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The Purification and Characterization of *O*-Acetylserine Sulfhydrylase-A from *Salmonella typhimurium**

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SUMMARY

O-Acetylserine sulfhydrylase-A is one of two highly specific enzymic activities which catalyzes the final step in L-cysteine biosynthesis in *Salmonella typhimurium*: O-acetyl-L-serine + sulfide → L-cysteine + acetate. The enzyme has been purified to near homogeneity and characterized. It is a pyridoxal phosphate-containing protein of $s_{20,w}$ 4.5 and molecular weight 68,000. Equilibrium sedimentation studies on native and carboxymethylated enzyme, analytical polyacrylamide disc gel electrophoresis in urea, pyridoxal phosphate analysis, tryptic peptide mapping, and amino acid analysis have shown that O-acetylserine sulfhydrylase-A consists of two identical subunits of molecular weight 34,000.

The spectrum of native O-acetylserine sulfhydrylase-A has a visible absorption maximum at 412 mμ due to pyridoxal phosphate. When O-acetyl-L-serine is added to the enzyme, the visible absorption maximum shifts to 470 mμ. This shift is dependent upon the concentration of O-acetyl-L-serine, and the equilibrium constant, determined spectroscopically, for this reaction is 6×10^{-7} M. The shift in spectral absorption does not occur in the presence of sulfide. The K_m for O-acetyl-L-serine, measured kinetically, in the presence of both substrates is 5×10^{-3} M.

In *Escherichia coli* and *Salmonella typhimurium* the synthesis of L-cysteine from L-serine and sulfide proceeds by the two-step pathway illustrated below (1):

$$\text{L-Serine} + \text{acetyl-CoA} \rightarrow O\text{-acetyl-L-serine} + \text{CoA} \quad (1)$$

$$O\text{-Acetyl-L-serine} + \text{sulfide} \rightarrow \text{L-cysteine} + \text{acetate} \quad (2)$$

The first reaction is catalyzed by the enzyme serine transacetylase, while the second is catalyzed by *O*-acetylserine sulfhydrylase (1).

Crude extracts of *S. typhimurium* contain two separate fractions of *O*-acetylserine sulfhydrylase activity, which can be readily resolved by gel filtration on Sephadex G-200 (2). The first, approximately 5% of the total of this enzymic activity, elutes from the gel just after the void volume together with all of the serine transacetylase activity. It has been previously established (2) that both the transacetylase and sulfhydrylase activities in this fraction reside in the same particle, the nature of which is discussed in the succeeding paper (3). Because of this association, we refer to this portion of the sulfhydrylase activity as "*O*-acetylserine sulfhydrylase-STA." The remaining 95% of the *O*-acetylserine sulfhydrylase activity elutes later from Sephadex G-200 and is not associated with serine transacetylase activity. This sulfhydrylase fraction is referred to as "*O*-acetylserine sulfhydrylase-A."

The present paper reports a purification to near homogeneity of *O*-acetylserine sulfhydrylase-A from *S. typhimurium* together with a description of its physical, chemical, and enzymic properties.

EXPERIMENTAL PROCEDURE

Materials—DL-Lanthionine, L-djenkolic acid, *N*-acetyl-L-cysteine, and L-cysteine were purchased from Sigma. *N*-Ethylmaleimide, acetyl-CoA, 1-fluoro-2,4-dinitrobenzene, DNP[1] amino acids, and enzyme grade ammonium sulfate were obtained from Mann. *O*-Acetyl-L-serine and *O*-acetyl-L-threonine were products of Yeda Research and Development, Ltd; *O*-acetyl-DL-homoserine, *O*-succinyl-DL-serine, and *O*-succinyl-L-homoserine were donated by Dr. D. Kerr and Dr. S. Guggenheim. ^{14}C-L-Serine and ^{14}C-L-cystine were purchased from New England Nuclear. β-Mercaptopyruvate was a gift of Dr. Marie Lipsett. Trypsin, Grade B, was obtained from Calbiochem and bovine serum albumin from Armour Pharmaceutical Company.

Acrylamide and *N*,*N*-methylenebisacrylamide, products of Eastman, were recrystallized from acetone prior to use. Urea was a product of Baker and was recrystallized from 95% ethanol

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[1] The abbreviation used is: DNP-, 2,4-dinitrophenyl-.

before use. All other chemicals were purchased from commercial sources and were of the highest grade generally available.

Assay for O-Acetylserine Sulfhydrylase—The assay for cysteine formation, described previously (1), was modified for easier manipulation and to give a 3- to 4-fold increase in sensitivity.

Fresh aqueous solutions of 1.0 M *O*-acetyl-L-serine (free base) and of 0.1 M sodium sulfide were prepared daily and stored in an ice bath. The reaction mixture of 0.2 ml at a final pH of 7.2 to 7.4 contained the following materials: Tris-HCl, 32 μmoles; *O*-acetyl-L-serine, 20 μmoles; sodium sulfide, 0.64 μmole; sodium EDTA, 0.16 μmole; and an appropriate amount of enzyme. Reactions were started by the addition of 0.18 ml of buffer-substrate mixture to 0.02 ml of solution containing 0.0024 to 0.024 unit of enzyme and were incubated at room temperature in capped glass test tubes, 13 × 100 mm. Enzyme or *O*-acetyl-L-serine was omitted from blanks.

After incubation for 4 min, 1.0 ml of freshly prepared 1 mM nitrous acid (1 part 0.1 M $NaNO_2$ to 99 parts 0.4 N H_2SO_4) was added to the reaction mixture, which was then vigorously agitated. Six minutes later, 0.1 ml of 2% ammonium sulfamate was added, and the solution was mixed. After 2 min, 1.6 ml of the following mixture were added with mixing: 1 part 2% $HgCl_2$ in 0.4 N HCl, 4 parts 6.88% sulfanilamide in 0.4 N HCl, and 2 parts 0.2% N-1-naphthylethylenediamine dihydrochloride in 0.4 N HCl. Full color was developed in 5 min and was stable for at least 15 min; absorbance at 540 mμ was measured in a Zeiss spectrophotometer.

In this modified assay, 0.2 ml of a 0.1 mM solution of cysteine gives an absorbance of 0.272, and color development is directly proportional to cysteine concentration. One unit of *O*-acetylserine sulfhydrylase is defined as that amount of enzyme catalyzing formation of 1 μmole of cysteine per min; therefore, 0.020 ml of an enzyme solution containing 1.0 unit per ml gives an absorbance of 1.09 at 540 mμ under the conditions of the assay. The rate of cysteine formation catalyzed by purified enzyme is linear with time up to 8 min, and the amount of product formed with time is proportional to the amount of enzyme added. Enzyme dilutions for assay were made in 0.05 M Tris-HCl, pH 7.6, containing 0.1 mg per ml of bovine serum albumin.

Preparation of O-Acetyl-^{14}C-L-serine and DNP-^{14}C-L-serine—*O*-Acetyl-^{14}C-L-serine was prepared from ^{14}C-L-serine as previously described (1). Radioautograms of thin layer chromatograms indicated that approximately 85% of the radioactivity in such preparations was in *O*-acetylserine, with the rest present as *N*-acetylserine and serine.

DNP-^{14}C-L-serine was prepared from ^{14}C-L-serine as described by Fraenkel-Conrat, Harris, and Levy (4). The preparation was dried, taken up in acetone, and purified by descending paper chromatography in water-saturated *n*-butyl alcohol. The chromatographed DNP-^{14}C-L-serine was eluted from the paper with 1% $NaHCO_3$ and radioactivity was determined in a gas flow counter. All of the radioactivity of this product was found to chromatograph, with authentic DNP-L-serine.

Chromatography—Thin layer chromatography for proof of product was performed on 250 μ thick silica gel plates obtained from Mann. Mixtures to be analyzed were first treated with an excess of *N*-ethylmaleimide to form the more stable maleimide derivative of L-cysteine (5). A chloroform-ethanol-acetic acid-water (50:32:10:8) solvent system was found to give good resolution of serine, *O*-acetylserine, *N*-acetylserine, and *N*-ethylmaleimide cysteine (1). Compounds were detected by either the chlorine-starch-potassium iodide technique of Rydon and Smith (6) or by ninhydrin spray.

Radioautography of thin layer plates was performed by covering the developed but unstained plate with a sheet of Kodak single coated medical x-ray film, emulsion side down, and exposing overnight.

Thin layer chromatography for NH_2-terminal amino acid analysis was performed on Eastman Chromagram silica gel sheets with fluorescent indicator. For development, the following solvent systems were used: *n*-butyl alcohol, butyl acetate-ammonia (1:2:3), and water-saturated *n*-butyl-alcohol for the ether-soluble amino acid derivatives. *t*-Amyl alcohol saturated with an aqueous solution of potassium phthlate at pH 6.0 was used for both the ether- and water-soluble derivatives. DNP-amino acids were detected by their yellow color and their fluorescence quenching. Descending paper chromatography of DNP-amino acids was performed on Whatman No. 1 paper with water-saturated *n*-butyl alcohol.

Tryptic Digestion and Peptide Mapping—Material to be digested was dialyzed against 1% ammonium bicarbonate, after which it was placed in a boiling water bath for 90 sec. Trypsin was added to the cooled enzyme solution at an enzyme-trypsin ratio of 50 (w/w). Incubation was carried out at 37° for 4 hours. The digest was then applied directly to Whatman No. 3MM paper (19.5 × 22.25 cm) and descending chromatography was carried out for 18 hours in the following solvent system: pyridine, 120 ml; *n*-butyl alcohol, 180 ml; acetic acid, 36 ml; water, 144 ml. After chromatography, the papers were dried and then subjected to electrophoresis at 2500 volts for 2 hours under Varsol in a pyridine-acetic acid-water (50:500:14,450) system at pH 3.6 (7). Peptides were detected by the cadmium ninhydrin stain (8). Arginine peptides were detected by the modified Sakaguchi reagent as described by Irreverre (9), and histidine- and tyrosine-containing peptides were detected by the Pauly reagent (10).

NH_2-terminal Amino Acid Analysis—NH_2-terminal amino acids were determined by the fluorodinitrobenzene method of Fraenkel-Conrat *et al.* (4) with several modifications. The entire procedure up to the final ether extraction was carried out in a 15-ml hydrolysis tube in order to minimize losses due to transfer. One- to 5-mg samples of protein in a volume of 1 ml or less were dialyzed against a concentration of sodium bicarbonate calculated to give approximately equal concentrations (w/v) of protein and sodium bicarbonate in the final dialyzed solution. After treatment with fluorodinitrobenzene and acidification, the reaction mixture was extracted with 10 10-ml portions of diethyl ether. The ether phase was discarded and the aqueous phase was frozen and lyophilized to dryness. One milliliter of constant boiling (5.7 N) HCl was added to the dried sample in the hydrolysis tube, which was then evacuated by repeated freezing and thawing under vacuum, sealed, and placed in an oven at 105° for 16 hours.

After cooling, the hydrolysate was diluted with 5 volumes of water and extracted with five 10-ml portions of ether. The ether extracts were combined and evaporated to dryness in a nitrogen stream, and the residue was dissolved in acetone for application to either thin layer plates or on 4-inch strips of Whatman No. 1 filter paper. In quantitative studies, DNP-amino acids were eluted from paper chromatograms with 1% $NaHCO_3$, and the absorbance at 360 mμ was determined.

Analytical Disc Gel Electrophoresis—Analytical polyacrylamide

disc gel electrophoresis was carried out at room temperature according to Davis (11). The buffer system used was that of Reisfeld and Small (12), with 4% acrylamide with 8 M urea or 7% acrylamide when urea was omitted. Proteins were stained with either 0.25% Amido black in 7% acetic acid or with a 1:40 dilution of a 1% aqueous solution of Coomassie blue in 10% trichloracetic acid (13).

Ultracentrifugation Studies—Ultracentrifugation was done in a Spinco model E analytical ultracentrifuge equipped with an electronic speed control. Sedimentation velocities were determined at 60,000 rpm with both schlieren optics and the ultraviolet photoelectric scanner (14). Molecular weights were determined at sedimentation equilibrium by the meniscus depletion method of Yphantis (15) with a 2-mm column height and scanning at 280 mμ. Determination of molecular weight of the enzyme in 6 M guanidine hydrochloride was made as described by Marler and Tanford (16). The partial specific volume, $\bar{v}$, of the native protein was estimated from the amino acid analysis (17).

Amino Acid Analysis—Amino acid analyses were performed on a Beckman model 120 B amino acid analyzer. Samples were hydrolyzed in 5.7 N HCl for 24, 48, and 72 hours at 105°. Following performic acid oxidation of the protein (18) and hydrolysis in 5.7 N HCl at 105° for 24 hours, half-cystine was determined as cysteic acid. Tryptophan and tyrosine were estimated spectrophotometrically by the method of Edelhoch (19).

Other Methods—Protein determinations were made by the method of Lowry *et al.* (20) with bovine serum albumin as a standard or, in the case of more purified preparations, by absorbance at 280 mμ. Carboxymethylation was carried out in 8 M urea and 0.01 M dithiothreitol with ^{14}C-iodoacetic acid (21). Following deproteinization with 0.3 N perchloric acid for 2 hours, pyridoxal phosphate was assayed by the method of Wada and Snell (22). Nitrogen determinations were performed by the micro-Kjeldahl method as modified by Ma and Zuazaga (23). Serine transacetylase activity and sulfide concentrations were determined as described previously (1). ^{14}C-L-cystine was reduced to ^{14}C-L-cysteine by incubating in 0.1 N ammonium hydroxide and 0.2 M 2-mercaptoethanol overnight at room temperature, following which the material was lyophilized and stored frozen in 0.01 N HCl.

Absorption spectra were measured in a Cary model 15 recording spectrophotometer. Starch gel electrophoresis was performed with the method of Smithies (24). Sonic oscillation was done in a Branson Sonifier, model LS75. Radioactivity was measured in a Nuclear-Chicago model C-110A gas flow counter.

PURIFICATION OF O-ACETYLSERINE SULFHYDRYLASE-A

The enzyme was purified from frozen *S. typhimurium* LT2 cells grown at 37° with forced aeration in a 300-liter fermentor on a 0.5% glucose-Vogel-Bonner salt medium (25) in which magnesium citrate was substituted for magnesium sulfate and with L-djenkolic acid (0.5 mM) as the sole sulfur source. Growth on L-djenkolic acid has previously been shown to derepress *O*-acetylserine sulfhydrylase (1) as well as certain other enzymes of the cysteine biosynthetic pathway (26), presumably by a slow release of cysteine. Purification was carried out at room temperature except as specifically mentioned, and standard buffer was 0.1 M Tris-HCl (pH 7.6)-0.01 M 2-mercaptoethanol.

I. Crude Extract—Three hundred and twenty grams of cells were thawed and resuspended in 640 ml of 0.05 M Tris-HCl (pH 7.6)-0.01 M 2-mercaptoethanol. Cells were sonically disrupted at 4° for 150 sec at 9 amps, and the material was centrifuged for 60 min at 13,000 × *g*. The supernatant layer was decanted off as crude extract, Fraction I.

II. Streptomycin Precipitation—Fraction I was diluted with an equal volume of 0.05 M Tris-HCl (pH 7.6)-0.01 M 2-mercaptoethanol and the pH was adjusted to 7.6 by the addition of 3.0 M Tris base. Then 0.5 volume of 10% streptomycin sulfate in 0.05 M Tris-HCl (pH 7.6)-0.01 M 2-mercaptoethanol was slowly added to the diluted crude extract with mixing. After 30 min at 4° the material was centrifuged at 13,000 × *g* for 30 min. The supernatant layer, Fraction II, was decanted off for further purification.

III. Ammonium Sulfate Fractionation—Crystals of ammonium sulfate were added slowly to Fraction II to 0.40 saturation (243 g per liter). After centrifugation at 13,000 × *g* for 10 min, the supernatant solution was made 0.70 saturated (by adding an additional 205 g per liter) with ammonium sulfate. The mixture was centrifuged, and the resulting precipitate was taken up in the smallest volume of standard buffer in which it would dissolve. This material (Fraction III) was then dialyzed against 8 liters of standard buffer for 20 hours.

IV. Sephadex G-100 Gel Filtration—Dialyzed Fraction III was applied to a Sephadex G-100-120 column (220 cm × 14.3 cm^2) equilibrated with standard buffer. The enzyme was eluted with the standard buffer at a flow rate of 1.0 ml per min, and 20-ml fractions were collected. Fractions with the highest specific activities were pooled as Fraction IV.

V. DEAE-Sephadex Chromatography—Fraction IV was applied to a DEAE-Sephadex A50-120 column (26 cm × 4.9 cm^2) equilibrated with standard buffer. After the column was washed with 1 volume of standard buffer, elution was carried out with a 2-liter linear gradient of 0 to 0.35 M NaCl in standard buffer at a flow rate of 0.5 ml per min. Ten-milliliter fractions were collected and assayed, and those with the highest specific activities were pooled. After dilution with 2 volumes of 0.02 M Tris-HCl (pH 7.6)-0.01 M 2-mercaptoethanol, this material was applied to a second DEAE-Sephadex A50-120 column (8 cm × 1.75 cm^2) equilibrated with standard buffer. The enzyme was eluted with a 250-ml linear gradient of 0 to 0.35 M NaCl in standard buffer at a flow of 0.15 ml per min. Fractions of 2.3 ml were collected and peak fractions were pooled as Fraction V. The elution pattern is seen in Fig. 1. In smaller scale preparations, the second DEAE-Sephadex column was omitted.

VI. Sephadex G-100, Superfine Gel Filtration—Ammonium sulfate was added to Fraction V to 0.70 saturation (472 g per liter), and, following centrifugation at 13,000 × *g* for 10 min, the precipitate was taken up in 3.0 ml of standard buffer. This was applied to a Sephadex G-100-40 column (70 cm × 3.15 cm^2) equilibrated with standard buffer, and the enzyme was eluted at a flow rate of 0.25 ml per min. Fractions of 3.0 ml were collected, and tubes with the highest specific activity were pooled (Fig. 2). Following dialysis against standard buffer, this preparation (Fraction VI) was stored at −20°. A summary of the purification appears in Table I.

RESULTS

Physical Properties of Purified O-Acetylserine Sulfhydrylase-A

When a portion of Fraction VI was subjected to analytical disc gel electrophoresis, a single yellow band was noted even

prior to staining. On staining, one protein component corresponding to the yellow band was observed. When large quantities of Fraction VI (100 to 300 μg per gel) were used, two slower running components could also be shown (Fig. 3, *A* and *B*). All of the *O*-acetylserine sulfhydrylase activity recovered by elution from unstained gels was associated with the dominant yellow component. For this reason, the two smaller components were assumed to represent contaminant proteins, although the possibility that they represent inactive forms of the enzyme could not be ruled out. An examination of disc gels of various dilutions of the enzyme suggested that the major component constituted 95 to 98% of the stainable material. In addition, both starch gel electrophoresis at pH 8.0 and cellulose acetate strip electrophoresis at pH 7.6 with 100 μg of sample and stained with Amido black revealed only a single protein component (Fig. 3, *C* and *D*).

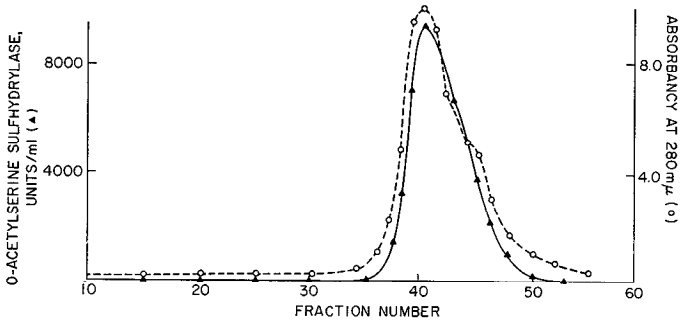

FIG. 1. Elution pattern of *O*-acetylserine sulfhydrylase-A from DEAE-Sephadex A-50 (Step V). Fractions of 2.3 ml were collected and assayed for absorbance at 280 mμ and for *O*-acetylserine sulfhydrylase activity.

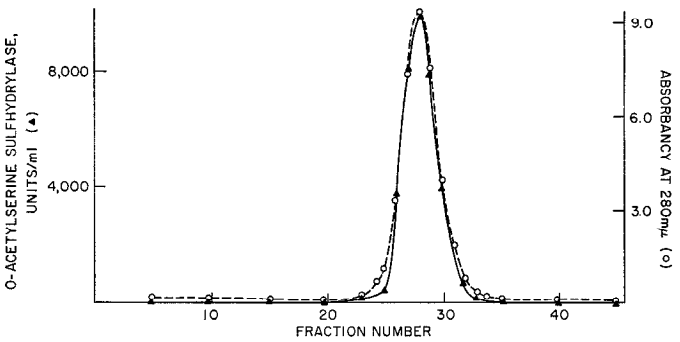

FIG. 2. Elution pattern of *O*-acetylserine sulfhydrylase-A from G-100-40 Superfine Sephadex (Step VI). Fractions of 3.0 ml were collected and assayed for absorbance at 280 mμ and for *O*-acetylserine sulfhydrylase activity.

Analytical disc gel electrophoresis in 8 M urea showed a slow moving, colorless, protein component in addition to a faster moving yellow band (Fig. 4*A*). When the enzyme was precipitated with either acid ammonium sulfate, 10% trichloracetic acid, or 0.3 M perchloric acid (to remove the pyridoxal phosphate), the yellow, faster moving component disappeared, leaving only the more slowly moving component (Fig. 4*B*). These results suggest that the slowly moving component represents enzyme resolved of pyridoxal phosphate while the faster component represents unresolved protein. Apparently, treatment with 8 M urea resolves only a portion of the enzyme so that both components are seen. When a special procedure to remove pyridoxal phosphate is used, however, the resolution of the enzyme is complete (as verified by pyridoxal phosphate assay), and only the slowly moving form is seen.

Sedimentation velocity studies of the purified enzyme showed a single, symmetrical sedimenting boundary with an $s_{20,w}$ of 4.5 (Fig. 5). No significant dependence of S on concentration was noted between 0.7 and 6.8 mg per ml of protein. Carboxymethylated protein and native enzyme treated with 1% sodium dodecyl sulfate both sedimented at 2.4 S. The molecular weight of native protein, determined by equilibrium sedimentation, is 68,000. A plot of log *c* against the square of the distance from the center of rotation (r^2) gave a straight line as shown in Fig. 6. Sedimentation equilibrium in 6 M guanidine-HCl gave a molecular weight of 32,000, and carboxymethylated protein had a molecular weight of 34,000.

In solution, the purified enzyme is intensely yellow. The absorption spectrum (Fig. 7) exhibited a peak at 412 mμ, presumably due to pyridoxal phosphate. The purified protein has a ratio of absorbance at 280 mμ to absorbance at 412 mμ of 3.5.

Chemical Properties

The purified enzyme has a dry weight nitrogen content of 16.3%, while a solution of enzyme contains 198 μg of protein nitrogen per A_{280} unit. Therefore, a solution containing 1.00 mg of protein per ml has an absorbance at 280 mμ of 1.21. The enzyme contains 0.0374 μmole of pyridoxal phosphate per A_{280} unit or 0.131 μmole of pyridoxal phosphate per A_{412} unit. This corresponds to an E_{412} of 7.6×10^3 M^{-1} cm^{-1} and 2.10 moles of pyridoxal phosphate per 68 000 g of protein.

TABLE I

Purification of O-acetylserine sulfhydrylase-A

Fraction and step	Volume	Protein Concentration	Units of *O*-acetylserine sulfhydrylase		Specific activity	Yield
			Per ml	Total		
	ml	*mg/ml*			*units/mg protein*	%
I. Crude extract	762	35.0	700	533,000	20	100
II. Streptomycin precipitation	2225	5.2	232	516,000	45	97
III. Ammonium sulfate fractionation	92.2	62.0	4,210	388,000	68	73
IV. Sephadex G-100-120 gel filtration	160	7.3	1,620	259,500	222	48.5
V. DEAE-Sephadex chromatography	16.1	7.2	7,040	113,000	972	21
VI. Sephadex G-100-40, superfine gel filtration	9.0	8.1	8,600	77,430	1100	14.5

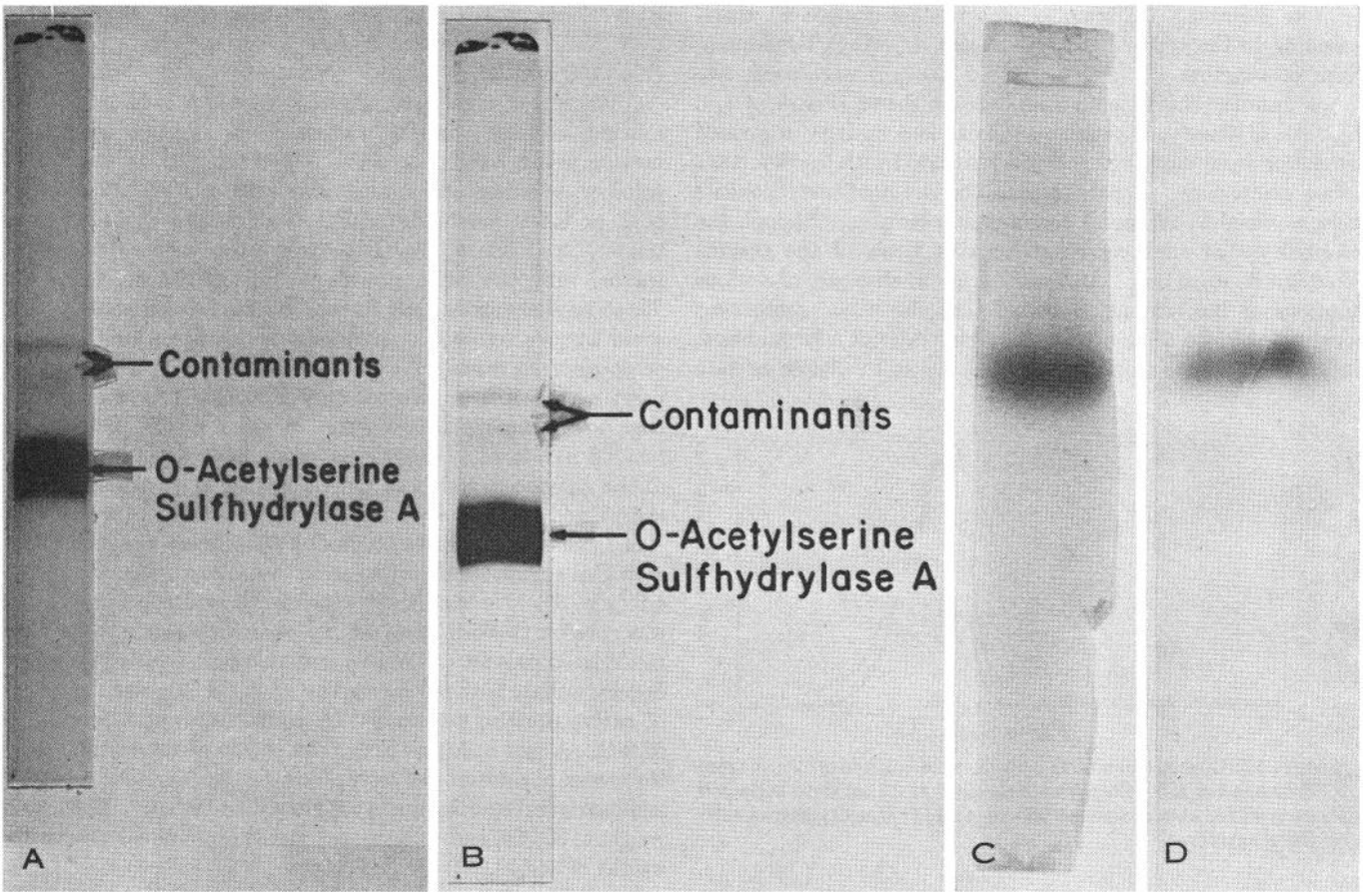

FIG. 3. *A* and *B*, polyacrylamide disc gel electrophoresis of purified *O*-acetylserine sulfhydrylase-A. To each 7% acrylamide gel, 100 μg of sample were applied. *A*, stained with 0.025% Coomassie blue in 10% trichloracetic acid; *B*, stained with 0.25% Amido black in 7% acetic acid. Direction of electrophoresis is toward the *bottom*. *C*, starch gel electrophoresis of purified *O*-acetylserine sulfhydrylase-A. Sample, 100 μg; electrophoresis at 150 volts for 16 hours at 4° in 0.05 M Tris-HCl, pH 8.0; stain 0.25% Amido black in 7% acetic acid. *D*, cellulose acetate strip electrophoresis of purified *O*-acetylserine sulfhydrylase-A (Fraction VI). Electrophoresis was carried out at 200 volts for 4 hours at 4° in 0.1 M Tris-HCl, pH 7.6; stain, 0.25% Amido black in 7% acetic acid. Direction of electrophoresis is toward the *bottom*.

The only NH_2-terminal amino acid found by the fluorodinitrobenzene method, with the use of both thin layer and paper chromatography, was serine. The result was verified by radioautography in which added DNP-^{14}C-L-serine was found to cochromatograph with the unknown DNP-amino acid from the protein. The recovery of DNP-serine was 0.27 mole/68,000 g of protein. In order to correct for loss of DNP-serine due to hydrolysis and subsequent purification steps, a known tracer amount of DNP-^{14}C-L-serine was added to the DNP-protein prior to hydrolysis with HCl. With a correction factor based on the recovery of DNP-^{14}C-L-serine (yield was 15.6%), a value of 1.85 moles of DNP-serine per 68,000 g of protein was calculated.

Since *O*-acetyl-L-serine is a substrate for *O*-acetylserine sulfhydrylase-A an attempt was made to rule out the possibility that the DNP-serine found was simply a result of enzyme-bound substrate reacting with fluorodinitrobenzene rather than the reaction of the latter with the NH_2-terminal residue of the enzyme. *O*-Acetylserine sulfhydrylase-A was incubated for 1 hour at 25° with a 100-fold excess (moles of substrates per mole of protein-bound pyridoxal phosphate) of *O*-acetyl-^{14}C-L-serine in 0.10 M Tris-HCl, pH 7.2. Following 3 days of dialysis against 0.05 M $NaHCO_3$, the incubation mixture was unexpectedly found to have approximately 1 mole of labeled substrate (or a derivative thereof) per mole of protein-bound pyridoxal phosphate. A similar incubation mixture was prepared but, prior to dialysis, the protein-substrate mixture was incubated with 8 M urea and 0.1 M 2-mercaptoethanol for 4 hours at 45°. After 5 days of dialysis against 0.05 M $NaHCO_3$, the dialyzed protein contained less than 0.1 mole of label per mole of originally protein-bound pyridoxal phosphate. (The treatment with urea removes most of the pyridoxal phosphate from the enzyme.) An NH_2-terminal amino acid determination on this material gave a value of 0.98 mole of DNP-serine per 68,000 g of protein. When corrected for losses as above, the yield of DNP-serine was 2.01 moles/68,000 g of protein.

Amino acid analysis of *O*-acetylserine sulfhydrylase-A gave the result shown in Table II. Partial specific volume ($\bar{v}$) was calculated from these data to be 0.74. Tryptophan and tyrosine content, determined spectrophotometrically, were, respectively, 5.0 and 10.1 residues per 68,000 molecular weight. The value for tyrosine was in reasonable agreement with that obtained by amino acid analysis of the hydrolyzed protein.

The tryptic peptide map of *O*-acetylserine sulfhydrylase-A is shown in Fig. 8. A total of 41 peptides were seen, 16 of which

contained arginine as evidenced by their reaction with the Sakaguchi reagent. Three peptides gave a Pauly reaction indicating the presence of tyrosine or histidine.

Following carboxymethylation of *O*-acetylserine sulfhydrylase-A in 8 M urea and 0.01 M dithiothreitol, the treated protein was dialyzed free of urea and reducing agent, and its specific radioactivity was determined. It was found that 2.4 moles of labeled reagent were bound per 68,000 g of protein. A tryptic peptide map of carboxymethylated protein was compared with a map obtained with enzyme that had been treated with 8 M urea and 0.01 M dithiothreitol but not with iodoacetic acid (Fig. 8). It was noted that a Sakaguchi-positive peptide from the non-carboxymethylated enzyme was absent from the map of the carboxymethylated protein, and in the latter a new Sakaguchi-positive peptide was obtained. This new peptide was shown by autography to be radioactive (Fig. 9). In addition, a single previously unobserved, ninhydrin-positive peptide without detectable radioactivity was noted on the map of the carboxymethylated material.

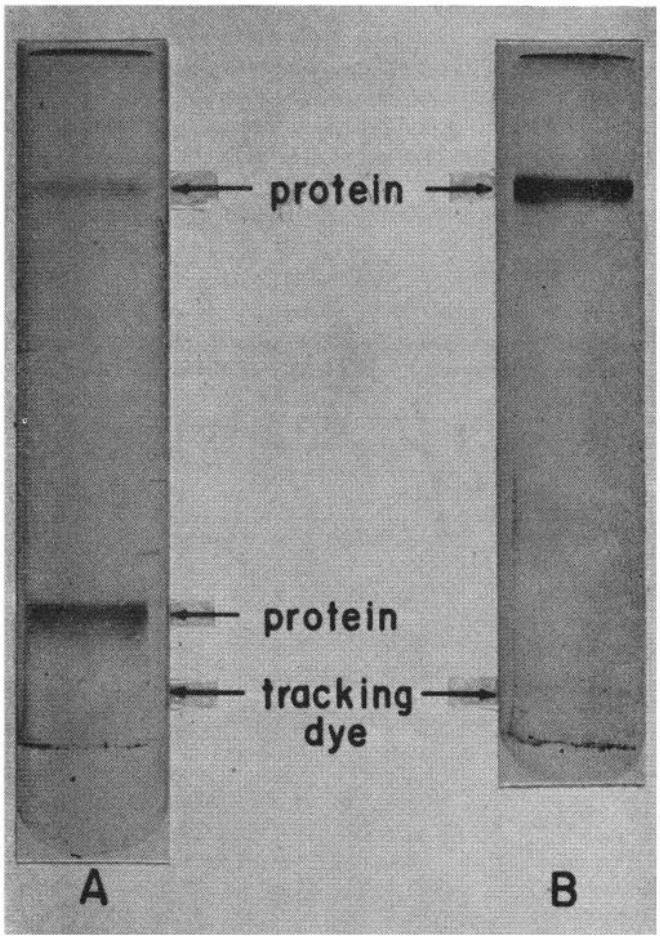

FIG. 4. Polyacrylamide urea disc gel electrophoresis of purified *O*-acetylserine sulfhydrylase-A (Fraction VI). Each sample contained 40 μg of protein and was stained with 0.025% Coomassie blue in 10% trichloracetic acid. *A*, sample received no treatment prior to application to 4% polyacrylamide urea gel. *B*, sample precipitated with 0.3 N perchloric acid, washed twice with water, and redissolved in 10 M urea prior to application to 4% polyacrylamide urea gel.

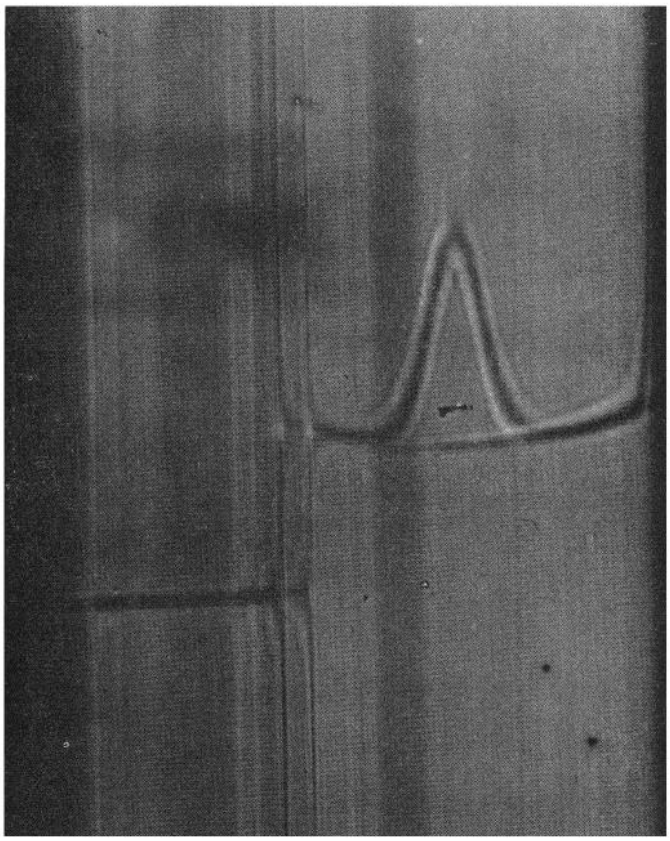

FIG. 5. Sedimentation of purified *O*-acetylserine sulfhydrylase-A for 12 min at 60,000 rpm. The protein concentration was 5.6 mg per ml in 0.1 M Tris-HCl, pH 7.6. The temperature was 22.3° and analyzer angle was 60°. Subsequent photographs of the schlieren pattern up to 72 min showed no additional sedimentable material. The $s_{20,w}$ is 4.5. Sedimentation is toward the *right*.

Enzymic Properties

Purified *O*-acetylserine sulfhydrylase-A has a specific activity of 1100 units per mg. It is free of detectable serine transacetylase activity. The enzyme is stable at room temperature for several days and retains 90% of its activity at −20° for 8 months. Heating the purified enzyme for 10 min results in the loss of only 5% of its activity at 62°, 50% at 67°, and 80% at 70°.

Proof of Product—The product of the *O*-acetylserine sulfhydrylase-A reaction was proved to be L-cysteine by the isolation and identification of its oxidized derivative L-cystine. Five hundred units of purified enzyme were added to 54 ml of 0.2 M Tris-HCl (pH 7.4)-0.001 M Na_2EDTA, containing 6 mmoles of *O*-acetyl-L-serine and 2.7 mmoles of Na_2S. The stoppered reaction mixture was incubated at 25° and aliquots were removed at time intervals and assayed for thiol. The reaction reached completion at 6 min with the formation of 2.7 mmoles of thiol. The product of the reaction was treated as described by Kredich and Tomkins (1), yielding 101 mg of material shown to be L-cystine by the following characteristics: (*a*) chromatographic mobility identical with known L-cystine by thin layer chromatography in two solvent systems and by paper chromatography; (*b*) melting point range of 261–264° (same as sample of authentic L-cystine); (*c*) specific optical rotation of −208° (0.1% in 1 N

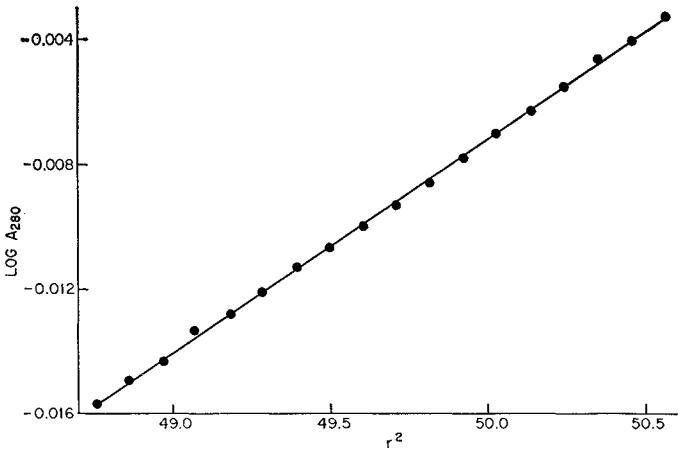

FIG. 6. Plot of log c against r^2 (r = distance from center of rotation) obtained for *O*-acetylserine sulfhydrylase-A in 0.1 M Tris-HCl, pH 7.6, by equilibrium sedimentation at 20,000 rpm for 10.5 hours at 20° with a 2-mm column height and scanning at 280 mμ. With $\bar{v}$ of 0.74, molecular weight was calculated to be 68,000.

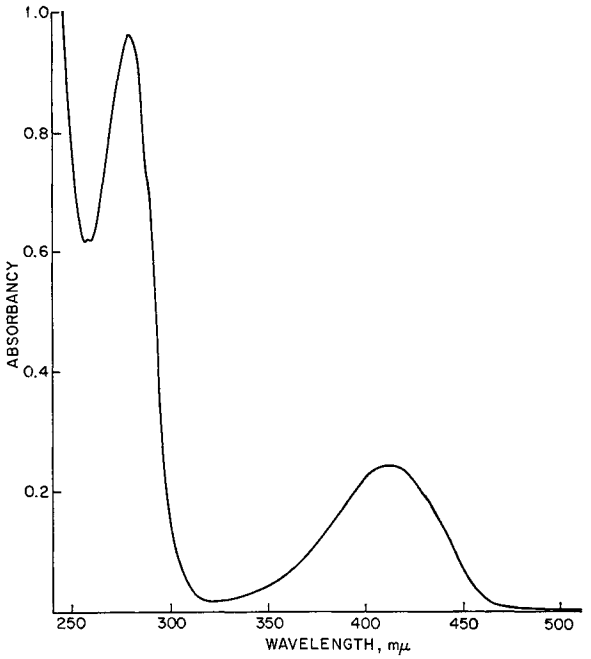

FIG. 7. Absorption spectrum of purified *O*-acetylserine sulfhydrylase-A. The tracing was done in a Cary 15 recording spectrophotometer. Protein concentration was 1.18 mg per ml in 0.1 M Tris-HCl, pH 7.6. Note the absorption peak with a maximum at 412 mμ due to pyridoxal phosphate.

HCl) (authentic L-cystine, −212°); (*d*) elemental analysis: C, 30.17%; H, 5.01%; N, 11.71%; S, 26.86% (analysis of authentic L-cystine: C, 30.28%; H, 5.18%; N, 11.41%; S, 26.87%).

Substrate Specificity—L-Serine, *N*-acetyl-L-serine, *O*-acetyl-L-threonine, *O*-succinyl-DL-serine, *O*-acetyl-DL-homoserine, and *O*-succinyl-L-homoserine gave little or no activity when substituted for *O*-acetyl-L-serine in the standard assay. β-Mercaptopyruvate, L-cysteine, and 2-mercaptoethanol could not replace sulfide in the reaction. This was determined by adding *O*-acetyl-^{14}C-L-serine to the incubation mixture and performing radioautography of thin layer chromatograms. By this technique, methyl mercaptan was found to react with *O*-acetyl-L-serine in the presence of enzyme to give a product which was tentatively identified as *S*-methylcysteine.

Kinetics—The K_m for *O*-acetyl-L-serine was 5×10^{-3} M at 25° (Fig. 10) and was independent both of sulfide concentration (between 5×10^{-4} M and 4×10^{-3} M) and of the order in which the reactants were added to start the reaction. At 1° the K_m for *O*-acetyl-L-serine was also 5×10^{-3} M. The K_m for sulfide at 25° was less than 1×10^{-4} M. L-Cysteine was not inhibitory in concentrations up to 3.7 mM.

Spectral Changes with O-Acetyl-L-serine—The absorption spectrum of the purified enzyme varied slightly in the pH range between 5.8 and 9.4 (Fig. 11). At pH values higher or lower than 7.6, an increase in absorbance between 310 and 380 mμ was noted, most prominent at 320 to 355 mμ, and the 412 mμ maximum shifted slightly to 405 mμ. Absorbance at 412 mμ remained unchanged, and there was no significant increase in absorbance at 470 mμ.

When *O*-acetyl-L-serine was added to *O*-acetylserine sulfhydrylase-A a marked change in the spectrum was noted (Fig. 12). The chief characteristics of this new spectrum were the appearance of an absorption peak with a maximum at 470 mμ and a diminution of the absorption peak at 412 mμ. In addition, a broad shoulder of increased absorbance between 360 and 300 mμ was noted. All of these effects were dependent on the concentration of added *O*-acetyl-L-serine. These spectral changes are probably due to the reaction of *O*-acetyl-L-serine with enzyme-bound pyridoxal phosphate. Neither L-serine nor *N*-acetyl-L-serine, at 10^{-4} M concentrations, affected the spectrum of the

TABLE II

Amino acid composition of O-acetylserine sulfhydrylase-A

Each value represents the result of a single analysis performed on samples hydrolyzed for the length of time indicated in the column heading.

Amino acid	Composition			Average or extrapolated integer
	24 hrs	48 hrs	72 hrs	
	residues/34,000 mol wt subunit			
Lysine	25.4	25.5	25.7	26
Histidine	2.7	3.1	3.1	3
Arginine	13.0	13.5	13.8	14
Aspartic acid	26.0	25.6	25.0	26[a]
Threonine	23.3	22.4	22.2	24[a]
Serine	16.8	16.1	16.2	17[a]
Glutamic acid	36.4	39.4	36.6	38
Proline	16.0	15.6	15.7	16
Glycine	33.1	32.8	32.0	33[a]
Alanine	31.1	30.6	30.6	31
Valine	18.1	19.7	19.9	20
Methionine	5.0	4.9	4.8	5[a]
Isoleucine	23.1	24.6	24.5	24
Leucine	31.3	32.0	31.4	32
Tyrosine	6.4	6.4	6.4	6
Phenylalanine	7.2	7.2	7.2	7
Cysteine	0.7[b]			
Tryptophan	5.0[c]			

[a] Extrapolated.

[b] Determined as cysteic acid after performic acid oxidation (18).

[c] Determined spectrophotometrically (19).

enzyme, whereas the effect of *O*-acetyl-L-serine was maximal at that concentration.

In order to calculate an equilibrium constant, K = (*O*-acetyl-L-serine) (enzyme)/(*O*-acetyl-L-serine-enzyme), the following assumptions were made: (*a*) the ΔA_{470} is linearly proportional to the amount of bound *O*-acetyl-L-serine; (*b*) at the maximum ΔA_{470} in the presence of a large excess of *O*-acetyl-L-serine all of the binding sites on the enzyme are saturated; (*c*) at low *O*-acetyl-L-serine concentrations, virtually all of the added substrate is bound to the enzyme. An extrapolation of the initial slope of the titration curve (Fig. 13) intercepts the maximum ΔA_{470} at an *O*-acetyl-L-serine concentration of 5.4×10^{-6} M in an experiment in which the enzyme concentration was 0.20 mg per ml. Thus, 1 mole of *O*-acetyl-L-serine binds to 37,000 g of protein, which corresponds with the pyridoxal phosphate content of the enzyme. With these data, we were then able to calculate the amounts of free and bound *O*-acetyl-L-serine at each point on the titration curve. A plot of $1/\Delta A_{470}$ against 1/free *O*-acetyl-L-serine concentration gives a straight line as seen in Fig. 13. The value of the equilibrium constant, K, obtained from the negative intercept on the abscissa is 6×10^{-7} M.

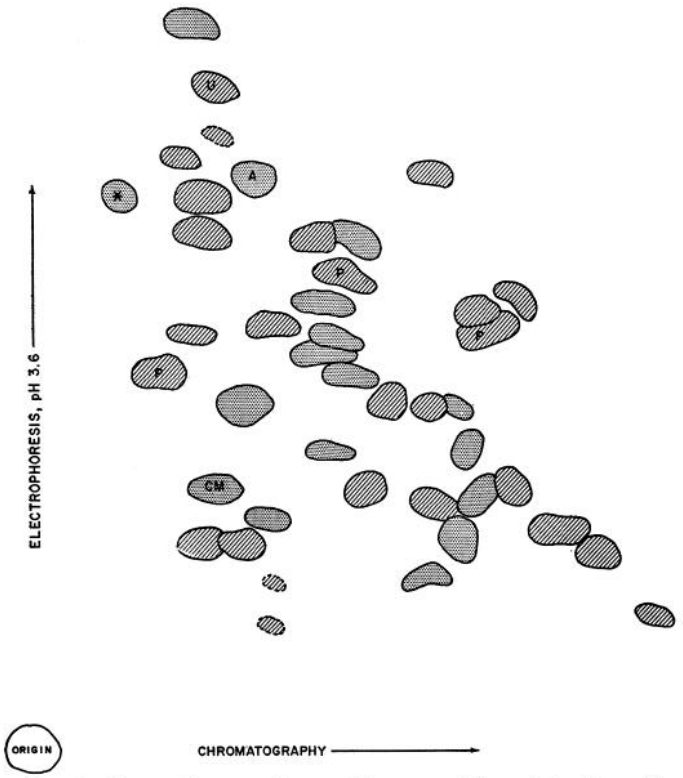

FIG. 8. Composite tryptic peptide map of *O*-acetylserine sulfhydrylase-A. This schematic representation is based on the following individual procedures: (*a*) separation of tryptic peptides of native enzyme followed by staining with Sakaguchi reagent, cadmium-ninhydrin reagent, or Pauly reagent; (*b*) separation of tryptic peptides of enzyme previously treated with urea and dithiothreitol and stained with Sakaguchi reagent followed by the cadmium-ninhydrin reagent; (*c*) separation of tryptic peptides of enzyme previously treated with urea and dithiothreitol and carboxymethylated with ^{14}C-iodoacetic acid. The unstained map was radioautographed for 48 hours and then stained with Sakaguchi reagent followed by the cadmium-ninhydrin reagent. *P*, peptides staining with Pauly reagent; *CM*, radioactively labeled peptide appearing in map of carboxymethylated enzyme; *U*, new unlabeled peptide appearing in map of carboxymethylated enzyme; *, peptide absent only in map of carboxymethylated enzyme; *A*, peptide staining with Sakaguchi reagent but not with cadmium-ninhydrin reagent; *dotted areas*, peptides staining with Sakaguchi reagent and cadmium-ninhydrin reagent; *cross-hatched areas*, peptides staining with cadmium-ninhydrin reagent but not with Sakaguchi reagent; *areas encircled by broken lines*, poorly staining peptides.

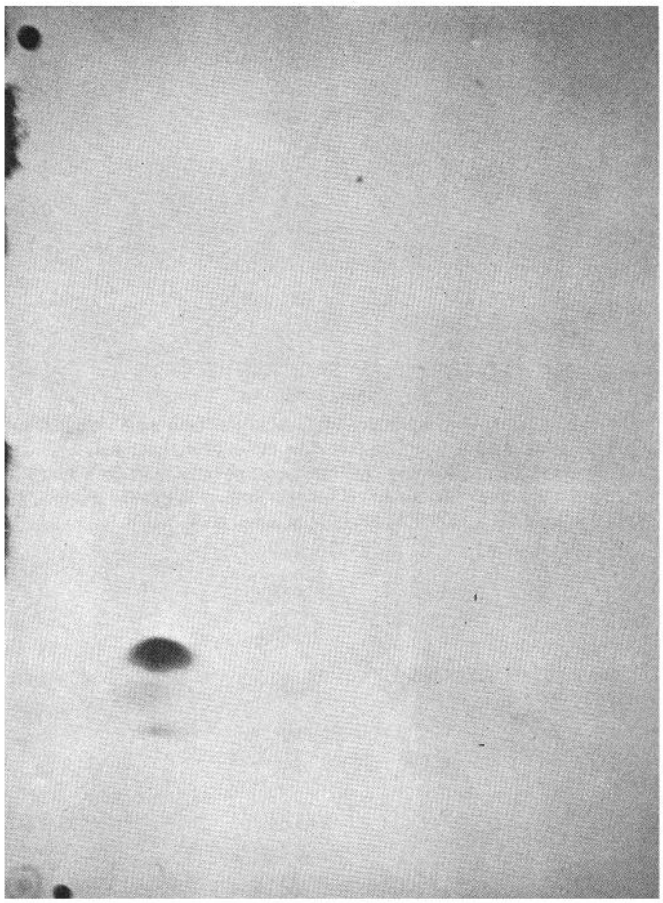

FIG. 9. Radioautograph of tryptic peptide map of ^{14}C-iodoacetic acid-treated *O*-acetylserine sulfhydrylase-A. Note the single predominant radioactive spot following 48 hours of exposure. This spot superimposes on the peptide marked *CM* in Fig. 8 which appeared following treatment of the enzyme with iodoacetic acid.

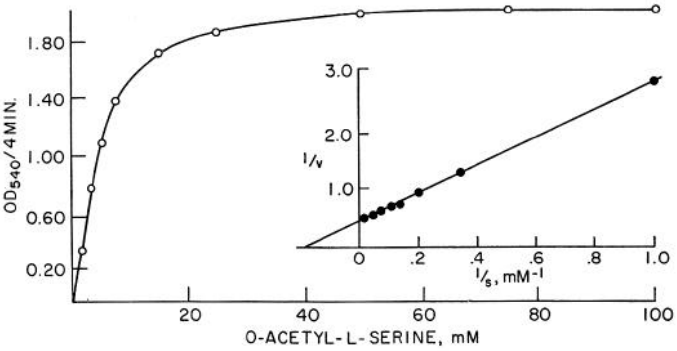

FIG. 10. Substrate concentration velocity curve at 25° for *O*-acetyl-L-serine with the use of purified *O*-acetylserine sulfhydrylase-A. The assay was performed as described under "Experimental Procedure" with varying concentrations of *O*-acetyl-L-serine. The K_m for *O*-acetyl-L-serine is 5×10^{-3} M at 25°.

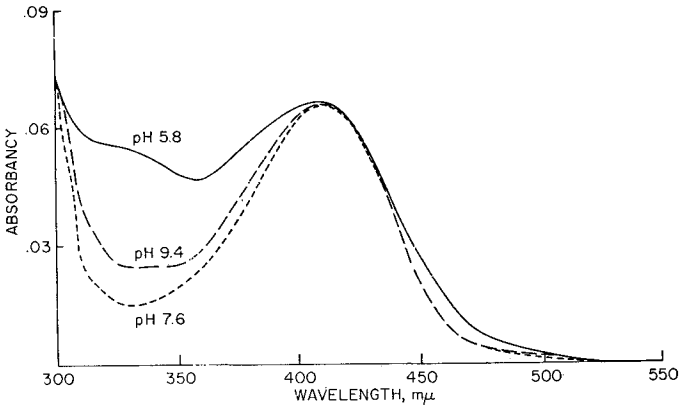

FIG. 11. Absorption spectra of *O*-acetylserine sulfhydrylase-A at pH 5.8, 7.6, and 9.4. The enzyme, at 0.24 mg per ml, was dialyzed against the following buffers and spectra read in a Cary 15 recording spectrophotometer: 0.5 M sodium phosphate buffer, pH 5.8; 0.1 M Tris-HCl, pH 7.6; and 0.1 M Tris-HCl, pH 9.4.

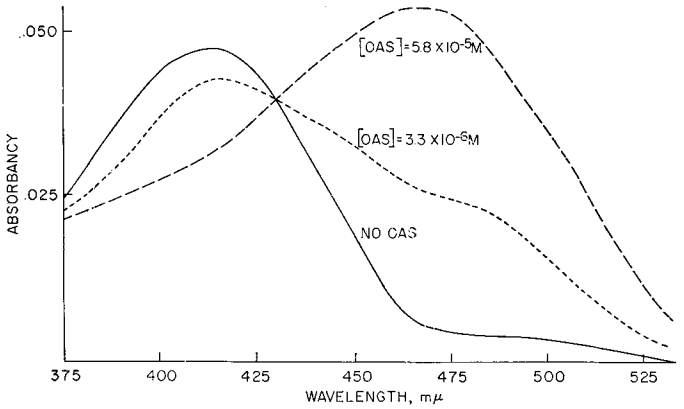

FIG. 12. Spectroscopic titration of purified *O*-acetylserine sulfhydrylase-A with *O*-acetyl-L-serine. The spectra were recorded in a Cary 15 recording spectrophotometer. Protein concentration was 0.23 mg per ml in 0.1 M Tris-HCl, pH 7.6. *O*-Acetyl-L-serine additions were made in 1- to 2-μl increments in a total volume of 1.0 ml at 25°. Only three spectra are shown here, but nine spectra were recorded at increasing concentrations of *O*-acetyl-L-serine (*OAS*).

A spectral analysis of *O*-acetyl-L-serine binding was carried out in the presence of sulfide, in order to duplicate the conditions used in the enzymic assay. The rapidity with which these substrates react to form cysteine in the presence of the high enzyme concentrations required for spectroscopic observation made it necessary to carry out the studies at 1° in optical cells of 10-cm light path. At this temperature, the K_m for *O*-acetyl-L-serine was 5 mM, and the K for the spectrally measured enzyme-substrate reaction was 5×10^{-7} M, about as at 25°. In the presence of 1 mM sulfide, however, the spectral shift from 412 mμ to 470 mμ did not occur at *O*-acetyl-L-serine concentrations as high as 0.1 M. Only after the sulfide had completely disappeared by reacting to form cysteine were the spectral changes characteristic of *O*-acetyl-L-serine binding observed. This result was independent of the order of addition of the reactants.

L-Cysteine, at concentrations of up to 0.01 M, had no effect on the spectrum of *O*-acetylserine sulfhydrylase-A. At concentrations of 0.1 mM, both L-cysteine and D-cysteine did, however, increase the apparent K for the *O*-acetyl-L-serine-induced spectral

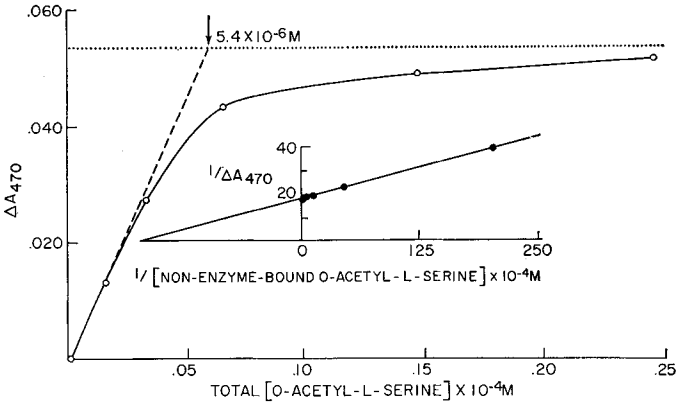

FIG. 13. Increment in absorption at 470 mμ of *O*-acetylserine sulfhydrylase-A as a function of increasing total *O*-acetyl-L-serine concentration. The data were obtained by a spectral titration as described in the preceding figure. The extrapolated initial slope of the curve intercepts the maximum ΔA_{470} at an *O*-acetyl-L-serine concentration of 5.4×10^{-6} M. Assuming a subunit molecular weight of 34,000, the molar concentration of subunits (0.20 mg per ml) was calculated to be 5.9×10^{-6} M. Thus 0.96 mole of *O*-acetyl-L-serine was bound per mole of subunit. In the *inset*, $1/\Delta A_{470}$ is plotted as a function of 1/[free *O*-acetyl-L-serine]. As described in the text, the equilibrium constant, K = (*O*-acetyl-L-serine)(enzyme)/(*O*-acetyl-L-serine-enzyme), is equal to 6×10^{-7} M.

shift about 4-fold. We have observed that *O*-acetyl-L-serine and L-cysteine react non-enzymatically to form *N*-acetyl-L-cysteine (probably with the formation of *S*-acetyl-L-cysteine as an intermediate). Although careful kinetic studies on this reaction have not been carried out, we have noted that the inhibitory effect of cysteine seems to increase with the time of incubation with *O*-acetyl-L-serine and enzyme. It is quite likely, therefore, that cysteine inhibits the spectral shift by reacting with *O*-acetyl-L-serine to form serine and *N*-acetylcysteine.

The spectral changes due to *O*-acetyl-L-serine binding were spontaneously reversible over a period of 10 to 20 hours, probably because of the instability of *O*-acetyl-L-serine at pH values above neutrality (1).

DISCUSSION

The data presented indicate that *O*-acetylserine sulfhydrylase-A from *S. typhimurium* is a pyridoxal phosphate-containing enzyme of molecular weight 68,000. The following evidence indicates that the protein is composed of two identical subunits of molecular weight 34,000, each containing 1 mole of pyridoxal phosphate: (*a*) equilibrium sedimentation of the carboxymethylated protein and of untreated protein in 6 M guanidine hydrochloride gave molecular weights of 34,000 and 32,000, respectively; (*b*) analytical disc gel electrophoresis of pyridoxal phosphate-free protein in urea gave a single component; (*c*) the pyridoxal phosphate content is 1 mole/31,700 g; (*d*) the amino acid analysis indicates that a 34,000 molecular weight subunit would result in a total of 41 tryptic peptides of which 14 would contain arginine (this is in good agreement with the tryptic peptide map which shows 41 total peptides, 16 of which contain arginine); (*e*) the amino acid analysis gave 0.70 mole of cysteine per 34,000, indicating that this is a minimum molecular weight, and a tryptic digest of carboxymethylated protein yielded only one major carboxymethylated peptide; (*f*) there is 1 mole of NH_2-terminal serine per 33,800 to 36,700 g of protein.

The kinetically determined K_m for *O*-acetyl-L-serine is 5×10^{-3} M, whereas the equilibrium constant for the substrate binding reaction, determined spectroscopically, is 6×10^{-7} M. The large disparity between these values could signify that the kinetic method does not give a valid indication of substrate binding affinity, or that the different conditions under which the two measurements were made influenced the results. In order to investigate the latter possibility, spectral analysis of the *O*-acetyl-L-serine binding reaction in the presence of sulfide was carried out. Although the results of this experiment did not indicate the cause of the above mentioned disparity, the data did reveal one interesting fact, *i.e.* that in the presence of sulfide the *O*-acetyl-L-serine-induced spectral change does not occur. Until the mechanisms of the reaction and of the spectral shift have been worked out, the interpretation of this data must remain open to speculation. It seems reasonable to assume, however, that *O*-acetyl-L-serine reacts with enzyme-bound pyridoxal phosphate to form a Schiff's base which is dehydrated to form pyridoxal phosphate-bound dehydroalanine. If this latter pyridoxal phosphate-bound compound were the source of the 470 mμ chromophore, then sulfide might prevent its accumulation either by blocking its formation or by rapidly reacting with it to form L-cysteine.

The relationship between *O*-acetylserine sulfhydrylase-A and *O*-acetylserine sulfhydrylase-STA is discussed in the following paper (3).

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The Purification and Characterization of *O*-Acetylserine Sulfhydrylase-A from *Salmonella typhimurium*

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