ENZYMATIC SYNTHESIS OF CYSTEINE AND S-METHYLCYSTEINE IN PLANT EXTRACTS[1]

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In a previous paper (Moore and Thompson, 1967), we have described the enzymatic formation of methionine from O-acetylhomoserine and methylmercaptan derived from the degradation of methylcysteine in Neurospora extracts. As a logical extension of this work and as a continuation of our interest in methylcysteine biosynthesis (Thompson and Gering, 1966), the enzymatic reaction of methylmercaptan with O-acetylserine to form methylcysteine (eq. 1) was investigated.

$$CH_3SH + \text{O-acetylserine} \rightarrow \text{methylcysteine} + \text{acetate} \qquad (1)$$

Crude extracts of Neurospora crassa, bakers' yeast (Saccharomyces cereviseae) and turnip leaves (Brassica rapa L.) readily promoted methylcysteine formation. The high activity of these extracts in catalyzing methylcysteine synthesis suggested to us that the "normal" function of the enzyme might be to synthesize cysteine (eq. 2).

$$\text{sulfide} + \text{O-acetylserine} \rightarrow \text{cysteine} + \text{acetate} \qquad (2)$$

The ability of these extracts to catalyze cysteine formation was readily ascertained. The possibility that cysteine synthesis and methylcysteine synthesis are promoted by one enzyme is supported (but not proven) by the similarity in

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the relative activities of O-acetyl-L-serine, L-serine, L-cysteine, D-serine and O-phospho-L-serine in both reactions.

The much greater activity (over 100 fold) with O-acetyl-L-serine, as compared to L-serine in these reactions, leads us to suggest that O-acetyl-L-serine may be the normal substrate. Evidence that O-acetylserine is the normal precursor of cysteine in bacteria (Kredich and Tompkins, 1966) lends credence to this suggestion.

METHODS AND MATERIALS: Yeast extracts were prepared by the autolysis of bakers' yeast that had been frozen in liquid nitrogen (Black, 1962). Turnip leaf extracts were obtained by grinding 10-30 gm of chopped cold leaves in a mortar with an equal weight of 0.2 M potassium phosphate buffer pH = 8.0. After centrifuging the debris at 20,000 x g for 30 min., the supernatant was dialyzed 18 hrs. against 0.01M potassium phosphate buffer pH 7.5 containing 10^{-3} M mercaptoethanol and 10^{-6} M pyridoxal phosphate (PALP) with one change of dialyzing solution.

The standard incubation mixture contained 100 μmoles of potassium phosphate buffer (pH = 8.0), 0.1 μmole of PALP, 10 μmoles of dithioerythritol, 25 μmoles of O-acetyl-L-serine (or other similar substrates), 0.5 μmole of $Na_2{}^{35}S$ (1.0 μc/μmole) or $^{14}CH_3SH$ (1.0 μc/μmole) and plant extract in a total volume of 2.25 ml. Control treatments included were one with boiled extract and one with no non-radioactive substrate (O-acetylserine etc.). Where cysteine was utilized as a substrate, boiled extract with cysteine was also included as a control and yielded the same results as boiled control without cysteine. $^{14}CH_3SH$ was dissolved in ethanol at a concentration of 8 μc/ml. Incubations were carried out in stoppered 13 x 100 mm test tubes for one hour at 25°C. The reaction was stopped by heating tubes in boiling water for 3 min. When $Na_2{}^{35}S$ was the substrate, 0.1 ml of 50% trichloracetic acid and 1.0 ml of 0.10 M Na_2S was added before heating. If there was sufficient precipitate to clog ion exchange resins, the sample was centrifuged and a suitable aliquot of the supernatant was removed. When the precipitate was insignificant, the entire incubation mixture was poured on a column (0.9 x 7 cm) of sulfonic acid resin in the hydrogen form. After the solution had penetrated the resin, labeled substrates were washed through the resin with 35 ml of 0.1 M mercaptoethanol and then 10 ml of water. Amino acids were eluted from the resin with 2N ammonia and the eluate tested for radioactive compounds by liquid scintillation in Bray's solution (Bray, 1960), using external standardization to determine efficiency.

Cysteine and methylcysteine were identified as follows. Aliquots of the resin eluate were dried with 1 μmole of unlabeled cysteine or methylcysteine and oxidized with performic acid (Moore, 1963). After destroying the excess H_2O_2 with HBr, the volatile acids were removed in vacuo at room temperature. When cysteic acid was the product, the residue was dissolved in water and put through a 0.3 x 2 cm column of sulfonic acid resin in acid form and the effluent and wash were dried. The material containing cysteic acid or methylcysteine sulfone was subjected to two dimensional paper chromatography in butanol-acetic acid-water (12:3:5) and phenol-water (8:3) (Thompson and Morris, 1959). Methylcysteine sulfone was located by its fluorescence after reaction with 1, 2 naphthoquinone 4-sulfonic acid (Morris and Thompson, 1965). The area of paper containing the methylcysteine sulfone was cut into small pieces (0.5 x 0.5 cm) into a scintillation vial and eluted with 5 ml of 50% ethanol by shaking for 24 hours. After chromatography of cysteic acid, this amino acid was located by spraying the chromatogram with 0.1% ninhydrin in ethanol and observing the color produced. The cysteic acid spot was cut into a scintillation vial. Five ml of 50% ethanol were placed in the vial and the color was bleached by placing the vial near a fluorescent light for 2-3 days. Radioactivity was determined as above.

Protein was measured by the method of Lowry et al. (1951).

Labeled compounds and unlabeled materials were obtained from commercial sources. O-Acetyl-L-serine contained traces (about 1%) of serine as determined by one-directional paper chromatography in phenol-water.

RESULTS: The initial work was carried out on *Neurospora* extracts but this was abandoned in favor of yeast and turnip extracts because of the greater ease of acquiring the latter plant materials. The data in Table 1 demonstrate the formation of cysteine and methylcysteine by yeast extracts. These data are the average of duplicates which agree closely and were obtained by counting the resin eluates. The identity of the amino acids was proven by paper chromatography of the oxidation products (see Methods and Materials). The recovery of methylcysteine sulfone and cysteic acid from paper chromatography was over 90% as compared to standards subjected to the same procedures.

The lower two levels of yeast extract were chosen such that there was a good correlation between the quantity of enzyme used and the quantity of cysteine or methylcysteine formed from O-acetylserine. At the intermediate level of extract, L-cysteine could be demonstrated to be a substrate with about 9% of the activity of O-acetylserine in both reactions (eq. 1 (an exchange reaction) and eq. 2). However, the highest level of extract (5600 μg of protein) was required to gain convincing evidence of the activity with L-serine, D-serine and O-phospho-L-serine. L-Serine has similar activities in cysteine and methylcysteine synthesis. Extrapolating from the cysteine or methylcysteine produced from O-acetylserine at the intermediate enzyme level, the activity of L-serine can be estimated to be about 0.5% of that with O-acetylserine. The activities with D-serine and O-phospho-L-serine were less than half that with L-serine for both enzymatic activities. The yeast extract appeared to be more active toward methylmercaptan than sulfide.

TABLE 1. THE FORMATION OF CYSTEINE AND METHYLCYSTEINE IN YEAST EXTRACTS

TREATMENT	PROTEIN	CYSTEINE[*] FORMED	PROTEIN	METHYLCYSTEINE[*] FORMED
	µg	mµmoles	µg	mµmoles
Complete[*]	112	89.9	56	121
Boiled extract	112	2.6	56	0.51
Minus O-acetyl-L-serine	112	2.6	56	0.45
Complete	224	172	112	218
Boiled extract	224	2.6	112	0.49
Minus O-acetyl-L-serine	224	2.6	112	0.59
Minus O-acetyl-L-serine plus L-cysteine	224	15.0	112	20.1
Boiled extract	5600	2.7	5600	0.59
Minus O-acetyl-L-serine	5600	5.9[#]	5600	13.1[#]
Minus O-acetyl-L-serine plus L-serine	5600	23.9	5600	72.4
Minus O-acetyl-L-serine plus D-serine	5600	11.0	5600	30.1
Minus O-acetyl-L-serine plus O-phospho-L-serine	5600	8.9	5600	37.7

[*] $Na_2{}^{35}S$ is the substrate for cysteine formation and $^{14}CH_3SH$ is the substrate for methylcysteine formation. Complete incubation mixture is given in "Methods and Materials" section.

[#] The "minus-O-acetyl-L-serine" control treatment formed more cysteine or methylcysteine than boiled control because substrate was released during incubation (probably by proteolysis).

Table 2 presents evidence for cysteine and methylcysteine synthases in turnip leaf extracts. As with yeast extracts, turnip shows best activity with O-acetyl-L-serine while L-cysteine has 10% of its activity in cysteine synthesis and 20% of its activity for methylcysteine synthesis. The relative activity with L-serine, in comparison to that for O-acetylserine was even less than that observed in yeast extracts for both cysteine and methylcysteine synthesis. We were unable to detect any activity with D-serine and O-phospho-L-serine in the turnip extracts for methylcysteine synthase, though there was slight activity with these substrates for cysteine synthase.

In the turnip extract, it took approximately eighty times as much protein to obtain methylcysteine synthase activity equivalent to cysteine synthase activity whereas with yeast more extract was required for cysteine synthase.

TABLE 2. THE FORMATION OF CYSTEINE AND METHYLCYSTEINE IN TURNIP LEAF EXTRACTS.

TREATMENT	PROTEIN	CYSTEINE* FORMED	PROTEIN	METHYLCYSTEINE* FORMED
	μg	mμmoles	μg	mμmoles
Complete*	5.6	93.4	223	49.0
Boiled Extract	5.6	2.5	223	0.16
Minus O-acetyl-L-serine	5.6	2.6	223	0.10
Complete	11.2	195	447	95.3
Boiled extract	11.2	2.7	447	0.18
Minus O-acetyl-L-serine	11.2	2.7	447	0.16
Minus O-acetyl-L-serine plus L-cysteine	11.2	20.1	447	22.7
Boiled extract	4470	1.05	4470	0.54
Minus O-acetyl-L-serine	4470	1.89	4470	1.35
Minus O-acetyl-L-serine plus L-serine	4470	2.80	4470	1.74
Minus O-acetyl-L-serine plus D-serine	4470	2.23	4470	1.41
Minus O-acetyl-L-serine plus O-phospho-L-serine	4470	2.26	4470	1.42

*See Table 1.

DISCUSSION: Giovanelli and Mudd (1967), and Wiebers and Garner (1967) have recently shown that spinach and yeast extracts respectively can synthesize cysteine from O-acetyl-L-serine. Wiebers and Garner showed that O-acetyl-L-serine was about ten times as active as L-serine in yeast whereas we observe over a hundred fold difference. This disparity in relative activities with O-acetylserine and serine has been found to be a consequence of the difference in sulfide level employed.

Cell-free synthesis of methylcysteine has previously been demonstrated in yeast extracts (Wolff, et al., 1955) using serine and methylmercaptan, but attempts to accomplish the same reaction in higher plants have failed (unpublished data of D. H. Turner and J. F. Thompson, 1961). The inactivity of serine in this reaction (Table 2) explains the cause of failure.

More recently, results from the administration of labeled cysteine, labeled serine and methyl-labeled methionine to radish leaves led to the conclusion that methylcysteine is formed by the methylation of cysteine (Thompson and Gering, 1966). At present, it is difficult to reconcile the latter *in vivo* results with the *in vitro* reaction demonstrated in this paper.

The similarity in the relative activities of O-acetyl-L-serine, L-cysteine, L-serine, O-phospho-L-serine and D-serine in the cysteine synthase and methylcysteine synthase reactions in yeast and turnip leaves could be construed as evidence that the same enzyme promotes both reactions and is supported by inability to separate the two activities in preliminary studies (five fold purification). However, a definitive answer must await additional information.

The lower activity with L-serine as compared to O-acetyl-L-serine in cysteine synthesis is interesting in view of the generally accepted concept that serine is the *immediate* precursor of cysteine in plants (Schlossman and Lynen, 1957, Bruggemann et al., 1962, and Meister, 1965), though in bacteria, O-acetyl-L-serine appears to be the normal immediate precursor of cysteine (Kredich and Tompkins, 1966). Evidence that O-acetyl-L-serine is the normal precursor of cysteine in higher plants or ever occurs in plants is under investigation.

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