ENZYMATIC SYNTHESIS OF CYSTATHIONINE BY EXTRACTS OF SPINACH, REQUIRING O-ACETYLHOMOSERINE OR O-SUCCINYLHOMOSERINE

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The known pathways of transsulfuration, the process whereby sulfur is transferred between homocysteine and cysteine, all involve cystathionine as an intermediate. In this process, both the direction of sulfur flow and the enzymatic mechanisms involved are different in different phyla. In mammals, transsulfuration proceeds only in the direction homocysteine → cysteine *via* the following reactions:

$$\text{homocysteine + serine} \xrightarrow[\gamma\text{-elimination}]{\beta\text{-replacement}^2} \text{cystathionine + water} \quad (1)$$

$$\text{cystathionine + water} \longrightarrow \text{cysteine + } \alpha\text{-ketobutyrate + ammonia} \quad (2)$$

Reaction (1) is catalyzed by cystathionine β-synthase (Selim and Greenberg, 1959); reaction (2) is catalyzed by a cystathionine γ-cleavage enzyme (Matsuo and Greenberg, 1958). In bacteria, transsulfuration proceeds only in the direction cysteine → homocysteine *via* the following reactions:

$$\text{cysteine + O-succinylhomoserine} \xrightarrow[\beta\text{-elimination}]{\gamma\text{-replacement}} \text{cystathionine + succinate} \quad (3)$$

$$\text{cystathionine + water} \longrightarrow \text{homocysteine + pyruvate + ammonia} \quad (4)$$

Reaction (3) is catalyzed by cystathionine γ-synthase (Rowbury, 1964; Delavier-Klutchko and Flavin, 1965a); reaction (4) is catalyzed by a cystathionine β-cleavage enzyme (Delavier-Klutchko and Flavin, 1965b). In fungi, pathways exist for both directions of sulfur transfer. Transsulfuration of homocysteine → cysteine proceeds *via* the combined reactions (1) and (2). Transsulfuration of

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[2]Since International Union of Biochemistry recommendations have not yet appeared on all four of the enzymes of transsulfuration, in general the terminology of Flavin (1963) is employed. This terminology refers to the particular carbon atom where the electronegative leaving group is situated.

cysteine → homocysteine probably proceeds via a γ-replacement reaction similar to reaction (3). Although the enzyme catalyzing this reaction in fungi has not been demonstrated, nutritional and other data (Nagai and Flavin, 1966) strongly suggest that O-acetylhomoserine is the source of "activated" homoserine. The conversion of cystathionine to homocysteine in fungi proceeds according to reaction (4).

Relatively little experimental work has been done on transsulfuration in higher plants. As part of a recently initiated study in this field, Giovanelli and Mudd (in preparation) have demonstrated that extracts of higher plants contain enzymes that cleave cystathionine. The rate of β-elimination (reaction 4) far exceeds that of γ-elimination (reaction 2). This note describes the synthesis of cystathionine according to reaction (3), by crude extracts of spinach. Synthesis proceeds equally well with either O-succinylhomoserine or O-acetylhomoserine. Synthesis of cystathionine from homocysteine and serine (reaction 1) could not be demonstrated.

EXPERIMENTAL: Extracts of an acetone powder of spinach were prepared as described by Giovanelli and Mudd (in preparation). Escherichia coli B cells (generously supplied by Dr. Herbert Weissbach) were ground with aluminum powder and the resulting extract clarified by centrifugation.

The standard reaction mixture contained the following components (in μmoles) in a final volume of 0.9 ml: potassium phosphate, pH 7.25 (90), pyridoxal phosphate (0.09), L-cystathionine (0.15), 1,4-dithio-meso-erythritol (1.5), L-cysteine-^{35}S, cysteine-^{14}C or $Na_2{}^{35}S$ (0.357), and enzyme equivalent to approximately 10 mg of protein. Supplementary amino acids were added where shown so that 4.5 μmoles of the L-isomer was present. Incubation was for two hours at 30° in a nitrogen gas phase.

The reaction was stopped by addition of 0.4 ml of a solution containing trichloroacetic acid (7.5%), L-cysteine (0.1 M) and L-cystathionine (0.75 mM). Protein was removed by centrifugation. An aliquot of 1.0 ml of supernatant solution was applied to a column of Dowex 50 H^+, which was then washed with water and eluted first with 0.4 N HCl, then with 3 N NH_4OH (Mudd et al., 1965). An aliquot of the NH_4OH eluate was oxidized with performic acid, subjected to electrophoresis in formic acid pH 1.6, and the distribution of radioactivity determined (Giovanelli and Mudd, in preparation). During chromatography on Dowex 50, $Na_2{}^{35}S$ is recovered in the effluent and washings. Most of the radioactivity originally present in cysteine is recovered in the HCl eluate. Cystathionine and other strongly basic compounds are recovered, relatively free of radioactive substrate, in the NH_4OH eluate. A small amount of radioactive cystine, formed by chemical oxidation of cysteine, is found in the ammonia eluate, but is readily separated from cystathionine in the subsequent steps of performic acid oxidation and electrophoresis.

Reduction with Raney nickel was performed essentially as described by Alderton (1953). Paper chromatograms were developed by the descending technique in solvents A, B or C. Solvent A contained methyl alcohol-pyridine-1.25 N HCl; 185 : 20 : 40. Solvent B contained n-butyl alcohol-glacial acetic acid-water;

120 : 30 : 50. Solvent C contained methanol-pyridine-water; 20 : 1 : 5. L-Cysteine-^{35}S and -^{14}C were prepared from correspondingly labeled cystine (Giovanelli and Mudd, in preparation). O-Succinyl-DL-homoserine was prepared by the method of Flavin and Slaughter (1965); O-acetylhomoserine was prepared essentially by the method of Sakami and Toennies (1942). O-Acetyl-L-serine and O-acetyl-L-threonine were from Yeda Research and Development, Rehovoth, Israel.

RESULTS: Figure 1 illustrates how the addition of various amino acids affects the products of cysteine-^{35}S metabolism. In the absence of added amino acid (Fig. 1A) no detectable peak of radioactivity migrated to the cathode. Addition of O-acetyl-L-homoserine (Fig. 1B) led to the formation of two new major peaks of radioactivity that migrated to the cathode. One of these migrated to the cystathionine area. The other peak of radioactivity is an unknown compound (designated compound X) that migrated more rapidly than cystathionine.

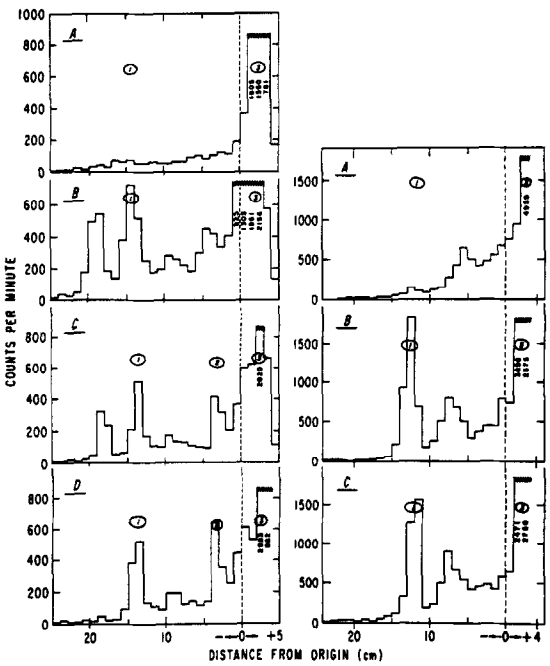

Fig. 1. Products of L-cysteine-^{35}S metabolism. A = no addition to standard reaction mixture; B = O-acetyl-L-homoserine added; C = O-acetyl-DL-homoserine added; D = O-succinyl-DL-homoserine added.

Fig. 2. Products of L-cysteine-U-^{14}C metabolism. A = no additions to standard reaction mixture; B = O-acetyl-L-homoserine added; C = O-succinyl-DL-homoserine added.

Compound X is the peak that migrates at approximately 19 cm. Enclosed numerals represent scale tracings of ninhydrin-positive areas corresponding to the oxidized products of: 1, cystathionine; 2, unknown; 3, cysteine. The direction of the cathode and anode is represented by - and + respectively.

Addition of O-acetyl-DL-homoserine (Fig. 1C) resulted in the same compounds being labeled, but to a less extent than with O-acetyl-L-homoserine. O-Succinyl DL-homoserine (Fig. 1D) was approximately as effective as O-acetyl-DL-homoserine in labeling cystathionine. However, compound X was not labeled in the presence of O-succinylhomoserine. No detectable peaks of radioactivity migrated to the cathode under the following conditions: (a) When the standard reaction mixture was supplemented with L- or DL-homoserine, O-acetyl-L-serine, L- or DL-serine, O-acetyl-L-threonine or L-threonine. (b) When a boiled enzyme was substituted for fresh enzyme in the presence of a reaction mixture supplemented with O-acetyl-L-homoserine. (c) When the amino acids L-serine-3-^{14}C and L-homocysteine were substituted for radioactive cysteine in the standard reaction mixture, or in the reaction mixture used to assay cystathionine β-synthase (Mudd et al., 1965).

The products of cysteine-U^{14}C metabolism are shown in Fig. 2. Formation of radioactive cystathionine is again dependent upon the addition of either O-acetylhomoserine (Fig. 2B) or O-succinylhomoserine (Fig. 2C). These observations thus confirm the expected incorporation of both carbon and sulfur atoms of cysteine into cystathionine. Carbon atoms of cysteine were not, however, incorporated into compound X. This latter observation suggested that H_2S, produced by enzymatic desulfuration of cysteine (Bruggeman et al., 1962), may be a more direct precursor than the sulfur of cysteine. This suggestion was supported by the observation that the rate of $Na_2{}^{35}S$ incorporation was approximately twenty times the rate of cysteine-^{35}S incorporation into compound X. As observed with cysteine-^{35}S, incorporation of $Na_2{}^{35}S$ into compound X specifically required the addition of O-acetylhomoserine.

A number of peaks migrating to the cathode at a rate slower than that of cystathionine were formed from cysteine-^{35}S, cysteine-^{14}C and $Na_2{}^{35}S$. The nature of these peaks is currently being studied.

The radioactive compound migrating with cystathionine in formic acid pH 1.6 was eluted and characterized as follows: (a) When subjected to electrophoresis in sodium carbonate-bicarbonate buffer pH 10.8, the compound migrated to the anode with the product obtained by oxidation of authentic cystathionine with performic acid. (b) When the radioactive compound synthesized from

cysteine-U-^{14}C was treated with Raney nickel the expected amino acids (alanine and α-aminobutyrate) produced from carrier cystathionine were formed. Only alanine contained radioactivity, and was characterized by cochromatography with authentic alanine in solvents B and C.

Table I shows the relative rates at which crude extracts of spinach or E. coli catalyze the synthesis of cystathionine in the presence of O-succinylhomoserine or O-acetylhomoserine. As demonstrated previously in Figs. 1 and 2, the relative rates of cystathionine synthesis by crude extracts of spinach were approximately equal with either O-acetylhomoserine or O-succinylhomoserine.

TABLE I

Relative rates of cystathionine synthesis by crude extracts of spinach and E. coli with O-acetylhomoserine and O-succinylhomoserine

Enzyme	Cystathionine synthesized mμmoles/mg protein/hr.	
	O-acetylhomoserine	O-succinylhomoserine
Spinach	0.73	0.63
E. coli	0.18	4.22

With a crude extract of E. coli, however, the relative rate of synthesis with O-succinylhomoserine and O-acetylhomoserine was 23/1. A corresponding ratio of 10/1 was reported by Nagai and Flavin (1966) for a preparation of cystathionine γ-synthase purified from Salmonella. The activity observed when extracts of spinach and E. coli were combined was additive. This suggests that the marked difference in relative activity observed with the two extracts in the presence of O-succinylhomoserine or O-acetylhomoserine was not due to enzymatic destruction of one of these amino acids. When extracts of E. coli were incubated with cysteine-^{35}S or $Na_2{}^{35}S$, in the presence of either O-acetylhomoserine or O-succinylhomoserine no accumulation of compound X was detected.

DISCUSSION: Demonstration in crude extracts of spinach of the synthesis of cystathionine predominantly by a γ-replacement (reaction 3), and its cleavage predominantly by a β-elimination (reaction 4) suggests that transsulfuration in higher plants may resemble that in bacteria in proceeding

predominantly in the direction cysteine → homocysteine. At present it is not clear whether the incorporation of radioactive cysteine into cystathionine represents a net synthesis or exchange. The following considerations are consistent with net synthesis. First, on theoretical grounds it seems unlikely that a reaction involving an "activated" form of homoserine would be readily reversible. Second, omission of cystathionine from a reaction mixture containing a crude spinach enzyme treated with Sephadex G-25 caused only a slight (35%) decrease in incorporation of cysteine-^{35}S into cystathionine. Some reduction would be expected because the spinach preparation is known to contain an active β-cleavage enzyme (Giovanelli and Mudd, in preparation).

It is clear that some important differences in the metabolism of sulfur in higher plants and bacteria exist. For example, whereas bacterial synthesis of cystathionine is relatively specific for O-succinylhomoserine, in the crude spinach system O-succinylhomoserine and O-acetylhomoserine are approximately equally effective. Another interesting difference is seen in the O-acetylhomoserine-dependent accumulation of compound X by the spinach but not by the E. coli system. Although O-acetylhomoserine is known to be a natural constituent of peas (Grobbelaar and Steward, 1958) the biochemical function of this compound has remained uncertain. A full appreciation of the biochemical significance of the reactions described awaits identification of compound X, and further purification of the enzyme systems involved.

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