SYNTHESIS OF HOMOCYSTEINE AND CYSTEINE BY ENZYME EXTRACTS OF SPINACH

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This communication describes the presence in extracts of spinach of an enzyme that catalyzes the reaction:

sulfide + O-acetylhomoserine → homocysteine + acetate (1)

This represents a new pathway for de novo synthesis of homocysteine. Homocysteine is further metabolized to ASR[1] and to methionine.

Evidence is also presented for de novo synthesis of cysteine in plants by another novel reaction:

sulfide[2] + O-acetylserine → cysteine + acetate[3] (2)

EXPERIMENTAL: Enzyme extracts were prepared from an acetone powder of spinach (Giovanelli and Mudd, 1966). The standard reaction mixture for determination of the rate of incorporation of $^{35}S^{=}$ into sulfur amino acids contained the following components (in μmoles) in a final volume of 0.9 ml: potassium phosphate, pH 7.25 (90), pyridoxal phosphate (0.09), DTE (5) $Na_2{}^{35}S$ (0.5), and enzyme. Supplementary amino acids were added where shown so that 4.5 μmoles of the L-isomer was present. O-Succinylhomoserine was added as the DL-isomer. Other amino acids were added as the L-isomer. Incubation was for two hours at 30° in a nitrogen gas phase.

The reaction was stopped by addition of 0.4 ml of cold 10% TCA, containing 5 μmoles of homocysteine. Protein was removed by centrifugation. The supernatant solution was applied to a column of Dowex 50 (H^+), that was first washed with water and then eluted with 3 N NH_4OH[4]. An aliquot of the NH_4OH eluate was counted to determine the total radioactivity incorporated into sulfur amino acids. The distribution of radioactivity was determined as follows. An aliquot of the NH_4OH eluate was oxidized with performic acid,

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[1] Abbreviations: ASR, S-adenosylhomocysteine; AMe S-adenosylmethionine; DTE, 1,4-dithio-meso-erythritol.

[2] It is not implied that sulfide ion is itself the active ionic species in either reactions 1 or 2.

[3] We have not yet attempted to identify the acetate which is presumed to be a product in both reactions 1 and 2.

[4] In a previous study (Giovanelli and Mudd, 1966) any radioactive homocysteine and methionine were discarded by prior elution with 0.4 N HCl.

subjected to paper electrophoresis in formic acid pH 1.6, and the distribution of radioactivity along the paper strip determined (Giovanelli and Mudd, 1966).

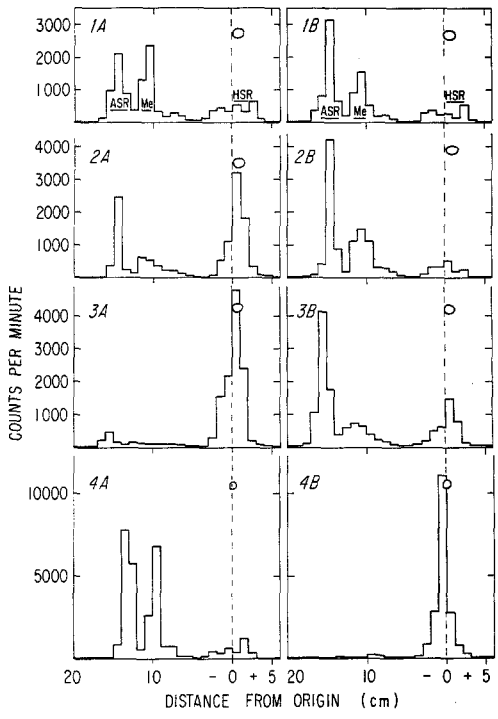
Homocysteine-^{35}S and cysteine-^{35}S were each characterized by the following criteria: (i) paper chromatography of the acid and its N-ethyl maleimide adduct, (ii) oxidation with hydrogen peroxide to the corresponding sulfonic acid. Methionine-^{35}S was characterized by the following criteria: (i) paper chromatography and electrophoresis, (ii) oxidation with hydrogen peroxide to methionine sulfoxide, (iii) conversion to the methylmethionine sulfonium salt.

We have previously reported (Giovanelli and Mudd, 1966) that crude extracts of spinach catalyze the incorporation of $^{35}S^{=}$ into an unknown compound, designated compound X. Compound X has now been characterized as ASR by the following criteria: (i) paper chromatography and electrophoresis, (ii) its absorption spectrum, (iii) oxidation with hydrogen peroxide to its sulfoxide, (iv) conversion to AMe by incubation with methyl iodide. The formation of AMe was confirmed by its neutral hydrolysis to 5'-methylthioadenosine and its alkaline hydrolysis to methionine. Details of the characterizations of each of the above compounds will be published elsewhere.

RESULTS: Figures 1-3 illustrate the relative distribution of radioactivity in sulfur amino acids formed by various enzyme preparations of spinach, and the effect of adenosine on this distribution.

With a crude enzyme preparation, either in the absence (Fig. 1A) or presence (Fig. 1B) of adenosine, radioactivity appeared predominantly in ASR. By contrast, when a crude enzyme preparation previously passed through a column of Sephadex G-25 was used, radioactivity accumulated predominantly in homocysteine (Fig. 2A). Addition of adenosine to the reaction mixture containing the Sephadex-treated enzyme (Fig. 2B) restored the predominant synthesis of radioactive ASR, with a corresponding decrease of radioactivity in homocysteine. In the presence of a 20-30% ammonium sulfate fraction, the major radioactive product was homocysteine (Fig. 3A). Addition of adenosine resulted in ASR as the major radioactive product (Fig. 3B).

Some properties of the homocysteine-synthesizing system were determined with the 20-30% ammonium sulfate fraction. The reaction is dependent upon the addition of enzyme and O-acetylhomoserine, and is stimulated by DTE (Table I). Omission of pyridoxal phosphate did not significantly affect the rate. No effect of 10 mM ATP was observed.



Figs. 1-3. Products of $^{35}S^{=}$ metabolism. The reaction mixture of Fig. 1 contains crude enzyme (7.8 mg protein), that of Fig. 2 Sephadex-treated crude enzyme (4.0 mg protein), and that of Fig. 3 a 20-30% ammonium sulfate fraction (12.7 mg protein). Both the "A" and "B" series contained the standard reaction mixture supplemented with O-acetylhomoserine. The reaction mixtures of the "B" series contained, in addition, 1.25 μmoles of adenosine.

Fig. 4. Effect of a pool of homocysteine on the products of $^{35}S^{=}$ metabolism. The standard reaction mixture supplemented with O-acetylhomoserine, and containing a crude enzyme extract, was used throughout. The reaction mixture of Fig. 4B contained, in addition, 5 μmoles of homocysteine.

Abbreviations used in figures: ASR, S-adenosylhomocysteine; HSR, homocysteine, Me, methionine. Scale tracings of the ninhydrin-positive area corresponding to the oxidized product of homocysteine are shown near the origin. The direction of the cathode and anode is represented by - and +, respectively.

Table I

Requirements for the synthesis of homocysteine

Conditions	Homocysteine synthesized
	mμmoles
Complete	12.6
Complete, enzyme boiled 7 min.	0.8
Minus O-acetylhomoserine	1.2
Minus DTE	9.2
Minus pyridoxal phosphate	12.3

The standard reaction mixture, containing a 20-30% ammonium sulfate fraction (1.4 mg protein), was used. Except where shown, the reaction mixture was supplemented with O-acetylhomoserine.

The effect of a number of amino acids in promoting the incorporation of $^{35}S^{=}$ into sulfur amino acids is shown in Table II. Only O-acetylhomoserine and O-acetylserine showed marked activity. The radioactive product formed in the presence of O-acetylserine was characterized as cysteine.

Table II

Substrate specificity for incorporation of $^{35}S^{=}$ into sulfur amino acids

Additions	^{35}S incorporated
	mμmoles
O-Acetylhomoserine	27.0
O-Succinylhomoserine	0.8
Homoserine	1.8
O-Acetylserine	77.0
Serine	1.3
O-Acetylthreonine	4.7
Threonine	0.8

The standard reaction mixture, containing a 20-30% ammonium sulfate fraction (7.3 mg protein) was used. The rates shown have been corrected for the small incorporation observed in the absence of added amino acid (Table I).

The possibility that radioactivity found in homocysteine was first incorporated into cysteine, and then transfered to homocysteine *via* transsulfuration through cystathionine was eliminated as follows. Incubation of the reaction mixture in the presence of a pool of cystathionine did not decrease the radioactivity appearing in homocysteine, and the isolated cystathionine pool contained virtually no radioactivity.

Methionine-^{35}S was also synthesized in the crude extract (Fig. 1A). In the Sephadex-treated extract (Fig. 2A) and the 20-30% ammonium sulfate fraction (Fig. 3A), its synthesis was markedly reduced. Addition of adenosine caused a small inhibition of methionine-^{35}S synthesis in the crude extract (Fig. 1B), and a small stimulation of methionine-^{35}S synthesis in the Sephadex-treated extract (Fig. 2B) and 20-30% ammonium sulfate fraction (Fig. 3B).

Addition of a pool of homocysteine to a reaction mixture containing a crude enzyme preparation changed the major radioactive product(s) from ASR and methionine (Fig. 4A) to homocysteine (Fig. 4B).

DISCUSSION: The combined observations reported above are consistent with a reaction sequence involving an initial synthesis by reaction 1 of homocysteine, which can be further metabolized to ASR and to methionine. ASR can be formed from homocysteine *via* an enzyme system previously reported in the

liver of several mammalian species (de la Haba and Cantoni, 1959), and in yeast (Duerre and Schlenk, 1962):

homocysteine + adenosine → ASR + water (3)

Apparently an adequate concentration of free adenosine is available in the untreated crude extract to permit the overall conversion of $^{35}S^{=}$ to ASR-^{35}S. Adenosine becomes limiting for reaction 3 when the crude enzyme preparation is either passed through a column of Sephadex G-25, or fractionated by ammonium sulfate.

The conversion of homocysteine to methionine is well established in mammalian and bacterial systems (Mudd and Cantoni, 1964). In higher plants, however, the mechanism of this conversion is poorly understood. The postulated methylation of homocysteine described here appears to require the presence of a low molecular weight factor in the crude extract. This factor could well be the source of the methyl group. The reason for the effect of adenosine on the synthesis of methionine-^{35}S is not clear.

Hitherto it has been generally considered that de novo synthesis of homocysteine proceeds from cysteine, via the intermediary formation of cystathionine. Previous studies (Giovanelli and Mudd, 1966) have indeed provided evidence for the operation of the transsulfuration pathway in higher plants. However kinetic analysis of the rate of labeling of various intermediates in the transsulfuration pathway suggests that de novo synthesis of homocysteine in Escherichia coli (Roberts et al., 1955) and Neurospora (Wiebers and Garner, 1960, 1966) may not proceed exclusively by this pathway, and that separate biosynthetic routes for the synthesis of cysteine and homocysteine may exist. Wiebers and Garner (1963) briefly reported on an enzyme system from Neurospora that synthesized homocysteine directly via the reaction:

sulfide + homoserine → homocysteine + water (4)

The enzyme system described here represents the first report of a direct sulfhydration of O-acetylhomoserine to homocysteine.

It therefore appears that de novo synthesis of homocysteine in plants may proceed by two alternate pathways - a direct sulfhydration pathway and a transsulfuration pathway. The observation (Giovanelli and Mudd, 1966) that crude extracts of spinach convert O-acetylhomoserine to ASR at a rate at

least ten times that of its conversion to cystathionine suggests that the direct sulfhydration pathway may play a key role in the de novo synthesis of homocysteine.

The apparent specificity of O-acetylhomoserine in the formation of homocysteine provides a possible physiological function of O-acetylhomoserine, which is a natural constituent of peas (Grobbelaar and Steward, 1958). O-Oxalylhomoserine, which is a natural constituent of many Lathyrus species (Przybylska and Pawelkiewicz, 1965), was relatively inactive in the synthesis of homocysteine. However, O-oxalylhomoserine was active in the incorporation of cysteine into cystathionine, catalyzed by enzyme extracts of spinach (Giovanelli and Mudd, unpublished experiments). An appreciation of the physiological significance of these O-acylamino acids awaits further knowledge on the natural distribution of these compounds and their enzymatic reactions throughout the plant kingdom.

The enzyme catalyzing the direct sulfhydration of O-acetylserine to cysteine (reaction 2) is probably similar to that recently reported in E. coli and Salmonella tryphimurium (Kredich and Tomkins, 1966). Large variations in the relative rate of synthesis of homocysteine-^{35}S and cysteine-^{35}S from O-acetylhomoserine and O-acetylserine, respectively, were observed in various ammonium sulfate fractions of spinach (Giovanelli and Mudd, unpublished experiments), suggesting that different enzymes are catalyzing the synthesis of these two amino acids.

The relationship between the enzyme systems reported here and those reported to catalyze the sulfhydration of homoserine (reaction 4) in Neurospora and of serine in spinach (Bruggemann et al., 1962) is currently being investigated.

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