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CYSTEINE BIOSYNTHESIS IN *PASTEURELLA MULTOCIDA*

CYSTEINE SYNTHASE, PURIFICATION AND PROPERTIES

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SUMMARY

The enzymatic synthesis of cysteine (from serine and sulfide) has been demonstrated in *Pasteurella multocida*. The enzyme, cysteine synthase (L-serine hydro-lyase (adding H_2S), EC 4.2.1.22) has been purified about 375-fold from acetone powder, by ammonium sulfate fractionation, acid treatment, calcium phosphate gel adsorption and chromatography on DEAE-cellulose. The purified preparation is not homogeneous, but contains a maximum of 7% impurities. The enzyme requires pyridoxal phosphate and is specific for L-serine and sulfide. The optimum pH is 8.0. The K_m estimated for L-serine is $1.3 \cdot 10^{-3}$ M. The enzyme is inhibited by hydroxylamine, hydrazine and cyanide and by sulfhydryl reagents (mercuric ions, iodoacetate, *p*-chloromercuribenzoate and arsenate). Monovalent cations enhance the activity.

NADPH-dependent sulfite reductase (hydrogen-sulfide: NADP oxidoreductase, EC 1.8.1.2) and aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) which catalyzes the formation of sulfite from cysteine sulfinic acid, have also been demonstrated in cell-free extracts.

INTRODUCTION

Pasteurella multocida utilizes sulfide, sulfite and thiosulfate for the synthesis of cysteine and other sulfur amino acids[1]. This paper describes the partial purification and some properties of L-cysteine synthase (L-serine hydro-lyase (adding H_2S), EC 4.2.1.22) (cysteine synthetase, serine sulfhydrase) which catalyzes the following reaction:

$$\text{L-Serine} + H_2S \rightarrow \text{L-cysteine} + H_2O$$

and presents some data concerning the metabolism of sulfite and cysteine sulfinic acid, in connection with the synthesis of cysteine in this bacteria. Cysteine synthase

Abbreviations: CSA, cysteine sulfinic acid; PCMB, *p*-chloromercuribenzoate.

has been purified to various extents from yeast[2], *Neurospora crassa*[3] and chicken liver[4].

MATERIALS AND METHODS

Reagents. NADPH, NADH, FAD and pyridoxal phosphate were obtained from Sigma Chemical Company. L-Serine and L-cysteine from Nutritional Biochemicals. DEAE-cellulose, from Serva, cysteine sulfinic acid (CSA) from Calbiochem, Carbowax from Union Carbide Chemicals Co. and Dowex AG 50 W-X8 from Bio-Rad Laboratories. The radioactive compounds were purchased from the Radiochemical Centre, Amersham, England and the New England Nuclear Corporation and had the following specific activities (mC/μmole): $Na_2{}^{35}S$, 1.4 and L-[1-^{14}C]serine, 5. Other reagents were of analytical grade.

Strain and cultures. P. multocida (strain Beaufort No. 28 from the Institute Pasteur, Paris) was grown in the liquid medium described previously[1]. After incubation for 16 h at 37°, aliquots of the liquid culture were grown at 37° in erlenmeyer flasks containing 2 l of medium, for 7 h with mechanical shaking. Cells were harvested by continuous-flow centrifugation at 34 000 × *g* in a Servall RC-2 centrifuge, and washed twice by centrifugation at 15 000 × *g*, with cold saline solution.

Preparation of cell-free extracts. Acetone powder was obtained from the wet cells[5] and stored at 4° *in vacuo*. Cell-free extracts were prepared from the acetone powder as described previously[6].

Enzyme assays. Cysteine synthase was usually determined by measuring colorimetrically the amount of cysteine formed[7,8] from L-serine and sulfide in the presence of pyridoxal phosphate[8]. In certain cases enzymic activity was determined by measuring the radioactivity of cysteine formed from labeled substrates. In these cases the incubation reaction was stopped by adding methanol. After centrifugation, the clear supernatant was passed through a small (1 cm × 2 cm) column of Dowex 50 W-X8 (H^+), concentrated at low temperature, chromatographed with butanol–acetic acid–water (60:15:25, by vol.)[9], radioautographed, counted, cochromatographed and identified as previously described[6,10,11]. Enzymic activity was also determined by measuring the radioactivity of [^{35}S]cysteic acid[2], the performic acid oxidation derivative of [^{35}S]cysteine[12]. Residual [^{35}S]sulfide was eliminated as barium sulfate[2].

L-Serine dehydratase was assayed by measuring colorimetrically the amount of pyruvic acid formed[13,14] from L-serine in the presence of pyridoxal phosphate. The reaction mixture contained, in μmoles: buffer phosphate (pH 8.2), 50; L-serine, 13; pyridoxal phosphate, 0.10; EDTA, 4.0; and extract in a final volume of 0.80 ml. Incubation was for 30 min at 37°. The reaction was stopped by adding methanol, the precipitate formed was removed by filtration and the supernatant was assayed for pyruvate[13,14]. In certain cases enzymic activity was assayed by measuring the radioactivity of [1-^{14}C]pyruvic acid formed from L-[1-^{14}C]serine. In these cases the method was the same as described above and pyruvic acid was identified in the radioautogram by the condensation reaction with 2,4-dinitrophenylhydrazine[15].

For both enzymes a unit of enzymatic activity was defined as the amount of enzyme catalyzing the formation of 1 μmole of product per h, under the conditions of the assay. Specific activity was expressed as units/mg of protein.

Sulfite reductase activity was shown by the specific oxidation of NADPH followed spectrophotometrically at 340 mμ with sulfite as substrate[16].

Aspartate aminotransferase was assayed by measuring the amount of sulfite formed[17,18] from CSA in the presence of α-ketoglutarate[19].

Protein was determined by the methods of LOWRY *et al.*[20] and WARBURG AND CHRISTIAN[21].

RESULTS

Purification of cysteine synthase. As shown in Table I, cell-free extract of *P. multocida* catalyzes the formation of cysteine from L-serine and sulfide, in the presence of pyridoxal phosphate. The product of reaction was identified as cysteine by comparison with authentic compound in respect to paper chromatographic behavior[9], reaction with ninhydrine[11] and nitroprusside spray reagents[22] and paper chromatographic behavior of the performic acid oxidation derivative, cysteic acid[12].

TABLE I

CYSTEINE SYNTHASE ACTIVITY IN CELL-FREE EXTRACT

The complete system contained 4.2 mg of protein and the following in μmoles: Tris buffer (pH 8.0), 50; L-serine, 13; Na_2S, 40; pyridoxal phosphate, 0.10; EDTA, 0.40. Final volume, 1.5 ml. Incubation was for 3 h at 37° and was stopped by adding 0.30 ml of 30% metaphosphoric acid. After centrifugation, cysteine was assayed in the supernatant. Heat inactivation was carried out by boiling the extract for 5 min.

Modifications of reaction mixture	*Cysteine formation (mμmoles)*
None	230
Less pyridoxal phosphate	80
Less L-serine	5
Less sulfide	2
Boiled extract	5

To purify cysteine synthase it was necessary to separate a very active L-serine dehydratase which was present in cell-free extracts. Table II shows that, for 9 μmoles of [1-^{14}C]pyruvate, only 0.5 μmole of L-[1-^{14}C]cysteine was produced from L-[1-^{14}C]-serine, in agreement with preliminary observations[23]. The low specific activity of pyruvic acid in this experiment, is probably due to Tris buffer, which is an inhibitor of L-serine dehydratase[24].

The purification of L-cysteine synthase was carried out according to the following procedure which was used routinely and found to be reproducible. All operations were carried out between 0° and 4°. All buffers, unless otherwise indicated, contained $5 \cdot 10^{-4}$ M EDTA and 10^{-5} M mercaptoethanol.

Step 1. Preparation of crude extract. Cell-free extract was extracted from 86 g of acetone powder, obtained from 452 g of cells (wet weight), collected from 160 l of culture. Cell-free extract was dialyzed for 3 h against 0.05 M Tris–HCl (pH 8.0). The dialyzed material was concentrated to 300 ml by Carbowax at 4°.

Step 2. Centrifugation at 100 000 × g. The solution from Step 1 (Fraction I) was centrifugated at 100 000 × *g* for 120 min in a Spinco model L ultracentrifuge. In this particular preparation only a 1.5-fold increase in the supernatant specific activity was achieved; the increase in specific activity was usually 5- to 6-fold.

TABLE II

CYSTEINE SYNTHASE AND L-SERINE DEHYDRATASE ACTIVITY IN CELL-FREE EXTRACTS

Expt. 1. The reaction mixture contained 4.1 mg of cell-free extract and the following in μmoles: Tris buffer (pH 8.0), 75; L-[1-^{14}C]serine, 27 ($1.01 \cdot 10^6$ counts/min); Na_2S, 40; EDTA, 8.0; pyridoxal phosphate, 0.20. Final volume, 1.7 ml. Incubation, for 3 h at 37°, was stopped by adding 7.4 ml of methanol. Other conditions are described in the text. *Expt. 2.* The reaction mixture contained 5.3 mg of cell-free extract and the following in μmoles: L-serine, 30; $Na_2{}^{35}S$, 65 ($1.41 \cdot 10^6$ counts/min); EDTA, 12; pyridoxal phosphate, 0.3. Final volume, 3.6 ml. Other conditions are as described above.

Expt No.	*Substrates*	*Products*	*Total activity*		*Specific activity*	
			(counts/min)	*(μmoles)*	*(counts/min per h per mg)*	*(μmoles/h per mg)*
1	L-[1-^{14}C]Serine	L-[1-^{14}C]Cysteine	18 400	0.5	1 500	0.04
	plus sulfide	*plus* [1-^{14}C]pyruvate	340 000	9.2	27 500	0.74
2	L-Serine	[^{35}S]Cysteine	11 500	0.5	700	0.03
	plus [^{35}S]sulfide	*plus* pyruvate		11.0*		0.70*

* Colorimetric assay.

Step 3. First $(NH_4)_2SO_4$ fractionation. $(NH_4)_2SO_4$ was slowly added with stirring in the cold room, and allowed to stand 3 h at 4° in the first fractionation and 16 h in the latter ones. All fractions were centrifugated at 20 000 × *g* for 30 min in a Servall RC-2 centrifuge.

To the supernatant solution (240 ml) obtained from Step 2 (Fraction II) solid $(NH_4)_2SO_4$ was added to 40% saturation. The resulting precipitate was removed by centrifugation and discarded, and $(NH_4)_2SO_4$ was added to the supernatant solution to 65% satn. The precipitate obtained by centrifugation was dissolved in 0.1 M Tris buffer (pH 8.0), and made up to 50 ml final volume with this buffer (Fraction III).

Step 4. Second $(NH_4)_2SO_4$ fractionation. Fraction III was brought to 35% satn. with $(NH_4)_2SO_4$. After centrifugation, the supernatant liquid was brought to 50% satn. in a similar manner. The precipitate obtained by centrifugation was dissolved in 0.1 M Tris buffer (pH 8.0), and dialyzed against 0.05 M phosphate buffer (pH 7.6) (Fraction IV).

Step 5. Acid treatment. Fraction IV (41 ml) was diluted with the same volume of cold distilled water, carefully adjusted to pH 5.7 with 0.5 M acetic acid, and allowed to stand 24 h at 2°. The precipitate formed was removed by centrifugation at 12 000 × *g* for 15 min and discarded. The supernatant layer was adjusted to pH 7.0 with Tris buffer (pH 8.0) (Fraction V). In this step, L-serine dehydratase was separated from L-cysteine synthase.

Step 6. Calcium phosphate gel adsorption. To Fraction V (96 ml), calcium phosphate gel (2 mg/mg of protein) was added with stirring for 20 min. The mixture was then centrifugated at 20 000 × *g* for 10 min; the supernatant was treated again by the same procedure. After centrifugation, the supernatant was discarded and the precipitates were combined and washed by centrifugation with cold distilled water. The enzyme was eluted 3 times, by stirring the precipitate for 10 min in 0.1 M Tris buffer (pH 8.0) saturated with 20% $(NH_4)_2SO_4$ containing 0.02 M EDTA. The gel was

removed each time by centrifugation at 5000 rev./min for 10 min. The pooled eluates were precipitated with $(NH_4)_2SO_4$ (85% satn.) and dialyzed against 0.005 M phosphate buffer (pH 8.5) (Fraction VI).

Step 7. DEAE-cellulose chromatography. Fraction VI (27 ml) was concentrated to 9 ml by Carbowax at 4° and then applied to a DEAE-cellulose column (2.5 cm × 21 cm) which had been equilibrated with 0.005 M phosphate buffer (pH 8.5). Elution with 0.005, 0.05 and 0.10 M phosphate buffer (pH 8.5) was carried out. 5-ml fractions were collected with the elution pattern shown in Fig. 1 and assayed for absorbance at

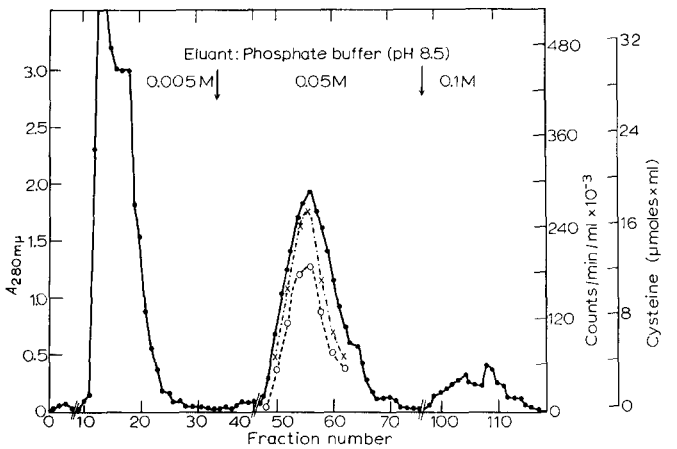

Fig. 1. DEAE-cellulose chromatography of calcium phosphate gel eluate. The DEAE-cellulose column was charged with 280 mg of protein. Activity was measured colorimetrically (Table I) with 0.10 ml of enzyme and incubation for 30 min, or with $Na_2{}^{35}S$, 65 μmoles ($1.4 \cdot 10^6$ counts/min) as substrate, measuring [^{35}S]cysteic acid radioactivity as described in the text. ●—●, absorption at 280 mμ; ○- - -○, μmoles of cysteine per ml; × . — . ×, counts/min per ml ^{35}S incorporated.

280 mμ and for cysteine synthase activity. Enzyme appeared at the second protein peak. It can be seen that enzymatic activity coincides with the level of proteins of that peak, in the colorimetric and radioactive assays.

Fractions containing the enzyme were pooled, and $(NH_4)_2SO_4$ (85% satn.) was added. The suspension, after standing for 16 h at 4°, was centrifugated and the precipitate was dialyzed against 0.05 M Tris buffer (pH 8.0). The purification data for a typical preparation are shown in Table III. The enzyme was purified about 375-fold and has a specific activity of about 13.8. The yeast enzyme has been purified 50-fold[2] and the enzymes of *N. crassa* and chicken liver have been purified about 100-fold[3,4].

Properties of the enzyme. Unless indicated otherwise, the properties of the enzyme were determined with the fraction obtained from the DEAE-cellulose column.

Stability and storage. No detectable loss of activity was observed when the purified preparation was stored at 4° for several months at pH 8.0 in Tris buffer. It was also stable after storage at −20°. Freezing and thawing did not significantly affect the activity but this was lost rapidly at 90°.

Purity. The purified preparation was concentrated by Carbowax and diluted with 0.05 M Tris buffer (pH 8.0). This material showed one component by analytical ultracentrifugation (Fig. 2) with traces of two others. The sedimentation coefficient

TABLE III

PURIFICATION OF CYSTEINE SYNTHASE

Fraction	*Total protein (mg)*	*Total activity (units)*		*Specific activity (units/mg)*		*Purification factor of synthase*
		Synthase	*Dehydratase*	*Synthase*	*Dehydratase*	
I. Crude extract	8100	300	13 800	0.037	1.70	
II. 100 000 × *g* supernatant	5570	290	11 000	0.052	2.00	1.4
III. 40–65% $(NH_4)_2SO_4$ precipitate	4585	1840	8 200	0.40	1.70	10.8
IV. 35–50% $(NH_4)_2SO_4$ precipitate	2050	1780	3 300	0.87	1.60	23.5
V. Acid treatment supernatant	500	1760	0 000	3.50	0.00	95
VI. Calcium phosphate gel eluate	280	1600		6.00		162
VII. DEAE-cellulose 0.05 M phosphate eluate	76.8	1060		13.80		375

($s_{20,w}$) value was 3.2 S at a concentration of 6.6 mg of protein per ml. The maximum amount of impurity was about 7%. When synthetic boundary cell was used, the presence of a contaminant was confirmed and the sedimentation coefficient ($s_{20,w}$) was 3.6 S, at a concentration of 3.3 mg/ml.

pH optimum. As shown in Fig. 3 the enzyme has activity between pH 7.0 and 8.9. The pH optimum with Tris buffer is 8.0.

Kinetics of enzyme reaction. The enzyme is saturated with concentrations of L-serine higher than $5.5 \cdot 10^{-3}$ M. When the rate of cysteine production was measured

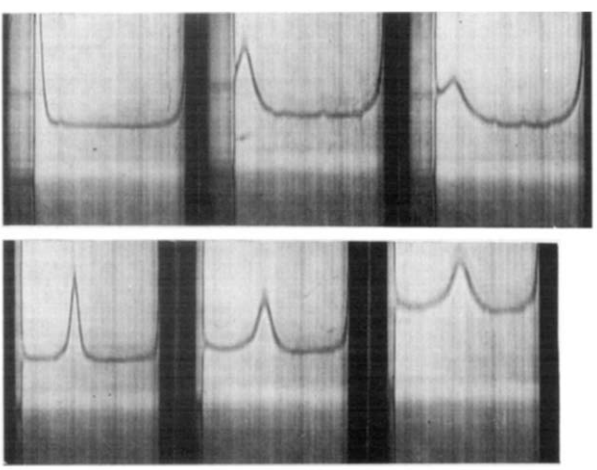

Fig. 2. Sedimentation pattern of cysteine synthase at 59 780 rev./min in a Spinco model E analytical centrifuge. Top, with standard cell, 6.6 mg of protein per ml in 0.05 M Tris buffer (pH 8.0). Bar angle of 50°. Photographs taken at 16-min intervals after speed equilibration. Bottom, with synthetic boundary cell, 3.3 mg of protein per ml. Initial angle of 50° was changed to 40° after 8 min. Photographs taken at 8-min intervals after speed equilibration.

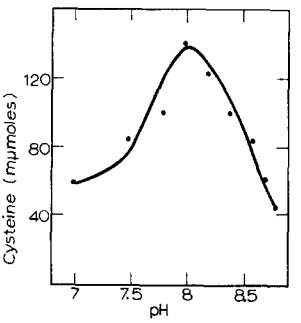

Fig. 3. Cysteine synthase activity as a function of pH. The reaction mixture contained 0.02 mg of enzyme. Incubation was for 30 min at 37°. Other conditions are as described in Table I.

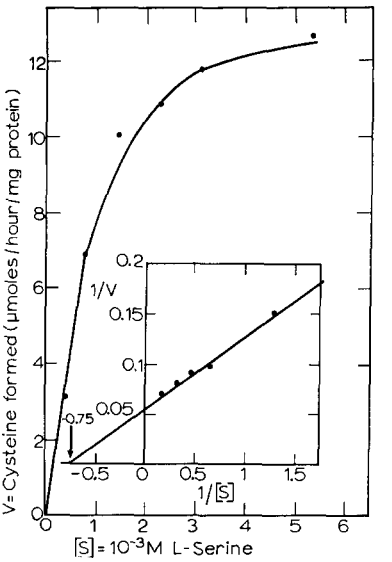

Fig. 4. Cysteine synthase as a function of substrate concentration. The reaction mixture contained 0.16 mg of enzyme. Incubation was for 30 min at 37°. Other conditions are as described in Table I.

at different concentrations of L-serine, Michaelis–Menten kinetics, were observed. The calculated K_m was $1.3 \cdot 10^{-3}$ M (ref. 25) (Fig. 4).

Substrate specificity. Related compounds which are not substrates are L-threonine, DL-α-alanine and glycine. With the crude extract, sulfite also seemed to act as substrate, probably due to the presence of sulfite reductase which reduces sulfite to sulfide. With the purified preparation neither sulfite, nor thiosulfate could replace sulfide.

Inhibitors. In view of the above requirements for enzyme reaction, inhibition

TABLE IV

EFFECT OF INHIBITORS ON CYSTEINE SYNTHASE ACTIVITY

The reaction mixture contained 0.08 mg of partially purified cysteine synthase. Final volume, 1 ml. Other conditions are described in Table I.

Inhibitor	*Final concn. (M)*	*Inhibition (%)*
PCMB	$6 \cdot 10^{-2}$	80
PCMB	$1 \cdot 10^{-3}$	41
Iodoacetate	$1 \cdot 10^{-2}$	45
Hydroxylamine	$2 \cdot 10^{-4}$	51
Hydrazine	$1 \cdot 10^{-3}$	62
Cyanide	$5 \cdot 10^{-4}$	55
Arsenate	$5 \cdot 10^{-2}$	94
Semicarbazide	$1 \cdot 10^{-1}$	87
Azide	$1 \cdot 10^{-1}$	63

TABLE V

EFFECT OF IONS ON CYSTEINE SYNTHASE

The reaction mixture contained 0.03 mg of partially purified cysteine synthase. Final volume 1 ml. Other conditions are described in Table I.

Ions	*Final concn. (mM)*	*Activity (%)*
None		100
Na^+	5	100
	10	106
Li^+	10	105
NH_4^+	10	110
	20	120
K^+	5	106
	10	117
	20	145
Mg^{2+}	10	100
Mn^{2+}	10	108
Cu^{2+}	1	52
Hg^{2+}	0.2	12
	2	0
SO_4^{2-}	10	100
PO_4^{3-}	10	90

by reagents capable of binding pyridoxal phosphate was expected[2,26]. Table IV shows that hydroxylamine, hydrazine and KCN significantly inhibit cysteine synthase. Semicarbazide and azide cause inactivation only at a comparatively high concentration. Inhibition by sulfhydryl reagents, arsenate, *p*-chloromercuribenzoate (PCMB) and iodoacetate are effective only at high concentrations. This fact suggests that the inactivation might result from the reaction with sulfide substrate, and not from a reaction with essential –SH groups. This type of reaction has been observed with cysteine desulfhydrase[4].

Effect of metal ions. Certain monovalent cations enhance the activity of the cysteine synthase (Table V). The order of effect of the cations was found to be $K^+ > NH_4^+ > Na^+ > Li^+$. Other enzymes have similar properties requiring pyridoxal phosphate[14,27]. The nature of this protective effect is unknown[27]. Divalent metal ions

TABLE VI

SULFITE REDUCTASE ACTIVITY IN CELL-FREE EXTRACT

The complete system contained 1.2 mg of cell-free extract and the following in μmoles: phosphate buffer (pH 8.0), 200; sulfite, 1.0; NADPH, 0.10. Final volume, 3 ml. NADPH oxidation was followed spectrophotometrically at 340 mμ for 5 min.

Modifications of reaction mixture	*NADPH oxidized (mμmoles)*
None	74
Add FAD	51
Less sulfite	26
Less extract	2
Replace NADPH with NADH	3

TABLE VII

ASPARTATE AMINOTRANSFERASE ACTIVITY IN CELL-FREE EXTRACT, WITH CSA AS SUBSTRATE

The reaction mixture contained enzyme, 0.27 mg, and the following in μmoles: phosphate buffer (pH 8.0), 250; CSA, 35; α-ketoglutarate, 15; pyridoxal phosphate, 0.10. Total volume, 2 ml. Incubation was for 15 min at 37°. The reaction was stopped by adding 0.50 ml of 1% KOH *plus* 1.0 ml of a saturated solution of $HgCl_2$ in ethanol. After centrifugation, sulfite was assayed in the supernatant. Heat inactivation was carried out by boiling the extract for 5 min.

Modifications of reaction mixture	*Sulfite formation (mμmoles)*
None	1400
Boiled enzyme	80
Less pyridoxal phosphate	400
Less CSA	50
Less α-ketoglutarate	90

Mn^{2+} and Mg^{2+} have no effect on the enzyme, but Cu^{2+} and Hg^{2+} inhibited enzyme activity. The anion PO_4^{3-} caused a 10% inactivation of the enzyme.

Metabolism of sulfite. Cell-free extracts of *P. multocida* reduce inorganic sulfite to sulfide. As shown in Table VI, this sulfite-reducing system is NADPH dependent and apparently does no require FAD, in contrast to *N. crassa* sulfite reductase[28]. It is probable that with a purified preparation the system would be dependent on FAD, as was observed in *Escherichia coli*[16]. NADH is unable to replace NADPH. The activity registered without sulfite may be due to NADPH oxidase.

Metabolism of CSA. Cell-free extracts of *P. multocida* catalyze the rapid formation of inorganic sulfite from CSA, as shown in Table VII. The reaction requires pyridoxal phosphate as activator and α-ketoglutaric acid. The enzyme, aspartate aminotransferase, is heat-labile and the activity in cell-free extracts remains stable for more than 6 months at −20°.

DISCUSSION

The enzymatic formation of cysteine from sulfide and L-serine has been found in bacteria[8,29], molds[2,28,30], spinach[8] and superior animals[8,26]. The enzyme, cysteine synthase, has been partially purified from yeast[2], *N. crassa*[3] and chicken liver[4]. In the present study a higher degree of purification, 375-fold (90% pure), has been achieved. The apparently lesser amount of enzyme registered in the first steps of the purification could be explained by the presence of an inhibitor which disappears with $(NH_4)_2SO_4$ precipitation to 40–65% satn. Acid treatment permits the separation of L-serine dehydratase. The properties of the purified preparation are similar to those of the yeast enzyme. The pH optimum, 8.0, found for the *P. multocida* enzyme, is lower than that found for the enzyme of chicken liver (pH 9.5)[4] and liver of hen's embryo and vitellin sack (pH 8.9)[26]. The K_m value for L-serine of *P. multocida* enzyme is $1.3 \cdot 10^{-3}$ M. Chicken liver and vitellin sack enzymes have $K_m = 3.5 \cdot 10^{-3}$ M for DL-serine[26] and *N. crassa* enzyme has $K_m = 7.8 \cdot 10^{-4}$ M for L-serine[3]. With the purified preparation, L-serine and sulfide are specific substrates. L-Homoserine, DL-α-aminobutyrate, hydroxypyruvate and D-serine are unable to act as substrates with the enzyme obtained from other sources[2,27].

The mechanism of cysteine biosynthesis in *P. multocida* is strikingly similar to that found in yeast[2] and *N. crassa*[3] but very different from the one described in *E. coli* and *Salmonella typhimurium*[31]. However, due to the conditions of culture used in this study, we cannot exclude the possibility that the serine transacetylase and *O*-acetylserine sulfhydrylase demonstrated in the latter bacteria[31], were repressed during growth by the presence of cysteine, though cysteine synthase was unaffected[29].

Sulfite reductase of *P. multocida* is NADPH dependent as it has been shown in a variety of microorganisms[19,28,29]. Sulfide remains the only end product detected to date for the sulfite-reducing system of *P. multocida* and other bacteria[19,32] and molds[28].

Cysteine sulfinic acid can be utilized by *P. multocida* as an alternate sulfur source, the metabolism of which gives rise to inorganic sulfite by the same mechanism described in other microorganisms[28].

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