The Formation of Sulphur-Containing Amino Acids in Germinating Seeds of Rape (*Brassica napus* L.)

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Sodium [^{35}S]sulphide was fed to batches of germinating rapeseed, in some instances with the addition of unlabelled cysteine. Both the total radioactivity and specific radioactivity of the free sulphur-containing amino acids were examined. Cysteine and homocysteine were rapidly labelled; label subsequently appeared in cystathionine and methionine. The results obtained indicated that both the sulphydration and trans-sulphuration pathways were operating. This conclusion was reinforced by the results of experiments in which batches of rapeseed were incubated with L-[^{14}C]homoserine. These showed the formation of labelled homocysteine, cystathionine and methionine. It was thought the trans-sulphuration pathway was making the greater contribution to the biosynthesis of methionine in germinating rapeseed.

The metabolism of sulphur-containing amino acids in higher plants has not been explored in detail. Most of our present knowledge of the metabolism of these amino acids has been obtained from studies with micro-organisms or animals (Davies, 1968). Thus, except for the work of Ellis (1963) on the biosynthesis of cysteine in beet discs, there appear to be no reports of experiments *in vivo* to determine the pathways of biosynthesis of the sulphur-containing amino acids in plant tissues.

Studies have been performed *in vitro* by Giovanelli & Mudd (1966, 1967, 1968) and Thompson & Moore (1968) to investigate the biosynthesis of these amino acids in plant extracts. These studies have shown that the two main pathways found to operate in bacteria, sulphydration (eqn. 1) and trans-sulphuration (eqns. 2 and 3), may also be operative in plants (Kredich & Tomkins, 1966; Delavier-Klutchko & Flavin, 1965; Giovanelli & Mudd, 1966, 1967).

Opinions seem to differ, however, about which of these pathways is the more important in plants (Giovanelli & Mudd, 1967; Kerr, 1971).

The present paper describes experiments in which [^{35}S]sulphide and L-[^{14}C]homoserine were fed to germinating rapeseed, and the subsequent fate of these compounds was then investigated. The results obtained are discussed in relation to the possible

routes for the biosynthesis of the sulphur-containing amino acids in these seeds.

Materials and Methods

Chemicals

Sodium [^{35}S]sulphide and L-[U- ^{14}C]homoserine were purchased from Amersham Searle Corp., Don Mills, Ontario, Canada. Na_2^{35}S was also purchased from New England Nuclear Corp., Dorval, Quebec, Canada. L-Cystathionine (A grade) was obtained from Calbiochem., Los Angeles, Calif., U.S.A. L-Cysteine and DL-homocysteine thiolactone were from Sigma Chemical Co., St. Louis, Mo., U.S.A. Other amino acids were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. All other chemicals were obtained from commercial sources and were of the highest purity available.

Plant materials and their treatment

Rapeseed (*Brassica napus* L. var. Target) was obtained from Dr. R. K. Downey of the Canada Department of Agriculture, Saskatoon. Before use in the feeding experiments, batches (5–7 g) of seed were first surface sterilized with 1% sodium hypochlorite solution for 3 min. The seeds were quickly washed with sterile water and sucked dry on a sintered-glass filter. They were then transferred to a sterile Petri dish containing 2 ml of sterile 0.05 M-potassium phosphate buffer, pH 7.35. The desired label was now added, and further additions of sterile phosphate buffer were made to keep the seeds moist until the experiment was finished. At this time, the

seeds were quickly washed with 1 litre of 0.5M-NaOH, followed by 1 litre of deionized water. They were then subjected to the extraction procedure described below.

Extraction of amino acids

The extraction procedure of Bielecki & Turner (1966) was modified as follows. First the seeds were homogenized in 100ml of methanol-chloroform-water (4:5:1, by vol.), by using a Virtis homogenizer operated at full speed for 2 min. This was followed by centrifugation at 20000g for 10 min in the SS34 head of the Sorvall RC2 refrigerated centrifuge operated at 2°C. The pellets were treated by boiling for 3 min in 80% (v/v) ethanol, and then homogenizing in a Virtis homogenizer for 2 min at full speed, and the homogenate was centrifuged in the Sorvall centrifuge under the conditions described above. The supernatant from this step was termed the ethanol extract.

The methanol-chloroform-water supernatant was stirred for 15 min with 50ml of chloroform plus 75ml of water by using a magnetic stirrer. The resulting mixture was centrifuged in the Sorvall centrifuge, under the conditions described above. The aqueous layer was collected and added to the ethanol extract. The chloroform layer was further extracted with 2 × 50ml of water, the aqueous layers were separated as described above and added to the ethanol extract. The final mixture of the ethanol extract, plus all aqueous layers was dried *in vacuo* at 50°C by using a Buchler rotary evaporator.

The dried material was now extracted with 2 × 25ml of di-ethyl ether, and the oil-free material dissolved in 10ml of 0.1M-HCl-0.07M-2-mercaptoethanol. This mixture was then fractionated on a column (1cm × 5cm) of Dowex 50W ion-exchange resin (X8; 200-400 mesh; H⁺ form). The column was washed with 50ml of deionized water, and then the acidic and neutral amino acids were eluted with 85ml of 0.2M-pyridine (Biemann & Deffner, 1961). The pyridine eluate was evaporated to dryness at 60°C *in vacuo* and dissolved in 10ml of the HCl-mercaptoethanol solution.

Thin-layer chromatography

T.l.c. plates were prepared by first homogenizing 20g of microcrystalline cellulose (Sigma cell; Sigma Chemical Co.) with 8g of MN-Silica gel G (Canadian Laboratory Supplies, Toronto, Ont., Canada), in 160ml of deionized water by using a Virtis homogenizer. This mixture was then spread (500μm thick) on glass plates (20cm²), which were dried at 105°C. For chromatographic separation, samples of the amino acid fraction were applied to the plates and dried with a stream of cold air and portions of mercaptoethanol were then overlaid on the same spots. The chromatograms were developed in chloro-

form-methanol-aq. 17% NH₃-mercaptoethanol (10:10:5:1, by vol.). The *R_F* values of ethionine, methionine, methylcysteine, homocysteine, cysteine and cystathionine in this system were 0.760, 0.700, 0.640, 0.526, 0.360 and 0.126 respectively.

Paper chromatography

Whatman 1MM paper was used, and the amino acid spots were developed in butan-1-ol-acetic acid-water (25:6:28, by vol.) for 40h under an atmosphere of N₂. Before the application of amino acids the extract was passed through a column of Dowex 50 ion-exchange resin and washed successively with 30ml of deionized water, 25ml of 0.05M-HCl and 30ml of water. Amino acids were then eluted from the column with 4M-NH₃. This latter procedure was necessary to remove any trace of mercaptoethanol, which would have interfered with the subsequent determination of sulphur-containing amino acids.

Identification and determination of amino acids

The sulphur-containing amino acids were identified by co-chromatography with authentic amino acids, followed by detection with ninhydrin spray [0.3g of ninhydrin in 100ml of 3% (v/v) acetic acid in butan-1-ol] and iodoplatinate spray (0.2g of platonic chloride and 4g of KI in 100ml of water). Amino acid spots were also subjected to oxidation with hydrogen peroxide as a further confirmatory test. The areas corresponding to methionine, cysteine or homocysteine were cut out from the chromatograms and eluted in 1M-HCl. They were then oxidized with 0.1 ml of 30% (v/v) hydrogen peroxide. The products of oxidation were then re-chromatographed on t.l.c. plates in the chloroform-methanol-aq. 17% NH₃-mercaptoethanol solvent. Authentic compounds were also oxidized in a similar manner and chromatographed together with the test compounds. The *R_F* values of oxidized methionine, homocysteine and cysteine were 0.377, 0.154 and 0.160 respectively. The oxidation and chromatography of cystathionine was carried out by the procedure of Mudd *et al.* (1965).

Quantitative measurements of the sulphur-containing amino acids were obtained by using the iodoplatinate colorimetric procedure of Awwad & Adelstein (1966), which is specific for sulphur-containing amino acids. Standard curves were produced for each of the sulphur-containing amino acids determined.

Measurement of radioactive amino acids

Labelled compounds on t.l.c. plates were located by radioautography on Kodak No-Screen X-ray film. The activity of the separated compounds was ascertained either by transferring the radioactive area to scintillation vials containing fluor and Cab-O-Sil, or

the cellulose acetate layer technique of Bielecki & Turner (1966) was used. In both cases, a toluene fluor mixture containing 0.05 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene and 5 g of 2,5-diphenyloxazole per litre of toluene was used, in the amount of 15 ml per scintillation vial. Radioactivity was measured with a Beckman LS-100 liquid-scintillation counter. Counting efficiency was greater than 70% in both cases. The same fluor system was used to measure the radioactivity in spots cut from paper chromatograms. These were inserted directly into 15 ml of fluor in a vial, and counted with approx. 70% efficiency.

Hydrolysis of rapeseed-meal protein

The insoluble residue left after the solvent extraction procedure was hydrolysed by the method of Finlayson (1965) and Bhatti *et al.* (1968).

The rapeseed meal from 7 g of seed was washed twice with 100 ml of 80% (v/v) ethanol, and sucked dry on a sintered-glass filter. The dry meal was then extracted with 500 ml of light petroleum (b.p. 30–60°C; Fisher Pesticide grade) and dried *in vacuo* for 24 h. Defatted meal (20 mg) was hydrolysed with constant-boiling HCl (b.p. 107°C) in a sealed tube, in an atmosphere of HCl vapour. The sealed-tube hydrolyses were carried out in an oven at 107±2°C for 15 h. The hydrolysates were then filtered on a sintered-glass filter, evaporated to dryness in a Buchler rotary evaporator at 60°C and freeze-dried. After freeze-drying the hydrolysates were dissolved in 6 ml of deionized water and fractionated on Dowex 50 (H⁺ form) as described above, with 4M-NH₃ as solvent.

Results

Extraction and chromatography of amino acids

Attempts to extract sulphur-containing amino acids from rapeseed by the methods of Bielecki &

Turner (1966) alone led to a 40% loss of sulphur-containing amino acids. Further extraction of the chloroform layer with water was found to be necessary to give a better recovery. Thus the procedure described in the Materials and Methods section gave a recovery of approx. 80% for the sulphur-containing amino acids. These calculations were based on recoveries of ³⁵S-labelled amino acids put through the complete extraction procedure. The amino acid fraction was always dissolved in solutions containing mercaptoethanol at all stages of experimentation before paper chromatography, to prevent the formation of disulphide compounds by oxidation of thiol-containing amino acids.

Continuous feeding experiments with sodium [³⁵S]-sulphide

The results of preliminary feeding experiments are summarized in Table 1. In each case, 4 μCi of Na₂³⁵S (2.3 mCi/mmol) was fed to 5 g of seeds which were then allowed to germinate for 2 h as described in the Materials and Methods section. When seeds were germinated in buffer containing Na₂³⁵S but no other additions, then 0.77% of the total radioactivity fed was incorporated into the amino acid and aqueous fractions. If however, the seeds were germinated in buffer also containing 0.5 mmol of unlabelled L-cysteine, then 1.44% of the total radioactivity fed was incorporated into these fractions. Further, in the presence of exogenous cysteine, the ³⁵S incorporated into the amino acid fraction was increased by more than twofold, compared with the experiments carried out in its absence (Table 1). The increase in the radioactivity incorporated into the sulphur-containing amino acids was due predominantly to an increase in the amount of labelled cysteine isolated. This was accompanied by a decrease in the radioactivity found in the other sulphur-containing amino acids (Table 1).

The reason for the large amount of radioactivity

Table 1. *Effect of exogenous cysteine on the uptake and metabolism of [³⁵S]sulphide by germinating rapeseed*

Seed was germinated in 0.05 M-potassium phosphate buffer, pH 7.35, containing 4 μCi of sodium [³⁵S]sulphide for 2 h. L-Cysteine (0.5 mmol) was either present or absent from the medium as indicated in the table.

Fraction	10 ⁻⁵ × Radioactivity (c.p.m./5 g of dry seed)		Distribution of ³⁵ S in sulphur-containing amino acids	
	+Cysteine	–Cysteine	+Cysteine	–Cysteine
Total free sulphur amino acids	6.63	2.48	—	—
Aqueous fraction	14.85	9.10	—	—
Cysteine	3.40	0.50	51%	20%
Cystathionine	1.62	0.90	24%	36%
Methionine	0.86	0.63	13%	25%
Homocysteine	0.75	0.45	11%	18%

Fig. 1. Incorporation of ^{35}S into the cysteine (●), cystathionine (■), homocysteine (▼) and methionine (○) of the free amino acid pool of germinating rapeseed

Seeds were incubated in 0.05M-potassium phosphate buffer, pH 7.35, containing $4\mu\text{Ci}$ of Na_2^{35}S + 0.5mmol of unlabelled cysteine for the periods of time indicated.

present in the aqueous fraction [eluted from Dowex 50 (H^+ form) resin (Table 1)] is not known, but it is likely that some ^{35}S sulphide is oxidized to ^{35}S -sulphate which in turn is reduced back to ^{35}S sulphide (Wetter, 1964). The ^{35}S sulphate would be expected to appear in the aqueous fraction, and thus increase the amount of radioactivity in this fraction.

Kinetics of ^{35}S incorporation into the sulphur-containing amino acids

Feeding experiments were also carried out during which a number of batches of rapeseed were allowed to germinate for time-periods up to 72h. The first such experiments performed used amounts of seed and label similar to those in the preliminary experiments described above. In addition, however, 0.5mmol of unlabelled cysteine was present in each experiment. The results obtained from this series are shown in Fig. 1, which shows that cysteine was the most heavily labelled amino acid. The total radioactivity present in cysteine declined rapidly during the first 24h. The amounts of radioactivity in the other sulphur-containing amino acids rose fairly rapidly during the first 5h of germination, but in contrast with that of cysteine they remained fairly constant after this.

A second series of experiments utilized 7g portions of seed which were incubated with 0.5mCi of Na_2^{35}S (specific radioactivity 25mCi/mmol), for the periods of time indicated in Figs. 2(a) and 2(b); no cysteine was added in these experiments. Fig. 2(b) shows that initially homocysteine was the most rapidly labelled amino acid. The specific radioactivity of the homo-

Fig. 2. Time-course of distribution of (a) total radioactivity or (b) specific radioactivity among cysteine (●), cystathionine (■), homocysteine (▼) and methionine (○) of the free amino acid pool of germinating rapeseed

Seeds were incubated in 0.05M-potassium phosphate buffer, pH 7.35, containing 0.5mCi of Na_2^{35}S , for the periods of time indicated.

cysteine pool appears to fall rapidly after about 3h, however, and it appears to be overtaken by that of cysteine. The specific radioactivity of cysteine falls in turn after about 5h, when there is a dramatic increase in the specific radioactivity of cystathionine. At about the same time, there is also a less spectacular increase in the specific radioactivity of homocysteine, which appears to be maintained for the duration of the experiments. It is notable that the specific radioactivity of methionine appears to increase in a regular manner throughout the entire course of the experiments.

The total radioactivity and specific radioactivity of cysteine and methionine isolated from the insoluble residue obtained from the previous experiments is shown in Table 2. The radioactivity in the insoluble fraction due to cysteine has reached a constant value by 1 h, and appears to stay at this value thereafter. The total and specific radioactivities of methionine appear to decline, however, after about 5 h germination.

Formation of cystathionine, homocysteine and methionine from homoserine in germinating rapeseed

Portions (6g) of seeds were incubated with 50 μ Ci of L-[U- 14 C]homoserine (31.8 mCi/mmol). Calculations showed that 8.2% and 21.1% of the total 14 C radioactivity originally fed was incorporated into the free sulphur-containing amino acids of the 4 h and 10 h samples respectively. The radioactivities of cystathionine, homocysteine and methionine in the 4 h and 10 h samples are given in Table 3. The activity of homocysteine remained relatively constant in both samples. In the 4 h sample the radioactivity of homocysteine is similar to that of cystathionine, but in the 10 h sample the radioactivity in cystathionine has increased by more than threefold, whereas that of homocysteine has stayed almost constant.

Discussion

Sulphide is considered to be the most reduced form of sulphur that is utilized by plants in the manufacture of organic sulphur-containing compounds such as amino acids (Roy & Trudinger, 1970). The results in the present paper show that when rapeseed samples were germinated in the presence of sodium [35 S]-sulphide, there was an immediate uptake of label with the subsequent formation of labelled cysteine, cystathionine, homocysteine and methionine (Table 1 and Figs. 1, 2a and 2b). When the experiments were carried out in the presence of a large excess of unlabelled cysteine, two effects were observed. First, the uptake of label into amino acid and non-amino acid fractions was greatly increased; the explanation of this increase is not known for certain. The second effect was a more specific one on the actual composition of the free amino acids (Table 1). Thus not only was the total amount of label entering the cysteine pool greatly increased, but the total amount of label entering cystathionine, homocysteine and methionine was greatly decreased compared with the control experiments containing no cysteine (Table 1). The most likely explanation for this occurrence is that cysteine is the first intermediate in the pathway of biosynthesis of the other sulphur-containing amino

Table 2. Total and specific radioactivities of cysteine and methionine isolated from hydrolysates of rapeseed meal

The seeds from which the meal was obtained had previously been incubated with 0.5 mCi of Na $_2^{35}$ S in 0.05 M-potassium phosphate buffer, pH 7.35, for the periods of time indicated in the table. Values in this table are not corrected for any hydrolytic losses of cysteine and methionine that may have occurred.

Time of feeding with Na $_2^{35}$ S (h)	10 $^{-5}$ $\times$ Total radioactivity (c.p.m./g of defatted rapeseed meal)		Sp. radioactivity (c.p.m./ μ mol)	
	Cysteine	Methionine	Cysteine	Methionine
1	5.62	4.14	221	72
3	4.39	4.75	174	88
5	5.15	5.05	214	99
9	6.35	4.57	251	84
13	4.88	3.83	156	72
21	5.76	3.72	220	68

Table 3. Conversion of L-[U- 14 C]homoserine into free 14 C-labelled sulphur-containing amino acids by germinating rapeseed

Seeds were fed on 50 μ Ci of labelled homoserine, in 0.05 M-potassium phosphate buffer, pH 7.35, for the periods of time indicated in the table.

Time of feeding with L-[U- 14 C]homoserine (h)	10 $^{-4}$ $\times$ Radioactivity (c.p.m./6g of seed)		
	Cystathionine	Homocysteine	Methionine
4	3.490	3.217	10.420
10	13.640	3.894	8.816

Scheme 1. Possible relationships between pathways involved in the biosynthesis of sulphur-containing amino acids present in germinating rapeseed

acids. Since cysteine will be actively metabolized in plants to give protein (Table 2), thioglucosides (Wetter & Chisholm, 1968) and cystathionine (Giovannelli & Mudd, 1966), it is reasonable that the amount of ^{35}S in labelled free cysteine, formed by the germinating seeds in the absence of exogenous cysteine, will be far lower than that found in cysteine produced in the presence of exogenous cysteine.

These results also argue for the presence of a bacterial type of biosynthetic pathway where cysteine acts as the primary sulphur donor (Young & Maw, 1958; Maw, 1967) rather than a mammalian pathway where homocysteine acts as the primary donor (Meister, 1965). A consideration of the results presented in Figs. 2(a) and 2(b) reinforces the idea that a bacterial type of trans-sulphuration pathway plays a major role in the biosynthesis of homocysteine and methionine. This can be deduced first from the high specific radioactivity of cystathionine between 5 and 21 h, and secondly, from the previous rapid rise and

decline in the specific radioactivity of cysteine, which appears to behave as the precursor of cystathionine.

However, the compound that is labelled most rapidly is homocysteine (Fig. 2b). Thus it would appear that at least during the first 4–5 h of germination the sulphydration pathway is operating, and may be responsible for the synthesis of significant amounts of homocysteine and methionine from homoserine and a sulphur source.

The results of the L-[U- ^{14}C]homoserine-feeding experiments (Table 3) indicate that initially homocysteine is produced at a rate sufficient to give a total radioactivity comparable with that of cystathionine at 4 h. After this time, however, much more radioactivity is incorporated into cystathionine, indicating that the trans-sulphuration pathway is becoming very active. Thus these results seem in accord with those obtained from the [^{35}S]sulphide-feeding experiments.

It has been noted (see the Results section) that the

synthesis of methionine seems to take place at a steady rate and is not subject to marked fluctuations. This is what one would expect of an end-product of a biosynthetic route, and if indeed both the trans-sulphuration and sulphydration pathways are operating, then they are obviously well integrated as far as the biosynthesis of methionine is concerned. Previous workers in this field have not suggested that both pathways might operate in any form of concerted or harmonious fashion (Giovanelli & Mudd, 1967, 1971). Scheme 1 is an attempt to compose these ideas on the two pathways into a rational metabolic scheme. It should be emphasized that no suggestion is being made of a compartmentation or separation of the two sets of reactions; indeed they may possibly be a function of one enzyme complex, as in some fungi (Kerr & Flavin, 1970).

A very noteworthy fact from Fig. 2(b) is that the sharp fall that occurs in the specific radioactivity of cystathionine after 9 h seems to be correlated with a sharp increase previously seen in the specific radioactivity of methionine. Work by Kerr & Flavin (1969) and Selhub *et al.* (1971) has indicated that the activity of cystathionine γ -synthase of *Neurospora* is inhibited by *S*-adenosylmethionine. Thus at least part of the explanation of the sharp fall in the specific radioactivity of cystathionine may possibly be the inhibition of cystathionine biosynthesis by *S*-adenosylmethionine. The formation of *S*-adenosylmethionine from methionine in higher plants has been shown to occur (Davies, 1966; Dodd & Cossins, 1968, 1969). Isolation of cystathionine γ -synthase from rapeseed, and investigation of the effect (if any) of *S*-adenosylmethionine or methionine on its activity, should show if such a feed-back control mechanism is operating.

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