Biochimica et Biophysica Acta, 481 (1977) 246—265

BBA 68048

SULPHUR METABOLISM IN *PARACOCCUS DENITRIFICANS*

PURIFICATION, PROPERTIES AND REGULATION OF SERINE TRANSACETYLASE, *O*-ACETYLSERINE SULPHYDRYLASE AND β-CYSTATHIONASE

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(Received May 11th, 1976)
(Revised manuscript received September 27th, 1976)

Summary

1. Serine transacetylase, *O*-acetylserine sulphydrylase and β-cystathionase were purified from *Paracoccus denitrificans* strain 8944.

2. Serine transacetylase was purified 150-fold. The enzyme has a pH optimum between 7.5 and 8.0, is specific for L-serine and is inhibited by sulphydryl-group reagents. The apparent K_m values for serine and acetyl-CoA are $4.0 \cdot 10^{-4}$ and $1.0 \cdot 10^{-4}$ M, respectively. Serine transacetylase is strongly inhibited by cysteine.

3. *O*-Acetylserine sulphydrylase was purified 450-fold. The enzyme has a sharp pH optimum at pH 7.5. In addition to catalysing the synthesis of cysteine, *O*-acetylserine sulphydrylase catalyses the synthesis of selenocysteine from *O*-acetylserine and selenide. The K_m values for sulphide and *O*-acetylserine are $2.7 \cdot 10^{-3}$ and $1.25 \cdot 10^{-3}$ M, respectively. The enzyme was stimulated by pyridoxal phosphate and was inhibited by cystathionine, homocysteine and methionine.

4. β-Cystathionase was purified approx. 50-fold. β-Cystathionase has a pH optimum between pH 9.0 and 9.5, is sensitive to sulphydryl-group reagents, required pyridoxal phosphate for maximum activity and has an apparent K_m for cystathionine of $4.2 \cdot 10^{-3}$ M. β-Cystathionase also catalyses the release of keto acid from lanthionine, djenkolic acid and cystine. Cysteine, *O*-acetylserine, homocysteine and glutathione strongly inhibit β-cystathionase activity and homocysteine and methionine represses enzyme activity.

5. *O*-Acetylserine lyase was identified in crude extracts of *Paracoccus denitrificans*. The enzyme is specific for *O*-acetyl-L-serine, requires pyridoxal phos-

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phate and is inhibited by KCN and hydroxylamine. The enzyme has a high K_m value for *O*-acetylserine (50—100 mM).

Introduction

In bacteria the metabolic pathway of sulphur from sulphide to methionine involves the following steps:

L-Serine + acetyl-CoA → *O*-acetylserine + Coenzyme A (1)
O-Acetylserine + sulphide → cysteine + acetate (2)
Cysteine + *O*-succinylhomoserine → cystathionine + succinate (3)
Cystathionine + H_2O → homocysteine + pyruvate + NH_3 (4)
Homocysteine + *S*-adenosylmethionine → methionine + *S*-adenosylhomocysteine (5)

The enzymes serine transacetylase, *O*-acetylserine sulphydrylase and β-cystathionase catalyse reactions 1, 2 and 4, respectively. These three enzymes have all been found in a variety of bacteria and plants [1—13]. In the bacteria studied to date the transfer of sulphur, via cystathionine, is unidirectional in the direction from sulphide to methionine [13].

This paper reports the purification, properties and regulation of serine transacetylase, *O*-acetylserine sulphydrylase and β-cystathionase from *Paracoccus denitrificans* strain 8944. It also reports on the substitution of sulphide by selenide in the synthesis of L-selenocysteine. Finally, the presence of *O*-acetylserine lyase, an enzyme reported previously only in plant extracts [14], is reported in extracts of *P. denitrificans*.

Materials and Methods

Acetyl-CoA was a gift from Boehringer und Soehne, G.m.b.H., Mannheim, Germany; DL-selenocystine, 5,5′-dithiobis-(2-nitrobenzoic acid), Coenzyme A, L-cystathionine, L-homoserine, $NADH_2$, pyridoxal phosphate and 1,2,4-triazole were obtained from The Sigma Chemical Co., London, U.K. β-Cyanoalanine was obtained from Calbiochem., Ltd., London, U.K. Triethylamine, aluminium selenide and 2,4-dinitrophenylhydrazine were purchased from British Drug Houses, Dorset, U.K. L-[3-^{14}C]Serine was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Silica gel thin-layer chromatography plates (20 × 20 cm, Type 6060 with fluorescent indicator) were obtained from Eastman Kodak Co., Rochester, N.Y., U.S.A.

Nitrogen (oxygen free) gas was obtained from Air Products, Ltd., U.K. Bis-(trimethylsilyl)trifluoroacetamide was obtained from The Sigma Chemical Co., London, U.K. and acetonitryl was obtained from British Drug Houses, Dorset, U.K. OV 1, OV 17 and Gas Chrom Q, 100—120 mesh, were obtained from Applied Science Labs., U.S.A.

Gas chromatographic identifications were carried out with a Pye Series 104 Gas Chromatogram with a flame ionization detector; the Pye Gas Chromatogram was linked to a Hewlett-Packard Integrator.

O-Acetyl-L-homoserine and *O*-acetyl-L-threonine were chemically synthesised by the method of Nagai and Flavin [15]. *O*-Acetyl-L-serine and *O*-acetyl-

D-serine were synthesised by the method of Sheehan and co-workers [16]. *N*-Acetyl-L-serine was prepared by the method of Kredich and Tomkins [1].

Infrared spectral studies were done with a Hilger-Watts Mk 2 Model H1200. KCl discs (13-mm diameter) of the compounds to be examined were pressed using a minidie (obtained from Spectroscopic Accessories Co., Kent, U.K.).

Assays

Serine transacetylase. Reaction mixtures (1 ml) contained, 100 μmol Tris · HCl buffer, pH 7.5, 1.0 μmol EDTA, 2 μmol acetyl-CoA, 2.5 μmol L-serine and a limiting amount of enzyme. Reactions were run in 1-ml cuvettes (1-cm light path) at 30°C. Reactions were started with the addition of L-serine and the initial rate of reaction was followed at 232 nm in a Pye Unicam SP 8000 spectrophotometer linked to an AR 25 linear recorder.

O-Acetylserine sulphydrylase. O-Acetylserine sulphydrylase activity was determined by a modification of the procedure of Kredich and Tomkins [1]. Assays were conducted at 30°C and reaction mixtures (1 ml) contained 10 μmol *O*-acetylserine, 10 μmol of Na_2S, 100 μmol Tris · HCl buffer, pH 7.5, 10 nmol of pyridoxal phosphate, 5 μmol dithiothreitol and enzyme. Reactions were started with the addition of enzyme and stopped by the addition of 0.2 ml 7.5% (w/v) trichloroacetic acid. Cysteine was estimated by the method of Gaitonde [17].

O-Acetylserine lyase. O-Acetylserine lyase activity was followed by measuring the release of pyruvate from *O*-acetylserine using 2,4-dinitrophenylhydrazine according to the method of Mazelis and Fowden [14].

β-Cystathionase. β-Cystathionase activity was measured by two methods: (a) the method of Rowbury and Woods [18]. Reaction mixtures (1 ml) contained 200 μmol Tris/phosphate buffer, pH 9.0, 10 nmol pyridoxal phosphate, 10 μmol L-cystathionine and a limiting amount of enzyme. Reactions were terminated after 15 min by the addition of 0.2 ml trichloroacetic acid (15%, w/v). Pyruvate was determined by the method of Friedemann [19]. (b) The second method which allows β-cystathionase activity to be continuously monitored spectrophotometrically, has been described previously [20]. Reactions were conducted in 1 cm light path cells in a thermoregulated Pye Unicam SP 8000 spectrophotometer at 30°C. Reaction mixtures (1 ml) contained 200 μmol Tris/phosphate buffer, pH 9.0, 10 nmol pyridoxal phosphate, 0.3 μmol NADH, 10 μmol L-cystathionine, 10 units lactate dehydrogenase and a limiting amount of enzyme. Reactions were initiated by the addition of cystathionine and the A_{340nm}, versus a water blank, was recorded continuously.

Measurement of selenocysteine. Selenocysteine was measured by the method described for the estimation of cysteine. DL-Selenocystine, obtained from commercial sources, was reduced to selenocysteine by incubation with excess dithiothreitol at pH 8.0 in Tris · HCl buffer. The ϵ_{560nm} for selenocysteine is approx. 1.5% of the ϵ_{560nm} cysteine $4.8 \cdot 10^3$). The A_{560nm} for selenocysteine was directly proportional to selenocysteine concentration and, in the absence of cysteine, could be used to measure selenocysteine concentration.

Preparation and identification of 2,4-dinitrophenylhydrazone derivatives of pyruvate and α-ketobutyrate. 2,4-Dinitrophenylhydrazone derivatives of keto

acids were prepared as described by Smith [21]. The keto acid derivatives were separated and identified by thin-layer chromatography.

Thin-layer chromatography and electrophoresis. Chromatograms were developed by ascending chromatography in either Solvent 1 or 2 for 8 h. Plates were dried, dipped in a 5% (w/v) solution of KOH in ethanol, and again dried. Keto acid dinitrophenylhydrazone derivatives appear as brown spots on a white background.

When cysteine or selenocysteine was analysed by thin-layer chromatography, the solutions were treated with excess *N*-ethylmaleimide for 10 min prior to application to the chromatogram. The cysteine-*N*-ethylmaleimide (and the selenocysteine-*N*-ethylmaleimide) adduct was thus protected from thiol oxidation and ran as a single spot separate from the remaining compounds examined in this study. Compounds were located with ninhydrin spray, ultraviolet fluorescence quenching, or by counting the radioactivity of small sections of the thin-layer chromatography plates.

Electrophoretic separation of reaction products was conducted on acid-washed 3 MM paper soaked in Solvent 3. Electrophoresis was run for 2 h with a voltage gradient of 42 V/cm. The solvents were: (1) *n*-butanol/ethanol/water (70 : 20 : 10, by vol.); (2) chloroform/ethanol/glacial acetic acid/water (50 : 32 : 10 : 8, by vol.); (3) 78 ml formic acid (90%, v/v) and 148 ml glacial acetic acid titrated to pH 1.8 with HCl and made up to 2.5 l with water.

Identification of reaction products of β-cystathionase by gas-liquid chromatography. A 50 ml reaction mixture was run for 16 h at 30°C, the reaction terminated by boiling for 3 min and the precipitated protein removed by filtration. The filtrate was applied to a column of Dowex 1-X2 (200—400 mesh) in the OH^- form, the column washed with distilled water until the pH returned to neutral, and the keto and amino acids eluted with 0.2 M HCl. The solution containing eluted keto and amino acids was evaporated to dryness and 1 mg of the resultant powder was derivatised in 0.1 ml of a 50/50 (v/v) mixture of bis(trimethylsilyl)trifluoroacetamide and acetonitryl in a sealed derivatisation tube at 150°C for 1 h; 0.5 μl of the resultant solution was examined by gas-liquid chromatography. Nitrogen (oxygen free) was used as the carrier gas. Glass columns 1.5 m long × 4 mm internal diameter were used. Two different column packings were used; Column 1 was packed with 5% (w/w) OV 17 on Gas Chrom Q, 100—120 mesh, and Column 2 was packed with 5% (w/w) OV 1 on Gas Chrom Q, 100—120 mesh. The carrier gas flow rate was maintained at 40 ml/min for both columns.

When column 1 was used a temperature program starting at 100°C and finishing at 250°C, with a temperature gradient of 5°C/min was employed. When column 2 was used a temperature program of an initial 5 min hold at 80°C, a temperature gradient of 5 ml/min, and a final hold at 250°C for 5 min was employed. The injector heater was set at 5 and the detector oven set at 250°C.

Enzyme purification. All steps of the purification procedures were conducted at 0—4°C and the buffer used throughout the purification of serine transacetylase and *O*-acetylserine sulphydrylase contained 20 mM Tris · HCl, pH 7.5, and 1 mM dithiothreitol, and the buffer used throughout the purification of β-cystathionase contained 20 mM Tris/phosphate, pH 9.0.

Cells of *P. denitrificans* strain 8944 were grown, harvested and a cell-free extract prepared as previously described [22].

Serine transacetylase. Solid $(NH_4)_2SO_4$ was added to the cell-free extract to give 50% saturation and the precipitated protein was collected by centrifugation at 30 000 × *g* for 20 min. Precipitated protein was dissolved in a minimal volume of buffer and dialysed against buffer overnight.

The dialysed $(NH_4)_2SO_4$ fraction was applied to a column of Sephadex G-200 (75.0 × 3.5 cm) equilibrated with buffer and the enzyme eluted with buffer at a flow rate of 0.45 ml/min. Fractions (5 ml) were collected, assayed for serine transacetylase and *O*-acetylserine sulphydrylase and those fractions containing serine transacetylase and no *O*-acetylserine sulphydrylase activity were pooled.

The pooled, active Sephadex G-200 fractions were applied to a column of hydroxyapatite (15.0 × 1.5 cm) equilibrated with buffer. The column was washed with 20 ml of buffer and the enzyme was eluted with a 500 ml linear KCl gradient (0—0.45 M KCl, dissolved in buffer), at a flow rate of 0.55 ml/min. Fractions (5 ml) were collected and assayed, and those fractions containing serine transacetylase activity were pooled and dialysed against buffer overnight. The pooled, active fractions were concentrated 10-fold by dialysis against a saturated solution of polyethylene glycol.

O-Acetylserine sulphydrylase. Protein precipitated between 35 and 75% saturated $(NH_4)_2SO_4$ was dissolved in a minimal amount of buffer and dialysed against buffer overnight. The $(NH_4)_2SO_4$ fraction was applied to a column of Sephadex G-200 (75.0 × 3.5 cm) equilibrated with buffer; the enzyme was eluted with buffer at a flow rate of 0.45 ml/min. Fractions (5 ml) were collected and assayed for *O*-acetylserine sulphydrylase activity; those fractions containing the highest specific activity were pooled. The pooled, active fractions were applied to a column of DEAE-cellulose (8.5 × 3.0 cm) equilibrated with buffer and then with 60 ml of 0.2 M KCl dissolved in buffer. The enzyme was eluted with a 500 ml linear gradient of 0.2—0.45 M KCl, dissolved in buffer, at a flow rate of 0.55 ml/min. Fractions (5 ml) were collected and assayed, and those fractions with the highest specific activity were pooled and dialysed against buffer overnight. The resultant fraction was applied to a second column of DEAE-cellulose (5 × 1 cm). The column was washed with 10 ml of 0.2 M KCl dissolved in buffer and the enzyme was eluted with 10 ml of 0.5 M KCl dissolved in buffer. The enzyme was dialysed against buffer overnight and then stored at −15° C.

β-Cystathionase. The cell-free extract was brought to 80% saturation with solid $(NH_4)_2SO_4$. Precipitated protein was recovered by centrifugation, dissolved in 15 ml of buffer dialysed overnight, applied to a column of Sephadex G-200 (90.0 × 3.5 cm) equilibrated with buffer and eluted with buffer. Fractions (5 ml) were collected and assayed, and active fractions were pooled and loaded onto a column of DEAE-cellulose (10.0 × 2.5 cm) and the column was washed with 50 ml of buffer. A KCl gradient (0—0.5 M KCl) was developed and the enzyme eluted at a flow rate of 0.55 ml/min. The fractions were assayed and the active fractions pooled, dialysed against buffer overnight, and then applied to another column of DEAE-cellulose (3 × 1 cm), equilibrated with buffer. The enzyme was eluted with 0.5 M KCl, in buffer, in a volume of 8 ml.

The enzyme was dialysed against buffer to remove the KCl and was then stored at −15° C.

Results

O-Acetylserine lyase

In initial experiments using cell-free extracts *O*-acetylserine sulphydrylase activity was not linear with time or protein concentration. Upon investigation, it was found that *O*-acetylserine was being removed from reaction mixtures due to the activity of *O*-acetylserine lyase, an enzyme found previously in plants. Mazelis and Fowden [14] reported *O*-acetylserine lyase activity in crude extracts of higher plants: the enzyme cleaves *O*-acetylserine by β-elimination into pyruvic acid, ammonia and acetate.

A brief study of *O*-acetylserine lyase in cell-free extracts of *P. denitrificans*, demonstrated that the bacterial enzyme resembles the plant enzyme in many of its properties. The enzyme is heat sensitive; pretreatment at 80° C for 3 min totally destroyed enzyme activity. The enzyme is specific for *O*-acetyl-L-serine; L-serine, *N*-acetyl-L-serine, *O*-acetyl-L-homoserine and *O*-acetyl-L-threonine did not act as substrates for the enzyme. The enzyme had a K_m in the range of 50—100 mM for *O*-acetylserine in four separate preparations. The enzyme is probably a pyridoxal phosphate-requiring enzyme since it was slightly stimulated by the addition of pyridoxal phosphate and was strongly inhibited by the addition of hydroxylamine and KCN. Since this enzyme removed *O*-acetylserine from the reaction mixtures used to determine *O*-acetylserine sulphydrylase activity it was necessary to remove *O*-acetylserine lyase from preparations prior to investigating the kinetic and regulatory properties of *O*-acetylserine sulphydrylase. Similarly L-serine deaminase activity, present in cell-free extracts, had to be removed from enzyme preparations used to investigate the properties of serine transacetylase since serine deaminase rapidly removed serine, one of the substrates of serine transacetylase, from reaction mixtures.

The release of pyruvate from cystathionine as catalyzed by cell-free extracts was not linear with either time or protein concentration. Furthermore, pyruvate, included in reaction mixtures in lieu of cystathionine, was removed from reaction mixtures (Table I). The cell-free extract was purified to remove the contaminating enzyme(s) responsible for the removal of pyruvate. The non-enzymic production of pyruvate from cystathionine was checked and was found to be insignificant.

During the purification steps serine transacetylase and *O*-acetylserine sulphydrylase activities were separated from *O*-acetylserine lyase and serine deaminase activities and were free from cross-contamination (Fig. 1). During the purification serine transacetylase was purified approx. 150-fold and *O*-acetylserine sulphydrylase was purified approx. 450-fold (Table II).

Properties

Serine transacetylase, *O*-acetylserine sulphydrylase and β-cystathionase activities were linear with time and protein concentration.

Kinetic constants were calculated from double reciprocal plots and these

TABLE I

ENZYMIC REMOVAL OF PYRUVATE FROM INCUBATION MIXTURES CONTAINING CELL-FREE EXTRACTS AND PARTIALLY PURIFIED ENZYME PREPARATIONS

Reaction mixtures were as described in Materials and Methods except that L-cystathionine was replaced by pyruvate as specified in the table. The cell-free extract and the partially purified enzyme preparation were used as the enzyme source. Reactions were conducted at 30°C and the amount of pyruvate remaining in the incubation mixtures was determined using Method 1.

Addition to assay	Enzyme source	nmol of pyruvate remaining in reaction mixtures after		
		0 min	15 min	30 min
Nil	Cell-free extract	0	3	7
100 nmol of pyruvate	Cell-free extract	98	67	52
200 nmol of pyruvate	Cell-free extract	207	157	123
Nil	Partially purified enzyme	0	0	2
100 nmol of pyruvate	Partially purified enzyme	103	100	101
200 nmol of pyruvate	Partially purified enzyme	209	206	204

values are summarised in Table III. Serine transacetylase activity was inhibited by serine concentrations above 2.5 mM. The rate of pyruvate release from cystathionine, as measured by both methods a and b increased with cystathionine concentration up to 9 mM. Double reciprocal plots of cystathionine concentration versus the rate of pyruvate release gave a K_m of $4.2 \cdot 10^{-3}$ M.

Serine transacetylase has a pH optimum between 7.5 and 8.0 and *O*-acetylserine sulphydrylase has a pH optimum, as determined by the rate of cysteine synthesis, at pH 7.5. However, because of the instability of *O*-acetylserine in alkaline conditions, reaction mixtures were maintained at pH 7.5. Maximum β-cystathionase activity was found to lie between pH 9.0 and 9.5 when a

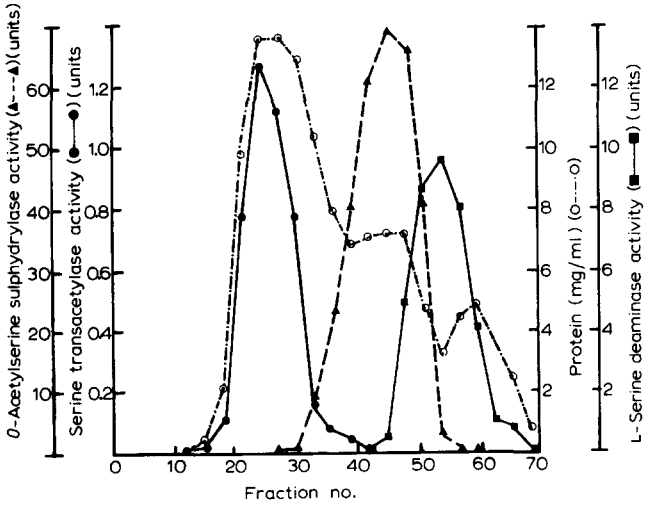

Fig. 1. Separation of serine transacetylase, *O*-acetylserine sulphydrylase and serine deaminase activities. The activities were separated by gel filtration on Sephadex G-200 after fractionation by $(NH_4)_2SO_4$. ○, protein; ●, serine transacetylase; ▲, *O*-acetylserine sulphydrylase; ■, serine deaminase.

TABLE II

PURIFICATION PROFILE OF *O*-ACETYLSERINE SULPHYDRYLASE

Units are μmol of cysteine synthesised per min.

Fraction No.	Purification step	Total protein (mg)	Enzyme activity		Recovery	
			Specific activity (units/ml)	Total (units)	Purification (-fold)	%
1	Crude homogenate	6590	3.31	21 812	0	100
2	$(NH_4)_2SO_4$ fractionation	1573	12.51	19 831	3.87	91
3	Sephadex G-200 gel filtration	108.4	150.4	16 300	45.44	75
4	DEAE-cellulose chromatography 1	8.3	1496	12 429	452	57
5	DEAE-cellulose chromatography 2	8.1	1498	12 133	452	56

number of buffer systems were used. In the presence of 0.2 M glycine/NaOH buffer, β-cystathionase activity was only 20% of the value in the Tris/phosphate or Tris/pyrophosphate buffers at the same molarity and pH. This inhibitory effect was due to glycine, which strongly inhibits β-cystathionase activity. Notwithstanding this effect, β-cystathionase activity decreased sharply at pH values above 9.5.

Serine transacetylase was apparently specific for L-serine; it did not catalyse the acetylation of L-homoserine, L-threonine, ethanolamine, taurine or D-serine, nor did it catalyse the exchange of the acetyl group from *O*-acetyl-L-homoserine to L-serine or L-homoserine. Similarly, *O*-acetylserine sulphydrylase was specific for *O*-acetyl-L-serine; L-serine, *N*-acetyl-L-serine, *O*-acetyl-D-serine, *O*-acetyl-L-threonine and *O*-acetyl-L-homoserine gave no activity when substituted for *O*-acetyl-L-serine. However, selenide did replace sulphide in the reaction and this is discussed below.

In contrast to the high degree of specificity of serine transacetylase and *O*-acetylserine sulphydrylase, β-cystathionase catalysed the cleavage and release

TABLE III

K_m VALUES FOR SUBSTRATES OF SERINE TRANSACETYLASE, *O*-ACETYLSERINE SULPHYDRYLASE AND β-CYSTATHIONASE

All kinetic constants were determined from replots of initial velocity. Values for constants were derived from more than two figures.

Enzyme	K_m (M)	
Serine transacetylase	Serine	$4.0 \cdot 10^{-4}$
	Acetyl-CoA	$1.0 \cdot 10^{-4}$
O-Acetylserine sulphydrylase	Sulphide	$2.7 \cdot 10^{-3}$
	O-Acetylserine	$1.25 \cdot 10^{-3}$
β-Cystathionase	Cystathionine	$4.2 \cdot 10^{-3}$

TABLE IV

SUBSTRATE SPECIFICITY OF THE PARTIALLY PURIFIED β-CYSTATHIONASE

Reaction mixtures were as described in Materials and Methods except that L-cystathionine was replaced by the compounds specified.

Substrate	Activity (%)
L-Cystathionine (10 mM)	100
L-Cysteine (10 mM) *	0
L-Cystine (5 mM)	28
DL-Lanthionine (20 mM)	136
L-Djenkolic acid (10 mM)	79
L-Homoserine (10 mM)	0
L-Homocysteine (5 mM) *	5
L-Glutathione (10 mM)	0
L-Methionine (10 mM)	0
β-Cyano-L-alanine (10 mM)	0

* Denotes assays containing 5 mM dithiothreitol.

of pyruvate from a number of substrates other than cystathionine. DL-Lanthionine was the most active substrate; β-cystathionase also catalysed the hydrolytic cleavage of L-djenkolic acid and L-cystine. L-Cysteine and L-homocysteine, in the presence of excess dithiothreitol, and L-homoserine and β-cyanoalanine were not substrates for the partially purified β-cystathionase when tested by both methods a and b (Table IV).

The effects of some common inhibitors on serine transacetylase and β-cystathionase activities are shown in Table V. *O*-Acetylserine sulphydrylase was not included in this study because both substrates and products of the reaction catalysed by the enzyme reacted directly with some of the inhibitors. Serine transacetylase was inhibited by sulphydryl-group reagents. The enzyme was also inhibited by hydroxylamine; this was perhaps due to the lowering of the acetyl-CoA concentration by the formation of the hydroxamate. β-Cystathionase was also inhibited by sulphydryl-group reagents, by phenylhydrazine and was completely inhibited by KCN (Table V).

TABLE V

EFFECT OF INHIBITORS ON SERINE TRANSACETYLASE AND β-CYSTATHIONASE

Serine transacetylase activity was measured as described in Materials and Methods. β-Cystathionase activity was measured by method a of Materials and Methods. The effects of inhibitors upon *O*-acetylserine sulphydrylase were not determined because of the reaction of some of the common inhibitors with both reactants and products of the reaction catalysed by the enzyme. n.d., not determined.

Compound	Activity (%)	
	β-Cystathionase	Serine transacetylase
N-Ethylmaleimide (10 mM)	6	12
p-Chloromercuribenzoate (5 μM)	5	5
Iodoacetamide (10 mM)	45	23
Hydroxylamine (10 mM)	0	36
KCN (10 mM)	0	15
Phenylhydrazine (5 mM)	0	94
NaF (10 mM)	84	n.d.

TABLE VI

EFFECT OF COFACTORS ON SERINE TRANSACETYLASE, *O*-ACETYLSERINE SULPHYDRYLASE AND β-CYSTATHIONASE

Enzymes were assayed as described in Materials and Methods: all additions to reaction mixtures were preincubated for at least 2 min prior to initiating the reactions by the addition of the respective substrates. n.d., not determined.

Additions	Activities (%)		
	Serine transacetylase	*O*-Acetylserine sulphydrylase	β-Cystathionase
None	100	100	100
$MgCl_2$ (10 mM)	97	102	97
EDTA (10 mM)	100	97	100
Minus pyridoxal phosphate	65	56	6
Dithiothreitol (5 mM)	n.d.	175	106
ATP (5 mM)	97	100	102
ADP (5 mM)	97	102	100

The effect of a number of cofactors upon enzyme activity is shown in Table VI. The addition of EDTA, Mg^{2+}, ATP or ADP to reaction mixtures had no effect upon any of the three enzymes. The activity of all three enzymes was reduced in reaction mixtures without pyridoxal phosphate. The rate of cysteine synthesis, catalysed by *O*-acetylserine sulphydrylase was stimulated by dithiothreitol; this may have been due to either (1) the reduction of thiol groups important to enzymic function, (2) the protection of the enzymically syn-

TABLE VII

EFFECT OF INTERMEDIATES OF THE SULPHUR METABOLIC PATHWAY ON SERINE TRANSACETYLASE, *O*-ACETYLSERINE SULPHYDRYLASE AND β-CYSTATHIONASE ACTIVITIES

Reaction mixtures for each enzyme were as described in Materials and Methods except that the additions were as specified in the Table. n.d., not determined.

Additions	Activity (%)		
	Serine transacetylase	*O*-Acetylserine sulphydrylase	β-Cystathionase
SO_4^{2-} (10 mM)	100	100	100
SO_3^{2-} (10 mM)	100	62	100
S^{2-} (10 mM)	n.d.	100	100
Coenzyme A (5 mM)	88	n.d.	94
L-Serine (10 mM)	n.d.	28	n.d.
O-Acetylserine (10 mM)	100	100	12
L-Cysteine (10 mM) *	0	100	6
L-Cystine (5 mM)	100	100	93
L-Cystathionine (10 mM)	96	21	100
L-Homocysteine (10 mM) *	66	33	5
L-Methionine (10 mM)	55	13	95
L-Glutathione (10 mM)	100	58	27
L-Homoserine (10 mM)	88	97	100
L-Cysteic acid (10 mM)	n.d.	22	n.d.

* Denotes those assays containing 5 mM dithiothreitol.

thesised cysteine from oxidation thus allowing a greater percentage of the synthesised cysteine to be measured by the method of Gaitonde [17]: (Gaitonde's method does not detect cystine) or (3) a combination of these factors.

Since serine transacetylase, *O*-acetylserine sulphydrylase and β-cystathionase are involved in the biosynthetic pathway of methionine, the effect of a number of intermediates of methionine biosynthesis were investigated to determine some of the regulatory properties of the enzymes (Table VII). Methionine and homocysteine inhibited serine transacetylase activity at relatively high concentrations only. L-Cysteine was an effective inhibitor of serine transacetylase activity inhibiting 78% of the enzyme activity at a concentration of $1 \cdot 10^{-5}$ M. In *Escherichia coli* and *Salmonella typhimurium* serine transacetylase activity is inhibited by 50% at a concentration of $1.1 \cdot 10^{-6}$ M [1]. Serine transacetylase activity was also inhibited slightly by coenzyme A, an end product of the reaction.

O-Acetylserine sulphydrylase activity was not affected by sulphate. Sulphite, homocysteine, methionine and cysteic acid competitively inhibited *O*-acetylserine sulphydrylase activity with respect to sulphide, and serine competitively inhibited enzyme activity with respect to *O*-acetylserine. *O*-Acetylserine sulphydrylase activity was not affected by either acetate, an end product of the reaction, or 1,2,4-triazole.

Cysteine, serine, homocysteine and reduced glutathione strongly inhibited β-cystathionase activity; methionine and homoserine were weaker inhibitors. L-Cystine slightly inhibited β-cystathionase activity probably due to competition with the substrate.

Except when L-cystine was the sole sulphur source, the specific activity of both serine transacetylase and *O*-acetylserine sulphydrylase remained approximately the same no matter what sulphur source was included in the growth medium. When *P. denitrificans* was grown with cystine as the sole sulphur source the specific activity of both enzymes was double that of the cells grown

TABLE VIII

EFFECT OF SULPHUR SOURCE DURING GROWTH UPON SERINE TRANSACETYLASE, *O*-ACETYLSERINE SULPHYDRYLASE β-CYSTATHIONASE ACTIVITIES

Crude extracts were prepared from 4 l of culture medium containing log phase cells as described in Materials and Methods. Crude extracts were dialysed for 12 h against 20 mM Tris · HCl buffer, pH 7.5 (for serine transacetylase and *O*-acetylserine sulphydrylase, and against 20 mM Tris/phosphate buffer, pH 9.0, for β-cystathionase). n.d., not determined.

Sulphur source during growth	Specific activity (units/mg of protein)		
	Serine transacetylase	*O*-Acetylserine sulphydrylase	β-Cystathionase
Sulphate	0.14	3.31	5.23
Sulphite	0.16	3.76	5.61
Sulphide	0.13	3.64	4.92
Sulphate plus L-homocysteine	0.11	3.38	3.87
Sulphate plus L-methionine	0.10	3.17	0.93
Sulphate plus 1,2,4-triazole	0.15	3.43	n.d.
L-Cystine	0.36	6.68	5.41
L-Cystine plus 1,2,4-triazole	0.41	6.49	n.d.
Sulphate plus L-Cystine	n.d.	n.d.	5.07

with sulphate as the sole sulphur source (Table VIII).

Addition of 1,2,4-triazole to growth media containing sulphate as the sole sulphur source had no effect on the specific activity of either enzyme. Neither serine transacetylase nor *O*-acetylserine sulphydrylase activities are affected by 1,2,4-triazole.

All of the *O*-acetylserine sulphydrylase activity was present in the soluble fraction of the cell. During the study to determine the effect of sulphur source on specific enzyme activity the specific activity of serine transacetylase, from cultures grown on the same sulphur source, varied. This was due to the variable release of the enzyme from the plasma membrane during sonication, a conclusion varified by examining the serine transacetylase activity associated with membrane vesicles prepared from cells of *P. denitrificans* grown in culture medium containing succinate as substrate, nitrate as the terminal electron acceptor and sulphate as the sole sulphur source [22]. Since sonication released serine transacetylase from the plasma membrane, the enzyme must have been loosely bound.

β-Cystathionase activity of dialysed cell-free extracts grown in the presence of sulphate, sulphite, sulphide, cystine or cystine and sulphate together, showed little variation. However, in extracts prepared from cells grown with sulphate, in the presence of L-homocysteine or methionine, the specific activity of β-cystathionase was significantly lower (Table VIII). The repression of β-cystathionase was significantly greater in those cells grown with methionine than those grown in the presence of homocysteine. Since methionine and homocysteine are known to inhibit β-cystathionase activity directly (Table VII) cell-free extracts were dialysed overnight to remove those potential inhibitors.

To determine the product of the reaction catalysed by serine transacetylase, L-serine was replaced with ^{14}C-labelled serine and the end products of the reaction were examined by thin-layer chromatography (Fig. 2). Although both *O*-acetylserine and *N*-acetylserine were detected it is known that *O*-acetylserine is chemically converted to *N*-acetylserine at pH values above 7.6 [1]. Moreover, *N*-acetylserine is quite stable under the same conditions [23]. Since there is

TABLE IX

REQUIREMENTS OF THE SERINE TRANSACETYLASE AND *O*-ACETYLSERINE SULPHYDRYLASE COUPLED-ENZYME SYSTEM FOR THE SYNTHESIS OF CYSTEINE

Reaction mixtures contained, in a total volume of 1.0 ml; 2.5 μmol acetyl-CoA, 10 μmol sulphide, 2.5 μmol L-serine, 100 μmol Tris · HCl buffer, pH 7.5, 10 nmol pyridoxal phosphate, 5 units purified serine transacetylase and 5 units purified *O*-acetylserine sulphydrylase. Reactions in the absence of sulphide, were run for 15 min at 30°C and then sulphide added and the reaction continued for a further 15 min.

System	Cysteine synthesised (μmol)
Complete	0.269
Complete minus acetyl-CoA	0.018
Complete minus sulphide	0.017
Complete minus L-serine	0.027
Complete minus pyridoxal phosphate	0.214
Complete minus serine transacetylase	0.015
Complete minus *O*-acetylserine sulphydrylase	0.017

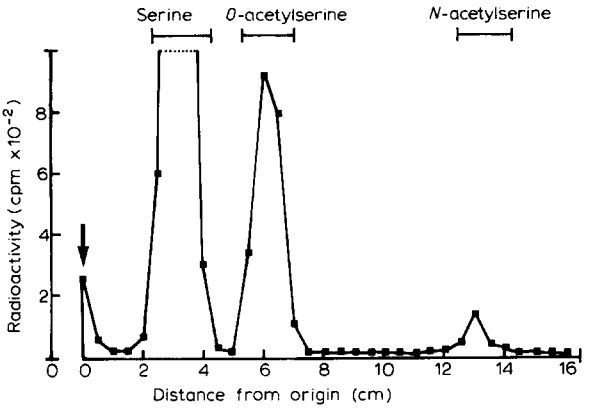

Fig. 2. Identification of the product of the serine transacetylase-catalysed reaction. Reaction mixtures were as described in the text. Aliquots of the reaction mixture were spotted onto thin-layer chromatography plates of silica gel, developed, dried, and the radioactivity of the plates counted.

significantly more *O*-acetylserine than *N*-acetylserine, and since *O*-acetylserine can be converted to *N*-acetylserine and not vice versa it is concluded that the physiological product of the reaction must be *O*-acetylserine. The end product was further identified by its ability to function as substrate for purified *O*-acetylserine sulphydrylase whereas *N*-acetylserine could not.

To determine the product of the reaction catalysed by *O*-acetylserine sulphydrylase a reaction mixture containing 10 mmol Tris · HCl buffer, pH 7.5, 0.5 μmol pyridoxal phosphate, 5 mmol *O*-acetylserine, 20 mmol Na_2S and 150 units of *O*-acetylserine sulphydrylase was run for 3 h at 30° C, stopped with 5 ml of 15% (w/v) trichloroacetic acid, and residual sulphide removed with a stream of nitrogen. The solution was reduced in volume by rotary evaporation at 30° C, filtered, and the pH of the solution adjusted to 10 with NaOH. The solution was filtered and bubbled with oxygen for 12 h; a fine, white precipitate formed. The precipitate was collected by filtration, dissolved in warm 1 M HCl, filtered, and recrystallised with NH_3/ethanol (5%, v/v) to pH 3.5. After standing at 4° C for 12 h the precipitate was collected by filtration. The final product was washed with ether and dried in vacuo. The precipitate was identified as L-cystine by infrared spectrophotometry. Following treatment with excess dithiothreitol (0.25 M) at pH 8.0, the compound gave a positive reaction with Gaitonde's reagent [17]; it also functioned as a substrate for purified cysteinyl-tRNA synthetase (from *P. denitrificans*, see ref. 24).

Since the enzymic product of the reaction catalysed by *O*-acetylserine sulphydrylase is a substrate for cysteinyl-tRNA synthetase, reacts with Gaitonde's reagent, and in the oxidised state exhibits the infrared spectrum of L-cystine, the product was concluded to be L-cysteine.

The enzymic release of pyruvate and homocysteine from cystathionine catalysed by β-cystathionase was confirmed by a variety of methods including: (1) thin-layer chromatography of the 2,4-dinitrophenylhydrazone derivatives of the keto acids extracted from reaction mixtures (Fig. 3), (2) high voltage paper electrophoresis of the products of reaction mixtures, (3) gas-liquid chromatography of the bis(trimethylsilyl)trifluoroacetamide derivatives of reaction

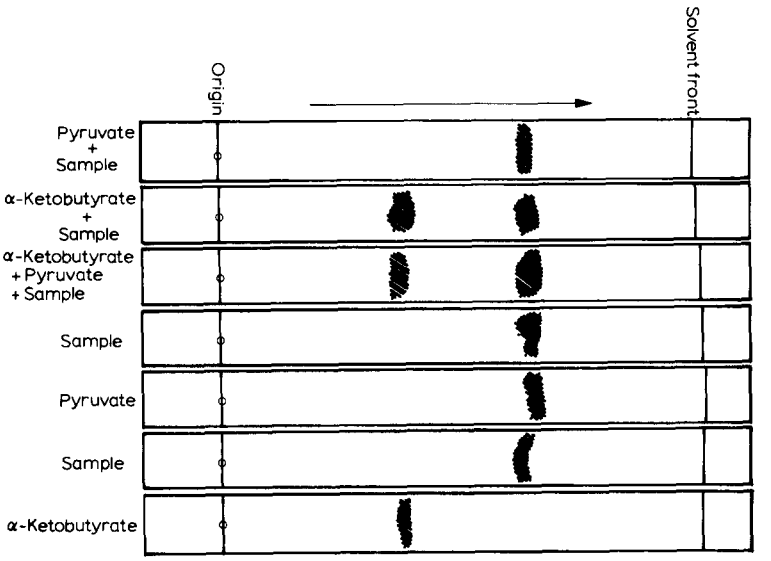

Fig. 3. Identification of pyruvate 2,4-dinitrophenylhydrazone by thin-layer chromatography. The keto acid formed during incubation mixtures containing β-cystathionase was isolated, purified and concentrated as described in the text. 2,4-Dinitrophenylhydrazone derivatives of pyruvate and α-ketobutyric acid were prepared as described in the text. Samples (10 μl) were streaked onto the chromatograms using a glass micropipette. The applied samples were dried and the chromatogram developed by ascending chromatography in Solvent 1 for 8 h.

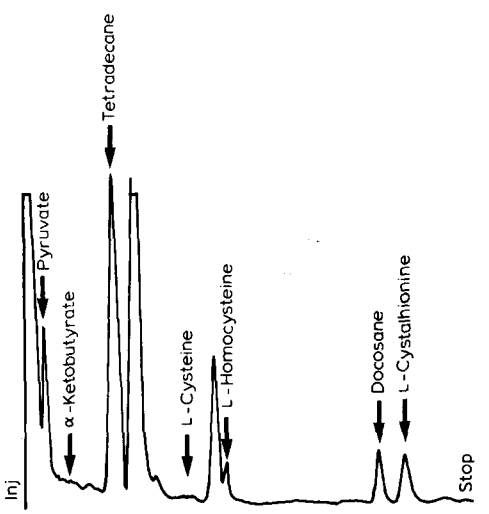

Fig. 4. Identification of the reaction products by gas-liquid chromatography. Reaction products were isolated, purified and derivatised as described in the text. 0.5 μl of the derivatised solution was subjected to gas-liquid chromatography. The 1.5 m × 4 mm glass column was packed with 5% (w/w) OV 17 on Gas Chrom Q, 100—120 mesh. The temperature program started at 100°C and increased at a rate of 5°C/min up to 250°C.

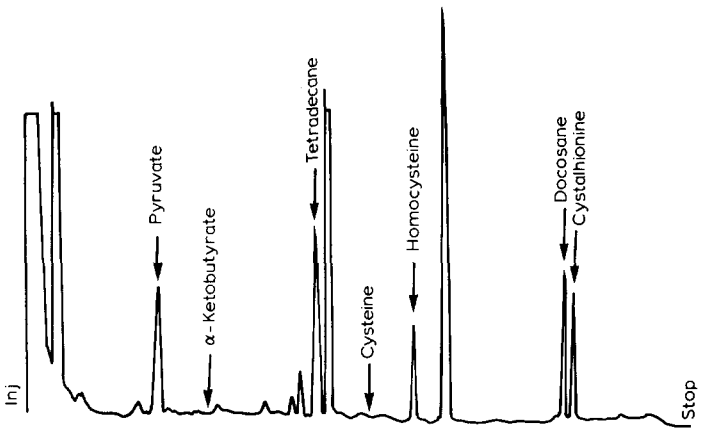

Fig. 5. Identification of the reaction products by gas-liquid chromatography. The isolation, purification and derivatisation were the same as described in Fig. 2. 0.5 μl of the derivatised solution was subjected to gas-liquid chromatography. The 1.5 m × 4 mm glass column was packed with 5% (w/w) OV 1 on Gas Chrom Q, 100—120 mesh. The temperature program consisted of a 5 min hold at 80°C, a temperature gradient of 5°C/min, and a final hold of 5 min at 250°C.

mixtures (Figs. 4 and 5) and, (4) enzymic oxidation, in the presence of lactate dehydrogenase, of the keto acid produced in reaction mixtures.

Cysteine and α-ketobutyrate, which are possible products of cystathionine metabolism, were not detected by any of the above techniques.

Properties of the serine transacetylase and O-acetylserine sulphydrylase coupled-enzyme system

Cysteine synthesis was detected in reaction mixtures containing [^{14}C] serine, acetyl-CoA, Na_2S, pyridoxal phosphate and both serine transacetylase and *O*-acetylserine sulphydrylase although the amount of cysteine synthetised was small. However, when a two-stage incubation was conducted, the amount of cysteine synthesised increased greatly (Table IX). This reconfirms an earlier finding that cysteine inhibits serine transacetylase activity at low concentrations (see Table VII). After adding *N*-ethylmaleimide, terminated reaction mixtures were evaporated to dryness and the residue dissolved in 0.5 ml water. A sample of the residue was subjected to thin-layer chromatography as described in Materials and Methods; the ^{14}C-labelled product was separated from serine and *O*-acetylserine, and ran with an R_F value identical with that of cysteine-*N*-ethylmaleimide (Fig. 6).

Synthesis of selenocysteine

Selenide acted as an alternative substrate for *O*-acetylserine sulphydrylase; selenide replaced sulphide in the reaction. This section reports the results of a brief investigation of the synthesis of selenocysteine from *O*-acetylserine and selenide, catalysed by purified *O*-acetylserine sulphydrylase.

Identification of selenocysteine

To 40 ml of Tris · HCl buffer, pH 7.5, containing 0.5 μmol pyridoxal phos-

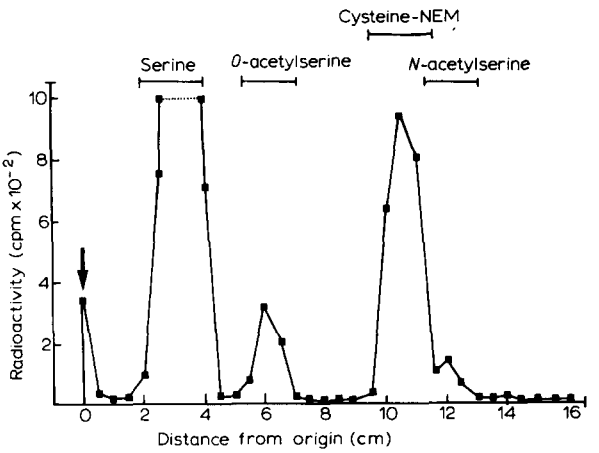

Fig. 6. Identification of the product of the serine transacetylase and *O*-acetylserine sulphydrylase coupled-enzyme system by thin-layer chromatography. Reaction mixtures were as described in the text except that serine was replaced by [^{14}C]serine. The products were separated as described in Materials and Methods. NEM, *N*-ethylmaleimide.

phate and 5 mmol *O*-acetylserine, were added 10 ml of a saturated solution of selenide and 200 units of purified *O*-acetylserine sulphydrylase. The reaction was run for 5 h at 30°C, was stopped by adding 5 ml of trichloroacetic acid (15%, w/v), and the remaining selenide removed with a stream of nitrogen. Selenocystine was precipitated and recrystallised from 1 M HCl as described above for cysteine. The purified product was identified as L-selenocystine by: (1) comparison of its infrared spectrum with the infrared spectrum of authentic selenocystine and, (2) by reducing it with excess dithiothreitol at pH 8.0, and confirming it as a substrate for purified cysteinyl-tRNA synthetase (see ref. 24).

Kinetics of selenocysteine synthesis

Selenide, but not selenite or selenate, replaced sulphide as substrate of purified *O*-acetylserine sulphydrylase. Since selenide and selenocysteine are toxic and extremely unpleasant compounds to work with, only a limited number of experiments involving selenide were conducted. The following experiments were conducted in duplicate and the results presented are means of duplicates; variation between duplicates was not greater than ±7%.

The rate of selenocysteine synthesis was linear as a function of time and enzyme concentration.

The addition of selenide to reaction mixtures containing sulphide inhibited cysteine synthesis but the kinetics of this inhibition were not investigated. In an experiment to determine the relative rates of selenocysteine and cysteine synthesis, increasing amounts of a saturated solution of selenide were added to reaction mixtures until no further increase in the rate of selenocysteine was detected; this ensured that the selenide concentration was not limiting, the concentration of the other components of the reaction mixtures was maintained. Under these conditions, the rate of selenocysteine synthesis was approx. 40% of the rate of cysteine synthesis from sulphide under the same conditions.

Discussion

Although *O*-acetylserine has not been isolated from a biological source, it has been widely used as a substrate for the enzymic synthesis of cysteine. Enzymes isolated from a variety of bacteria [1,2,9,25] and plants [5,8,11,26] all utilise *O*-acetylserine and sulphide for the in vitro synthesis of cysteine. The occurrence of serine transacetylase in bacteria and plants, and the fact that *O*-acetylserine is the most active substrate for the enzymic synthesis of cysteine, strongly suggests that *O*-acetylserine is the most likely substrate for cysteine synthesis. Cell-free extracts of *P. denitrificans* synthesised cysteine from *O*-acetylserine and sulphide at a much greater rate than from serine and sulphide.

The reaction catalysed by *O*-acetylserine sulphydrylase is a fairly specific one. Only when *O*-acetylserine was used as the substrate providing the carbon skeleton was there significant synthesis of cysteine.

Purified *O*-acetylserine sulphydrylase from *P. denitrificans* is stimulated by pyridoxal phosphate. Although this stimulation has not been demonstrated in many organisms *O*-acetylserine sulphydrylase is believed to be a pyridoxal phosphate-requiring enzyme. The β-cystathionase of *P. denitrificans*, like that of *E. coli* [3] and *Proteus morganii* [27] and γ-cystathionase from fungi [28] and mammalian liver [29] required pyridoxal phosphate as a cofactor.

O-Acetylserine sulphydrylase from *P. denitrificans* has kinetic constants similar to those found in other organisms. Becker and co-workers [3] obtained two different K_m values for the binding of *O*-acetylserine to the enzyme isolated from *S. typhimurium*; a value of $5 \cdot 10^{-3}$ M was obtained using a colorimetric assay method, while using a spectrophotometric method, a value of $6 \cdot 10^{-7}$ M was obtained. Becker and co-workers [3] were unable to explain these vastly different values. Ngo and Shargool [30] obtained a K_m value for *O*-acetylserine of $1.7 \cdot 10^{-6}$ M with *O*-acetylserine sulphydrylase from rape seed. The two lower K_m values above may be more realistic in the living organism. Support for this idea comes from the fact that in spite of *O*-acetylserine being the most likely substrate for the biosynthesis of cysteine in vivo [5,11,31,32] it has not, to date, been isolated from any organism. Furthermore it is acknowledged that the K_m of an enzyme for a substrate is an indication not only of the affinity of the enzyme for that substrate, but also for the availability of the substrate in vivo [33].

It has been reported that 1,2,4-triazole inhibits the growth of *S. typhimurium* and that this inhibition is overcome by the addition of serine, cysteine or methionine [34,35]. This growth inhibitory effect is due to a lowering of serine transacetylase activity by 1,2,4-triazole which causes a lowering of the intracellular concentration of *O*-acetylserine. Jones-Mortimer and co-workers [10] have reported that in *E. coli* *O*-acetylserine induces the enzymes of sulphate assimilation. The inhibition by 1,2,4-triazole is therefore believed to be due to a lowering of serine transacetylase activity which lowers the intracellular concentration of *O*-acetylserine, and thus removes the inducer of enzymes in the initial steps of sulphate assimilation. In *P. denitrificans* neither the growth nor the specific activity of serine transacetylase is affected in cells grown in the presence of 1,2,4-triazole.

Kredich [25] reported that serine transacetylase of *S. typhimurium* was not

repressed or derepressed by either cysteine or sulphur starvation; it was, however, inhibited by cysteine. In *E. coli* [9,10], *Rhodopseudomonas spheroides* [9] and *S. typhimurium* [1] the formation of *O*-acetylserine sulphydrylase is strongly repressed by the presence of cysteine in the growth medium. This is not, however, a general phenomenon since the levels of the enzyme in *Pseudomonas aeruginosa* and *Bacillus megaterium* are not correlated with the nature of the sulphur source in the growth medium [9].

Both serine transacetylase and *O*-acetylserine sulphydrylase appear to be constitutive enzymes in *P. denitrificans* and are controlled by feedback inhibition. Serine transacetylase is strongly inhibited by cysteine; this appears to be a general property of serine transacetylase [2,5]. Feedback inhibition of serine transacetylase regulates the amount of *O*-acetylserine available for the synthesis of cysteine and therefore plays an important role in regulating the intracellular concentration of cysteine; regulation of the concentration of cysteine is important since quite low cysteine concentrations inhibit respiration (unpublished results).

O-Acetylserine sulphydrylase is also under strict control by feedback inhibition. Accumulation of cystathionine, homocysteine, methionine and cysteic acid, all metabolites of cysteine, inhibit *O*-acetylserine sulphydrylase activity. *O*-Acetylserine sulphydrylase is also inhibited by serine; this is important since intracellular accumulation of serine would occur if there was a deficiency of acetyl-CoA.

β-Cystathionase is also controlled by feedback inhibition by homocysteine and methionine.

The fact that both serine transacetylase and *O*-acetylserine sulphydrylase are constitutive enzymes in *P. denitrificans* provides a means for removing sulphide, a compound toxic to respiration, should it be produced intra- or extracellularly and converting it to a less toxic compound.

In *S. typhimurium* serine transacetylase and *O*-acetylserine sulphydrylase are, under certain conditions, associated in a multifunctional complex, cysteine synthetase [2]. In plants Smith [5,26] showed that serine transacetylase and *O*-acetylserine sulphydrylase do not associate, by the observation that a fraction of serine transacetylase activity is firmly bound to mitochondria, whereas all the *O*-acetylserine sulphydrylase activity is present in the soluble fraction. In *P. denitrificans* no evidence was found to indicate that the two enzymes were associated in a protein complex. On the contrary, a situation similar to that in plants was found.

β-Cystathionase is repressed in cells grown in the presence of homocysteine and methionine. The repression caused by homocysteine may be due to either methionine formed from homocysteine by the growing organism, or to homocysteine itself. Homocysteine also controls β-cystathionase activity by direct feedback inhibition.

Cystathionine can be degraded in either of two ways: (1) cleavage between the sulphur atom and the three-carbon chain (β-elimination) resulting in the formation of homocysteine, pyruvate and ammonia, and (2) cleavage between the sulphur atom and the four carbon chain (γ-elimination) resulting in the formation of cysteine, α-ketobutyrate and ammonia.

There is a stable and highly active β-cystathionase present in extracts of *P.*

denitrificans. With cell-free extracts or purified preparations no evidence for mechanism 2, the mechanism which operates in animals and fungi, was found. The possibility remains, however, that an enzyme catalysing the γ-elimination is destroyed during preparation of enzyme extracts.

The relatively high pH optimum of β-cystathionase activity has been reported previously for *E. coli* [36], *S. typhimurium* [20] and *P. denitrificans* strain 11 [37].

Similar effects of inhibitors upon β-cystathionase have been reported for β-cystathionase isolated from *E. coli* [36]. β-Cystathionase from both *E. coli* and *P. denitrificans* is sulphydryl-group reagent sensitive and is inhibited by cyanide.

This paper includes the first report of the synthesis of selenocysteine by a purified enzyme preparation although its synthesis by whole cells of yeast has been reported previously [38].

O-Acetylserine lyase activity has been reported previously only in plant extracts [14] but it is now reported for the first time in bacterial extracts. Since β-cystathionase, cysteinyl-tRNA synthetase and methionyl-tRNA synthetase are inhibited by *O*-acetylserine [24] the function of *O*-acetylserine lyase may be to regulate the intracellular concentration of *O*-acetylserine and therefore play a role in the overall regulation of sulphur metabolism in *P. denitrificans*.

Acknowledgements

J.N.B. was the holder of an 1851 Royal Commission Scholarship. We thank Dr. W. Greenaway for performing the gas-liquid chromatography examinations.

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