluted with carrier and degraded by the method described in the previous paper (Matsuo and Yamazaki, 1963) to determine the location of ^{14}C in the sinigrin molecule as shown below.



c) DNFB= 2, 4-Dinitrofluorobenzene.
d) DNP= 2, 4-Dinitrophenyl.

In the ^{14}C and ^{15}N double labelled tracer experiments, homomethionine was proved to be the direct precursor of sinigrin as indicated in Table II.

Table II. ^{14}C and ^{15}N Double Labelled Tracer Experiments.

			Precursor				Allylthiourea		
Metabolic period hour	Fresh wt. of plants g.	Amt. fed mg.	Total act. μC.	Sp. act. μC./mmol.	Atoms% excess ^{15}N	$\frac{^{14}C}{^{15}N}$ e)	Sp. act. μC./mmol.	Atoms% excess ^{15}N f)	$\frac{^{14}C}{^{15}N}$ e)
DL-Homomethionine-2-^{14}C, ^{15}N.									
3	210	32.4	6.7	33.4	32.4	1.0	0.24	0.22	1.1
24	212	32.4	6.7	33.4	32.4	1.0	0.58	0.32	1.8
DL-Allylglycine-2-^{14}C, ^{15}N.									
24	200	64.3	21.4	38.1	51.0	0.75	0.0054	0.11	0.05

e) $^{14}C/^{15}N = \frac{\mu C./mmol.\ of\ ^{14}C}{Atoms\%\ excess\ of\ ^{15}N}$

f) The value corrected for the nitrogen atom derived from the ammonia used in the preparation of allylthiourea.

Homomethionine was incorporated into sinigrin without randomization and with higher incorporation ratio than that of methionine, and the amino group of homomethionine was also incorporated into the molecule of

sinigrin being attached with the intact carbon chain. The results of the double tracer experiments showed that when the precursor was fed for 3 hours, $^{14}C/15_N$ ratio of allylthiourea obtained was unchanged from the initial ratio. However the 24 hour feeding experiment gave a final $^{14}C/15_N$ ratio by a factor of 1.8. The similar result was observed by Underhill (1965), when DL-γ-phenylbutyrine-2-^{14}C, ^{15}N was incorporated into the aglycone of gluconasturtiin by 24 hour feeding, giving $^{14}C/15_N$ ratio 3.81 in comparison with the initial ratio 1.84, whereas the $^{14}C/15_N$ ratio remained unchanged when the precursor was given in L-form. This result was explained as follows: since D-form was converted to the corresponding α-keto acid by a D-amino acid oxidase, D-amino acid labelled with ^{14}C and ^{15}N was transformed into L-form labelled with only ^{14}C and the incorporation of ^{15}N was decreased. In our experiment of short time feeding (3 hours), $^{14}C/15_N$ ratio in the precursor of DL-form was almost equal to the ratio of the metabolite. This shows that the dilution of L-precursor did not give rise so rapidly. On the other hand, when allylglycine was fed to the plant, a remarkable difference of $^{14}C/15_N$ ratio was observed between the initial precursor and the metabolite.

It appears therefore that the aglycone moiety of sinigrin is directly derived from homomethionine, and allylglycine is not the direct precursor, thus the formation of the double bond in sinigrin molecule would be caused by the elimination of methanethiol at the latest stage of biosynthesis.

Glucoibervirin, a mustard oil glucoside of Iberis sempervirens L., has a structure which would be caused by the addition of methanthiol to the double bond of sinigrin. As the both glucosides are occuring in the same plant (I. sempervirens) (Kjaer, 1960), it would not be unreasonable to assume that glucoibervirin is the immediate precursor of sinigrin.

In the previous paper (Matsuo and Yamazaki, 1964), we reported acetate-2-^{14}C and malonate-2-^{14}C were incoporated into sinigrin with the same labelling pattern. But now we have found that malonate is more predominant as a precursor than acetate. The incorporation of ^{14}C from sodium acetate-2-^{14}C was decreased by the addition of sodium malonate, and sodium malonate-2-^{14}C was not effected by sodium acetate. The result of the competitive feeding experiment between malonate and acetate is shown in Table III.

Table III. Competitive Feeding Experiment.

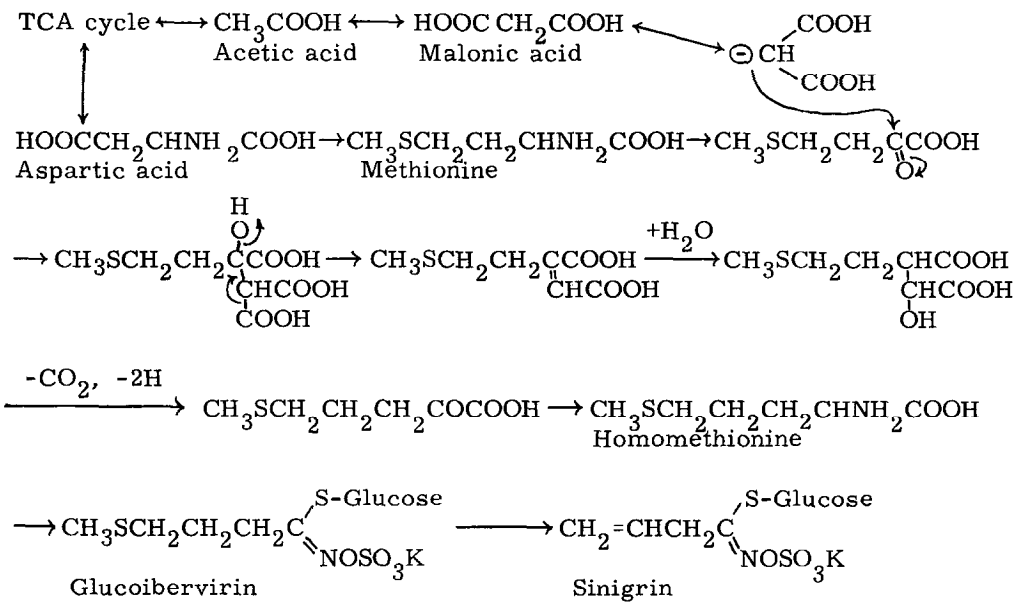
Exp. No. Substrate	Fresh wt. of plants g.	Metabolic period hour	Allylthiourea: Specific activity μC./mmol.	Allylthiourea: Relative[g] specific activity %
(1) Na acetate-2-^{14}C, 0.1mmol.(20μC.)+Na malonate, 0.1mmol.	115	3	0.22	45
(2) Na acetate-2-^{14}C, 0.1mmol. (20μC.).	115	3	0.45	91
(3) Na malonate-2-^{14}C, 0.1mmol. (20μC.)+Na acetate, 0.1mmol.	115	3	0.47	94
(4) Na malonate-2-^{14}C, 0.1mmol. (20μC.).	115	3	0.50	100

g) Rerative sp. act. = $\frac{\text{Sp. act. (μC./mmol.) of allylthiourea in the other exp.}}{\text{Sp. act. (μC./mmol.) of allylthiourea in the exp. (4).}} \times 100$

It seems that homomethionine should be biosynthesized from methionine and malonate. Chisholm and Wetter (1964) proposed that the condensation product of methionine and acetic acid would be α-(2-methylthioethyl)malic acid, which would then be converted to β-(2-methylthioethyl)malic acid. Underhill(1965) showed that gluconasturtiin was biosynthesized from L-γ-phenylbutyrine, derived from phenylalanine and acetic acid, and the

competitive experiments supported β-benzylmalic acid would be a precursor of L-γ-phenylbutyrine. On the other hand, methionine was degraded to 3-methylthiopropionamide by horseradish peroxidase (Mazelis and Ingraham, 1962), but the amide was not incorporated into sinigrin as shown in Table I.

By the results of present experiment, we propose the biosynthetic scheme of sinigrin as follows:



ACKNOWLEDGEMENTS

We are grateful to Prof. S. Shibata, of University of Tokyo, for his encouragement, to Dr. Y. Kasida and Dr. T. Hino, of this Institute, for their advices and to Prof. A. Kjaer, of Royal Veterinary and Agricultural Collage, for his kind supply of the authentic sample of homomethionine for comparison.

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