Sulfuration of O-Acetylhomoserine and O-Acetylserine by Two Enzyme Fractions From Spinach

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We have previously reported (Giovanelli and Mudd, 1967) the synthesis of homocysteine and cysteine by extracts of spinach via the following reactions:

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

O-acetylserine + sulfide → cysteine + acetate (2)

In this communication the biosynthesis of methionine, ethionine, S-methylcysteine and S-ethylcysteine is shown to proceed when sulfide is replaced by methylmercaptan or ethylmercaptan:

O-acetylhomoserine + methylmercaptan → methionine + acetate (1')

O-acetylhomoserine + ethylmercaptan → ethionine + acetate (1'')

O-acetylserine + methylmercaptan → S-methylcysteine + acetate (2')

O-acetylserine + ethylmercaptan → S-ethylcysteine + acetate (2'')

The term "sulfuration" is used to describe the general process of sulfur incorporation, as illustrated by the above reactions.

It is further shown that two separable enzyme fractions (A and B) catalyze these reactions. At the stage of purification attained, fraction A is active with both O-acetylhomoserine and O-acetylserine. Fraction B is active with O-acetylserine only.

Experimental: Reaction 2 was assayed by the colorimetric procedure described by Kredich and Tomkins (1966). The reaction mixture contained the following components (in μmoles) in a final volume of 0.45 ml: potassium phosphate (45), pyridoxal phosphate (0.15), Na_2S (1.8), O-acetyl-L-serine (30). The rates reported for this reaction are higher than those previously reported (Giovanelli and Mudd, 1967), due to attainment of more optimal conditions. All other reactions were assayed by the rate of incorporation of the appropriate radioactive substrate into amino acids, in reaction mixtures containing the following components (in μmoles) in a final volume of 0.45 ml: enzyme, potassium phosphate (45), pyridoxal phosphate (0.045) and dithiothreitol (5). To this standard reaction

mixture were added the following substrates, where appropriate: O-acetyl-L-homoserine (30), O-acetyl-L-serine (30), $Na_2{}^{35}S$ (3.6), $^{14}CH_3SH$ or $CH_3.CH_2{}^{35}SH$ (0.3 with O-acetylhomoserine, 26 with O-acetylserine).

The concentration of substrate present in all reactions approached saturation, so that the rates determined approximated maximal velocities (V_{max}). The concentrations of methylmercaptan and ethylmercaptan noted above reflect the marked difference in K_m of these substrates, depending on the O-acylaminoacid substrate present (Giovanelli and Mudd, unpublished experiments). All reactions had a final pH of 7.25, and were incubated at 30° in a nitrogen gas phase.

Reaction mixtures for the determination of the rate of incorporation of radioactive substrate into amino acids were stopped by addition of 0.8 ml of cold 5% trichloroacetic acid and the resulting precipitate was removed by centrifugation. The total radioactivity incorporated into amino acids was determined in the supernatant solution as follows. Radioactivity incorporated via reaction 1 was determined on a column of Dowex 50 H^+, as previously described (Giovanelli and Mudd, 1967). In all other reactions, unreacted radioactive substrate ($^{14}CH_3SH$ or $CH_3.CH_2{}^{35}SH$) was removed by bubbling a stream of nitrogen through the supernatant solution for 30 minutes. An aliquot of this solution was then assayed for the amount of radioactivity incorporated into amino acids.

Na_2S and $Na_2{}^{35}S$ were purified from commercial material by microdiffusion (Conway, 1957) and stored at 0° in stoppered 1 ml tubes in an atmosphere of nitrogen. Sulfide was determined with 5,5'-dithiobis-(2-nitrobenzoic acid) (Kredich and Tomkins, 1966). $^{14}CH_3SH$ and $CH_3.CH_2{}^{35}SH$ were obtained from commercial sources and were used without further purification. The source of all other chemicals has been described previously (Giovanelli and Mudd, 1966).

Enzyme extracts were prepared from an acetone powder of spinach (Giovanelli and Mudd, 1966). Fractions described in Fig. 1 were prepared from protein which precipitated in the following ranges of ammonium sulfate saturation: 20-27, 27-31, 31-36, 36-40, 40-44, 44-50, 50-55, 55-65 and 65-80. Protein precipitating in the range 30-36% saturation with ammonium sulfate was refractionated (see Fig. 2) with the following ranges of ammonium sulfate saturation: 20-30, 30-36, 36-40, 40-44, 44-50 and 50-80. Before assay, each enzyme fraction was passed through a column of Sephadex G-25, which had been previously equilibrated with a solution containing potassium phosphate (0.1 M), ethylenediaminetetraacetic acid (0.1 mM) and 2-mercaptoethanol (0.14mM) final pH 7.25.

Methionine-^{14}C, S-methylcysteine-^{14}C, ethionine-^{35}S and S-ethylcysteine-^{35}S were each characterized by the following criteria: (i) paper chromatography and electrophoresis, (ii) oxidation with hydrogen peroxide to the corresponding sulfoxide.

Results: Fig. 1 shows the distribution of activity of reactions 1, 1', 2 and 2' in fractions precipitated by increasing concentrations of ammonium sulfate. Two distinct peaks of activity were observed. One peak (fraction A) contained the major portion of activity associated with O-acetylhomoserine (reactions 1 and 1'), and was localized in the ammonium sulfate fractions precipitating between approximately 30-40% saturation.

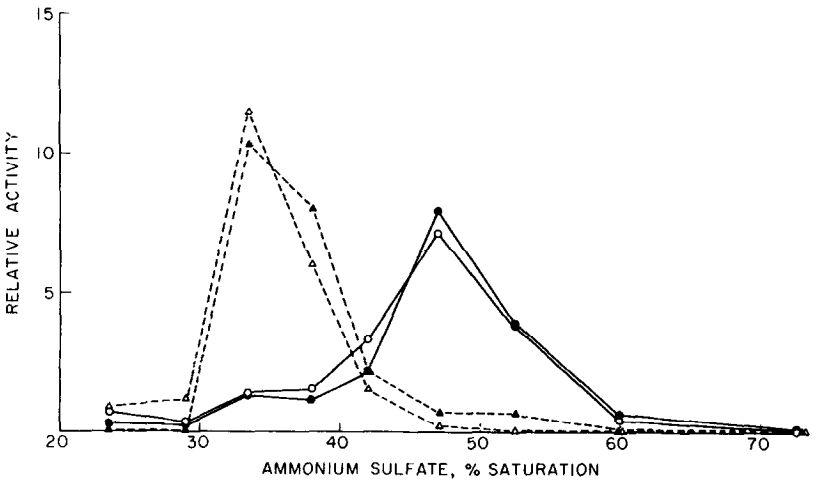

Fig. 1. Distribution of activity with O-acetylhomoserine and O-acetylserine

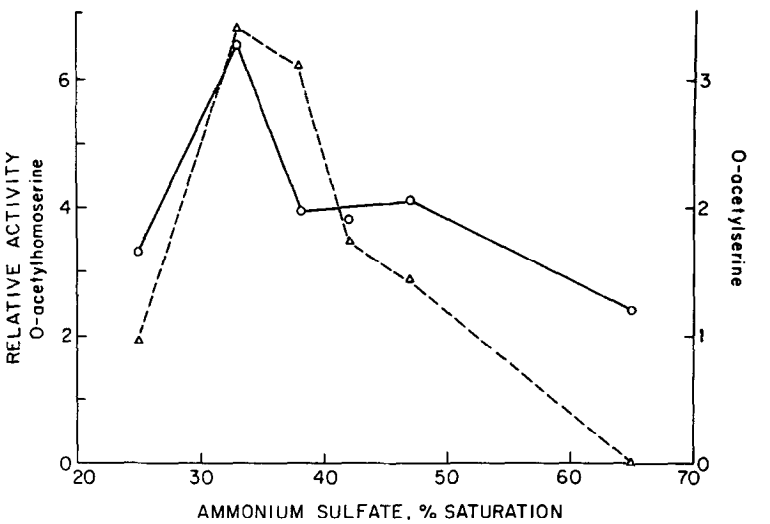

Fig. 2. Refractionation of Protein precipitating in the range 30-36% ammonium sulfate saturation.

--▲--, O-acetylhomoserine + sulfide; --△--, O-acetylhomoserine + methylmercaptan; —●—, O-acetylserine + sulfide; —○—, O-acetylserine + methylmercaptan. The values of "relative activity" were derived by dividing the % total recovered activity by the change in ammonium concentration used to precipitate that particular fraction (see Experimental). The values of the abscissa were obtained from the midpoints of the range of each ammonium sulfate fraction. For example, in Fig. 1 the relative activity value of 8 for O-acetylserine + sulfide was obtained by dividing the % total activity (48.0) in the ammonium sulfate fraction precipitating between 44-50% by 6, the change in ammonium sulfate concentration used to precipitate that fraction. This relative activity value of 8 was then plotted against an ammonium sulfate saturation value of 47%. This procedure is considered to provide a valid representation of the distribution of activity when the changes in ammonium sulfate concentrations used to precipitate the fractions were not constant.

The second peak (fraction B) contained the major portion of activity associated with O-acetylserine (reactions 2 and 2'), and was localized in the fractions precipitating between approximately 40-60% saturation. The distribution of activity determined with sulfide (reactions 1 and 2) corresponded very closely with that determined with methylmercaptan (reactions 1' and 2').

A comparison of the relative rates of sulfuration of O-acetylhomoserine and O-acetylserine by fractions A and B is presented in Table I.

Table I

Comparison of the rates of sulfuration of O-acetylhomoserine and O-acetylserine by fractions A and B

	O-Acetylhomoserine			O-Acetylserine		
	Sulfide	Methyl-mercaptan	Ethyl-mercaptan	Sulfide	Methyl-mercaptan	Ethyl-mercaptan
Fraction A	0.7	1.3	0.3	70	26	2.8
Fraction B	(0.1)	(0.1)	(0.06)	765	197	21.5

Fractions A and B correspond to protein fractions precipitating in the range 30-36% and 44-50%, respectively, of ammonium sulfate saturation. Values are reported as mμmoles/mg protein/minute. Values shown in parentheses are at the limits of sensitivity of the assays, and should be regarded only as approximate.

The results of this table demonstrate that: (i) Sulfuration of O-acetylserine is catalyzed at a much higher rate than that of O-acetylhomoserine. (ii) The relative rates for each sulfur substrate are different with O-acetylhomoserine and O-acetylserine. Thus methylmercaptan is the most active substrate with O-acetylhomoserine, whereas sulfide is the most active substrate with O-acetylserine. With both O-acylaminoacids, ethylmercaptan is relatively the least active substrate. (iii) Fraction B is active predominantly with O-acetylserine, showing negligible activity with O-acetylhomoserine (less than 1% of that with O-acetylserine). Fraction A is active with both O-acylaminoacids. Even though most of the activity with O-acetylhomoserine resided in fraction A (Fig. 1), Table I shows that this fraction was, in fact, more active with O-acetylserine than with O-acetylhomoserine. During a second fractionation of fraction A with ammonium sulfate, the major portion of the O-acetylserine-dependent

activity precipitated with the fractions containing the O-acetylhomoserine activity (Fig. 2). This suggests that the activity of fraction A with O-acetylserine cannot be explained by a contamination with fraction B, and suggests the presence of two species of proteins active with O-acetylserine. Whether one of these species is identical to the O-acetylhomoserine-dependent enzyme will be determined only by further investigation.

In addition to O-acetylhomoserine and O-acetylserine, the following amino acid substrates were assayed with sulfide and methylmercaptan: O-Succinyl-DL-homoserine, O-oxalyl-L-homoserine, L-homoserine, O-phosphoryl-L-serine, N-acetyl-L-serine, L-serine, O-acetyl-L-threonine and L-threonine. In the presence of fractions A or B, these amino acids reacted at rates less than 4% of these observed with O-acetylhomoserine or O-acetylserine, respectively.

The combined results demonstrate the presence of at least two enzyme fractions catalyzing the sulfuration of O-acetylserine or of O-acetylhomoserine. The close correspondence between the distribution of activity determined with sulfide (reactions 1 and 2) and with methylmercaptan (reactions 1' and 2') further suggests that both enzymes are able to use either sulfide or methylmercaptan as substrates.

Discussion: Fraction B appears to be similar to O-acetylserine sulfhydrase demonstrated in Salmonella typhimurium and Escherichia coli (Kredich and Tomkins, 1966; Wiebers and Garner, 1967). Enzymes similar to fraction A, that catalyze the sulfhydration (reaction with sulfide) of either O-acetylhomoserine or O-succinylhomoserine have been reported in fungi and bacteria, respectively (Kerr and Nagai, 1967; Flavin and Slaughter, 1967; Wiebers and Garner, 1967). The possible significance of the sulfhydration of O-acetylserine (reaction 2) and O-acetylhomoserine (reaction 1) in higher plants has been discussed in a previous publication (Giovanelli and Mudd, 1967).

The results reported here demonstrate that methylmercaptan and ethylmercaptan can replace sulfide in the reaction with O-acetylserine or O-acetylhomoserine. Methylmercaptan has long been known to occur naturally in higher plants (Nakamura, 1925). However, neither the mechanism of its synthesis nor utilization in plants has been investigated. The present work focuses attention on the possible biochemical role of methylmercaptan in the biosynthesis of S-methylcysteine and methionine.

S-Methylcysteine and its sulfoxide are normal constituents of a number of plants (Thompson, 1967). On the basis of isotope feeding experiments with whole tissues, two mechanisms have been proposed for the synthesis of this amino acid in higher plants. Thompson and Gering (1966)

proposed that this compound is formed in radishes by transmethylation of cysteine. Sugii, et al., (1963), on the other hand suggested synthesis in garlic plants by the following reaction:

serine + methylmercaptan → S-methylcysteine + water (3)

An enzyme catalyzing reaction 3 has been reported in yeast (Wolff, et al., 1956). The spinach enzyme, by contrast, is inactive with serine, and provides a novel pathway for the participation of methylmercaptan in the biosynthesis of S-methylcysteine.

The direct formation of methionine from methylmercaptan has been demonstrated in both Neurospora (Moore and Thompson, 1967) and S. typhimurium (Flavin and Slaughter, 1967). In both organisms the reaction appears to be of limited physiological significance. The role of this reaction in higher plants will depend in part on whether these forms can generate methylmercaptan ultimately from sources other than methionine.

References

Conway, E. J. (1967). "Microdiffusion analysis and volumetric error," Crosby Lockwood, London, 4th edition, p. 244.
Flavin, M., and Slaughter, C. (1967). Biochem. Biophys. Acta 132, 400.
Giovanelli, J., and Mudd, S. H. (1966). Biochem. Biophys. Res. Comm. 25, 366.
Giovanelli, J., and Mudd, S. H. (1967). Biochem. Biophys. Res. Comm. 27, 150.
Kerr, D., and Nagai, S. (1967). Fed. Proc. 26, 387.
Kredich, N. M., and Tomkins, G. M. (1966). J. Biol. Chem. 241, 4955.
Moore, D. P., and Thompson, J. F. (1967). Biochim. Biophys. Res. Comm. 28, 474.
Nakamura, N. (1925). Biochem. Z. 164, 31.
Sugii, M., Nagasawa, S., and Suzuki, J. (1963). Chem. Pharm. Bull. (Tokyo) 11, 135.
Thompson, J. F. (1967). Ann. Rev. Plant Physiol. 18, 59.
Thompson, J. F., and Gering, R. K. (1966). Plant Physiol. 41, 1301.
Wiebers, J. L., and Garner, H. R. (1967). J. Biol. Chem. 242, 5644.
Wolff, E. C., Black, S., and Downey, P. F. (1956). J. Am. Chem. Soc. 78, 5958.