Phytochemistry, 1964, Vol. 3, pp. 57 to 64. Pergamon Press Ltd. Printed in England

BIOSYNTHESIS OF MUSTARD OIL GLUCOSIDES. II. THE ADMINISTRATION OF SULPHUR-35 COMPOUNDS TO HORSE-RADISH LEAVES*

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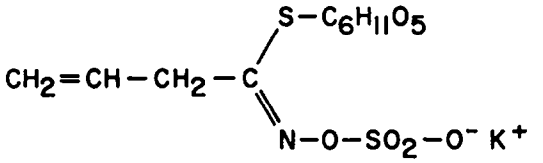
(*Received* 8 *July* 1963)

Abstract—Sulphur-35 from labelled sulphate, sulphide, thiosulphate and methionine was incorporated into sinigrin isolated from mature horse-radish leaves. Sulphate-S was more readily incorporated than was that from sulphide, thiosulphate or methionine. When inorganic sulphur-35 was supplied, about 80 per cent of the radioactivity of sinigrin was in the bisulphate moiety and 20 per cent in the isothiocyanate. However, with methionine-^{35}S as the tracer the isothiocyanate portion contained 90 per cent of the radioactivity. The data suggest that methionine sulphur is incorporated into sinigrin by a different pathway than are the various forms of inorganic sulphur.

HORSE-RADISH (*Armoracia lapathifolia* Gilib.) contains two isothiocyanates or mustard oils which have been identified as allyl and β-phenylethyl isothiocyanate.[1,2] The former represents about 85 per cent of the total in the root of this plant.[2] The thioglucoside which contains the aglycone, allyl isothiocyanate is known as sinigrin (I). This molecule contains two atoms of



I

sulphur, one in the bisulphate residue and the other associated with the glucose molecule. Enzymatic hydrolysis of sinigrin with myrosinase[3,4] yields allyl isothiocyanate which contains the reduced sulphur atom, potassium bisulphate containing the oxidized sulphur atom, and D-glucose.

Recently Underhill *et al.*[5] have studied the incorporation of some ^{14}C-labelled compounds into the isothiocyanate and carbohydrate portions of the molecule. However, the mode of

* Presented in part before the 3rd annual conference of the Canadian Society of Plant Physiologists, Quebec City, Quebec, June 1962.
Issued as N.R.C. No. 7692.

[1] A. KJAER, *Progr. in Chem. Org. Nat. Prods.* **18**, 122 (1960).
[2] O. ISAAC and E. KOHLSTAEDT, *Arch. Pharm.* **295**, 165 (1962).
[3] Myrosinase is a crude enzyme mixture which contains sinigrin sulphohydrolase (myrosulphatase) and merosinigrin glucohydrolase (thioglucosidase).[4]
[4] R. D. GAINES and K. J. GORING, *Arch. Biochem. Biophys.* **96**, 13 (1962).
[5] E. W. UNDERHILL, M. D. CHISHOLM and L. R. WETTER, *Can. J. Biochem. Physiol.* **40**, 1505 (1962).

sulphur incorporation into the sinigrin molecule has not been examined. This report describes preliminary experiments on the incorporation of various forms of ^{35}S into the sinigrin of horse-radish leaves.

RESULTS

Incorporation of Sulphur-35 from Sodium Sulphate

The first experiment was of an exploratory nature in which a horse-radish plant was fed 1 l. nutrient solution containing 273 μc of sodium sulphate-^{35}S (4·3 mg). During a period of a week about 85 per cent of the total radioactivity administered was taken up. The plant was then separated into leaf and root portions and each was fractionated separately. Table 1 presents the results of this experiment. About 50 per cent of the radioactivity incorporated appeared in the methanol extract and 16 per cent in the residue, the remainder was unaccounted for. The sinigrin and the fraction designated as acids accounted for most of the ^{35}S in the

TABLE 1. A COMPARISON OF THE INCORPORATION OF SULPHUR-35 FROM SODIUM SULPHATE* INTO VARIOUS FRACTIONS ISOLATED FROM THE LEAF AND ROOT OF HORSE-RADISH

Fraction	Leaf		Root	
	Activity, μc	Specific activity, mμc/mg	Activity, μc	Specific activity, mμc/mg
Methanol extract	70·1	6·8	47·1	16·9
Lipids	0·3	0·6	0·2	0·9
Amino acids	0·4	0·7	0·6	2·7
Acids	19·8	7·8	21·4	12·3
Sugars and neutrals	0·8	0·2	0·1	0·1
Sinigrin	18·2	79·0	4·2	70·0
Residue	24·2	1·1	14·1	1·6

* 273 μc of $Na_2{}^{35}SO_4$ administered of which 85 per cent was taken up.

methanol extract. The acids contained a good deal of the unmetabolized sulphate-^{35}S, but no attempt was made to identify the other radioactive compounds in the fraction. The sinigrin fraction was radioactive and there appeared to be no significant difference in the biosynthesis of this compound in either the root or the leaf.

The incorporation of sulphur-35 fed as sodium sulphate for varying metabolic periods is shown in Table 2. The radioactive recoveries were in the neighbourhood of 100 per cent when based on the methanol extract and the residue. The various fractions isolated from the former accounted for about 70 per cent of the total activity. The values in Table 2 are expressed as relative specific activities because of varying amounts of radioactivity administered to batches of leaves of different weight. The value is obtained by dividing the specific activity of each fraction (expressed as mμc/mg) by the specific activity of the dry weight of the leaves (also expressed as mμc/mg) by assuming that the activity was uniformly distributed.

The zero time experiment was carried out to determine which fractions would contain unmetabolized sulphate ion. A known amount of radioactive sodium sulphate was added after the leaves had been boiled in methanol and then fractionated in the regular manner.

Inorganic sulphate appeared only in the residue and the acid fraction (Table 2). The same experiment also provided an estimate of the sulphate content of the leaf. The estimation was made on the residue either by precipitation as $BaSO_4$ or by a colorimetric method.[6] By employing the isotope dilution technique, the sulphate content of horse-radish leaves for this experiment was determined to be 2·5 g SO_4^{2-}/100 g dry weight. This value is of the same order as found for brussels sprouts (2·34 g SO_4^{2-}/100 g), red cabbage (2·04 g), and spinach (2·61 g).[7]

The specific activities of the lipid and the sugar fraction are low and do not vary to any great extent. The amino acid fraction contains considerable activity which decreases with time, a trend which continues into the longer term experiment reported in Table 1. These

TABLE 2. THE EFFECT OF TIME ON THE INCORPORATION OF SULPHUR-35 FROM SODIUM SULPHATE INTO VARIOUS FRACTIONS OF HORSE-RADISH LEAVES

	Metabolic period, hr			
	0*	2	6	18
Wt. of leaves fed, g	52	66	71	104
$Na_2{}^{35}SO_4$ fed, μc	98	113	119	106
$Na_2{}^{35}SO_4$ fed, mg	4·3	4·3	4·3	5·2
	Relative specific activity†			
Methanol extract	1·0	1·0	1·2	1·7
Lipids	0	0·06	0·06	0·03
Amino acids	0	1·5	0·3	0·8
Acids	2·9	2·9	1·6	0·6
Sugars and neutrals	0	0·04	0·03	0·05
Sinigrin	0	2·6	7·0	5·9
Residue	1·2	0·8	0·9	0·7

* The $Na_2{}^{35}SO_4$ was added to the hot methanol–leaf mixture.
† Specific activity of fraction/specific activity of leaves on a dry wt. basis.

results suggest that the sulphur-containing amino acids are synthesized quite rapidly and are then gradually incorporated into proteins or employed in other reactions requiring these acids. As was demonstrated previously the acids are grossly contaminated with sulphate and therefore the specific activities for this fraction have little significance. The residue, which is the insoluble material left after the hot methanol extraction, has a high specific activity which decreases with length of metabolic period (see Tables 1 and 2). Much of this activity can be accounted for as unmetabolized $^{35}SO_4^{2-}$, while a small percentage is associated with the protein found in the residue. Sinigrin has the highest activity and reaches a maximum between 2 and 18 hr. The drop in specific activity in the experiment of 18 hr duration is undoubtedly due to the larger amount of leaf material used. The values represent 7, 20, and 17 per cent incorporation of sulphur-35 for the 2, 6, and 18-hr metabolic periods respectively.

[6] R. J. BERTOLACINI and J. E. BARNEY, JR., *Anal. Chem.* **29**, 281 (1957).
[7] C. LONG, Editor, *Biochemists' Handbook*, p. 1040, E. and F. N. Spon Ltd., London (1961).

The intramolecular distribution of sulphur-35 in sinigrin, isolated from the above feeding experiments, are presented in Table 3. The high specific activity of the thioglucoside suggests that sulphur-35 from sulphate is readily incorporated. The lower specific activity of the last experiment (18 hr) is due to the large dilution of $^{35}SO_4^{2-}$ by the sulphate already present in the leaf (104 g as compared to 71 g). If the dilution value is calculated after making this correction the discrepancy disappears. The ratio of reduced sulphur-35 (the isothiocyanate sulphur) to oxidized sulphur-35 (the bisulphate sulphur) increases with the length of the metabolic period.

TABLE 3. THE INTRAMOLECULAR DISTRIBUTION OF SULPHUR-35 IN SINIGRIN ISOLATED FROM HORSE-RADISH LEAVES TO WHICH $Na_2{}^{35}SO_4$ HAD BEEN ADMINISTERED FOR VARIOUS PERIODS OF TIME

	Metabolic period			
	2 hr	6 hr	18 hr	7 days
Sinigrin				
Specific activity*	14·9	35·8	19·1	26·4
Dilution value†	3·8	1·5	1·7	—
Distribution				
Per cent in isothiocyanate	16	19	33	36
Per cent in sulphate	84	81	67	64

* Specific activity = μc/mmole.

† Dilution value = $\frac{\text{specific activity of } SO_4}{\text{specific activity of sinigrin}}$.

Incorporation of Sulphur-35 from Other Sulphur Compounds

Several other ^{35}S-labelled compounds gave results similar to those obtained for sodium sulphate (Table 4). A notable exception is the sulphur-35 distribution in the fractions when methionine was fed. The lipid fraction contained very little radioactivity for any substrate. The sulphur-35 content of the amino acid and the sugar fractions were much higher for methionine-^{35}S than for any of the other substrates. Although the amino acid fraction contains unmetabolized methionine, about one-half of the radioactivity is due to other unidentified compounds. The radioactive material in the sugar fraction was not investigated further at this time. The incorporation of sulphur-35 from sulphide, inner-labelled thiosulphate, and methionine into sinigrin is similar (see Table 4). On the other hand, sulphur-35 from outer-labelled thiosulphate is not nearly as effectively incorporated into sinigrin.

A time-course experiment, similar to the one for sulphate was conducted with sulphide. Approximately 26 per cent of the sulphur-35 was incorporated into the thioglucoside in about 18 hr. This compares to the incorporation obtained with sulphate after 6 hr. These preliminary results would suggest that sulphide-S is more slowly incorporated into the sinigrin molecule than is sulphate-S.

The intramolecular distribution of sulphur-35 in sinigrin is tabulated in Table 5 and it should be compared with Table 3. The incorporation for the sulphur-35 from various sources is similar excepting for outer-labelled thiosulphate. The distribution of sulphur-35 in sinigrin is similar for the three inorganic anions tested thus suggesting that sulphur from these

compounds is incorporated into thioglucoside by a common pathway. The sulphur in methionine is distributed quite differently in that the sulphur from an organic source is more easily incorporated into the isothiocyanate moiety than is sulphur from an inorganic source.

TABLE 4. THE INCORPORATION OF SULPHUR-35 FROM SODIUM SULPHIDE, SODIUM THIOSULPHATE, AND METHIONINE IN VARIOUS FRACTIONS OF HORSE-RADISH LEAVES

	Sulphide	Thiosulphate		Methionine
		Outer-	Inner-	
Wt. of leaves fed, g	82	72	68	86
^{35}S-compound fed, μc	147	52	51	45
^{35}S-compound fed, mg	0·43	7·2	0·9	15·7
Metabolic period, hr	6	3	3	6
	Relative specific activity			
Methanol extract	1·5	1·7	1·8	2·4
Lipids	0·06	0·05	0·05	0·1
Amino acids	0·6	0·2	0·2	6·5
Acids	lost	0·02	0·07	0·4
Sugars and neutrals	0·04	0·05	0·05	1·2
Sinigrin	1·8	1·0	1·9	2·0
Residue	0·8	0·7	0·8	0·4

TABLE 5. THE INTRAMOLECULAR DISTRIBUTION OF ^{35}S IN SINIGRIN ISOLATED FROM HORSE-RADISH LEAVES TO WHICH THE COMPOUNDS SHOWN WERE ADMINISTERED*

	Sulphide	Thiosulphate		Methionine
		Outer-	Inner-	
Sinigrin				
Specific activity†	9·8	2·2	4·4	3·3
Per cent incorporation‡	7·2	3·7	6·6	10·2
Distribution				
Per cent in isothiocyanate	24	20	16	91
Per cent in sulphate	76	80	84	9

* The metabolic period was the same as that designated in Table 4.

† Specific activity = μc/mmole.

‡ Per cent incorporation = $\frac{\text{total activity in sinigrin}}{\text{total activity administered}} \times 100$.

DISCUSSION

The isothiocyanate-S obtained from sinigrin must undergo a number of reduction steps when derived from sulphate. Although very little work has been done on the reduction of sulphate in higher plants, there is evidence to indicate that it is carried out in a manner similar

to that employed by micro-organisms. Recent reviews[8,9] discuss this reduction process and Fig. 1 summarizes the steps which are involved in the reduction and oxidation of sulphur. The reduction of sulphate begins with its activation by way of adenosine-5'-phosphosulphate (APS) to 3'-phosphoadenosine-5'-phosphosulphate (PAPS). The active sulphur is then reduced to sulphite and finally to sulphide. Sulphide according to the cycle can undergo the oxidation step through thiosulphate, sulphite and APS back to sulphate. Inorganic sulphur from the cycle can be converted into organic sulphur by the formation of cysteine from the condensation of sulphide with serine by the enzyme serine hydro-lyase (adding H_2S).[10]

The incorporation of sulphur from sodium sulphate into sinigrin of horse-radish leaves might undergo reactions as shown in Fig. 1. The biosynthesis of the bisulphate moiety can be visualized to occur by the transfer of sulphate from PAPS to a suitable acceptor ("X") by a sulphotransferase.[9] The origin of the isothiocyanate sulphur undoubtedly is more

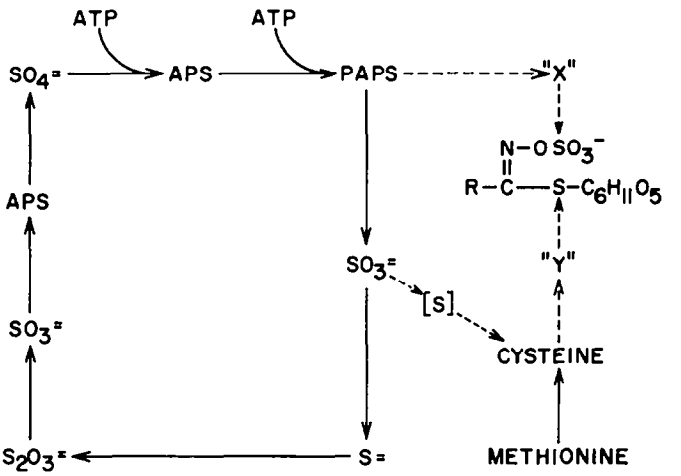


FIG. 1. A PROPOSED SCHEME FOR THE INCORPORATION OF SULPHUR INTO SINIGRIN IN HORSE-RADISH. ATP, adenosine triphosphate; APS, adenosine-5'-phosphosulphate; PAPS, 3'-phosphoadenosine-5'-phosphosulphate; R, allyl side chain.

complex. Sulphur from any of the inorganic anions tested could be oxidized or reduced to PAPS and then reduced further to sulphite and finally to an active sulphide [S]. The next step would involve a reaction analogous to the one in yeast[10] in which either cysteine or some other organic compound is formed. This organic sulphur can now be transferred to a suitable acceptor, "Y", and finally by a series of unknown steps appear in the isothiocyanate-S.

Since sulphide condenses with serine in yeast to give cysteine, one would expect that sulphur-35 from sodium sulphide might be incorporated into the isothiocyanate moiety much more effectively than into the sulphate moiety. However, the results indicate that this is not the case and in addition they suggest that sulphide is first oxidized to sulphate and then incorporated into sinigrin. Fromageot and Perez-Milan[11] have shown indirectly that this is

[8] L. G. WILSON, *Ann. Rev. Plant Physiol.* **13**, 201 (1962).
[9] J. D. GREGORY and P. W. ROBBINS, *Ann. Rev. Biochem.* **29**, 347 (1960).
[10] K. SCHLOSSMANN and F. LYNEN, *Biochem. Z.* **328**, 591 (1957).
[11] P. FROMAGEOT and H. PEREZ-MILAN, *Biochim. Biophys. Acta* **32**, 457 (1959).

what happens in whole tobacco leaves. This, therefore, is the reason for showing active sulphur [S] instead of inorganic sulphide. The results for both inner- and outer-labelled thiosulphate suggest that both sulphurs are oxidized to sulphate and then reduced and incorporated as already described. The methionine-sulphur appears almost exclusively in isothiocyanate and one can visualize this occurring by way of cysteine, as the sulphur in the latter is derived from the breakdown of methionine.[12]

MATERIALS AND METHODS

Cultivation of Plants and Administration of Labelled Compounds

Horse-radish (*Armoracia lapathifolia* Gilib.) plants were grown in a modified Hoagland solution from root cuttings in gravel culture. The plants were grown either in the greenhouse or in a growth chamber with a day-length of 18 hr and a light intensity of 15,000 lx. Leaves of plants that were 2 to 3 months old were employed for biosynthetic studies. The radioactive material was administered to the leaves as described in a previous publication.[5] The amount of radioactive material used is designated in the appropriate tables.

Radioactive Materials

The radioactive samples employed in this investigation were obtained from various commercial sources.

Counting of Sulphur-35

The samples were counted as solid samples at infinite thinness with a gas flow detector equipped with Micromil windows.[13] The radioactive solutions were plated on stainless-steel planchets over a 2 cm^2 area; in order to ensure even spreading a drop of 0·1 % detergent solution was added to the sample on the planchet. The sample was then dried under a stream of warm air. The plated materials were corrected for self-absorption by using an appropriate correction curve. The latter was prepared by plating the same amount of activity (as sodium sulphate-^{35}S) with increasing amounts of glucose as diluent. From these counts a correction curve relating activity with the weight of material on the planchet was prepared. Corrections were made for radioactive decay.

Isolation of Sinigrin

The isolation procedure previously described[5] has been considerably modified to obtain increased yields of crystalline sinigrin. The initial extraction was carried out as before[5] and the lipid and amino acid fractions isolated. The acid effluent from the Amberlite IR-120 (H^+) column was neutralized with 5 N KOH and then concentrated *in vacuo*. The concentrate was then passed through a Dowex-2 × 8 (Cl^-) column. The dimensions of the latter column were determined by taking into account the amount of 5 N KOH employed, i.e. 1 meq of base requires 1 cm^3 of Dowex-2. After application to the column it was washed with water and the latter added to the effluent (designated as the sugar and neutral fraction). The sinigrin was then eluted from the Dowex column with 0·1 N potassium salicylate and the eluate was collected with a fraction collector in 5-ml samples in order to separate acids from the thio-glucoside. Salicylate was employed as it effectively displaced the bisulphate ion (found in

[12] Y. MATSUO and D. M. GREENBERG, *J. Biol. Chem.* **230**, 545 (1958).
[13] The equipment employed was obtained from Nuclear Chicago.

sinigrin) from this resin.[14] The sinigrin-containing tubes were located by spotting on paper and spraying with ammoniacal silver nitrate. The sinigrin usually appeared with the salicylate and the tubes containing the thioglucoside were bulked and acidified to pH 2 with 5 N H_2SO_4. The precipitate of salicylic acid was removed by filtration and the last traces were eliminated by extracting the acid solution with diethyl ether. The acid solution was again neutralized with 5 N KOH and evaporated to dryness *in vacuo* and then crystallized as previously reported.[5]

During this investigation it was found that the crystalline sinigrin thus isolated was grossly contaminated with potassium nitrate. The nitrate was removed by using a resin column (1 cm diameter) prepared as follows: 6 cm^3 of Amberlite IR-120 (H^+) was packed on top of 3 cm^3 of Amberlite IR-45 (OH^-). Before crystallization the dried methanol extract was dissolved in distilled water (1 g in 50 ml) and passed through the above column at approximately one drop per second or fast enough to retain the nitrate. The acid solution from the column was again neutralized with KOH and evaporated to dryness *in vacuo*. The dry residue was dissolved in 90% ethanol from which the sinigrin crystallized as the potassium salt. Carbon and hydrogen analyses verified that this sample contained no nitrate.

Enzymatic Hydrolysis of Sinigrin

Enzymatic hydrolysis, which yields D-glucose, allyl isothiocyanate, and sulphate was employed to separate the two sulphur atoms found in sinigrin. The thioglucoside was hydrolysed for approximately 12 hr with myrosinase in 0·02 M citrate buffer, pH 4·0.[15] The mixture was steam distilled and the first 60 ml of distillate was collected in 10 ml of 6 N NH_4OH. The ammoniacal distillate was boiled in a water-bath for 2 hr and then counted to give the radioactivity of the sulphur atom found in the isothiocyanate molecule. The activity of the bisulphate portion of the thioglucoside was obtained by counting the residue.

Acknowledgements—The author would like to thank Mr. J. W. Kirkpatrick for his excellent technical assistance and Dr. R. L. Eager of the University of Saskatchewan for his generous gift of inner- and outer-labelled thiosulphate. The author also would like to thank Mr. J. H. Wasson, who grew the plant material employed for this investigation.

[14] R. M. WHEATON and W. C. BAUMAN, *Ind. Eng. Chem.* **43**, 1088 (1951).
[15] L. R. WETTER, *Can. J. Biochem. Physiol.* **33**, 980 (1955).