BBA 66441

PURIFICATION AND PROPERTIES OF RAT LIVER CYSTEINE SULFINATE DECARBOXYLASE

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(Received June 28th, 1971)

SUMMARY

Rat liver L-cysteine sulfinate (2-amino-3-sulfinopropionate) decarboxylase (L-cysteine sulfinate carboxy-lyase, EC 4.1.1.29) was purified 300-fold. The purification was achieved by the use of $(NH_4)_2SO_4$ fractionation between 35–45% satn., gel filtration on Sephadex G-100, and DEAE-cellulose column chromatography. This is the most active and stable cysteine sulfinate decarboxylase reported to date. Observations made by polyacrylamide-gel electrophoresis, molecular sieving on Sephadex G-100, and analytical ultracentrifugation appear to show that the purified enzyme was approaching homogeneity. The sedimentation coefficient and the molecular weight were estimated to be approx. 4.76 S and 66 000, respectively. The cysteine sulfinate decarboxylase activity was measured in Tris–maleate, imidazole, and phosphate buffers at pH 6.8, and the maximal activity was observed in the phosphate buffer. The purified cysteine sulfinate decarboxylase has an apparent K_m of 0.2 mM and does not catalyze the decarboxylation of cysteate (2-amino-3-sulfonopropionate). Cupric and mercuric ions are the only metal ions tested which showed some inhibition of the enzyme activity. Dithiothreitol and 2-mercaptoethanol were found as effective as glutathione as activators for cysteine sulfinate decarboxylase.

INTRODUCTION

CHATAGNER *et al.*[1] in 1954 first reported the presence of L-cysteine sulfinate (2-amino-3-sulfinopropionate) decarboxylase (L-cysteine sulfinate carboxy-lyase, EC 4.1.1.29) in rat liver. The cysteine sulfinate decarboxylase is a soluble enzyme[2–6] with a pH optimum between 6.8 to 8.0 (refs. 2, 3 and 6) requiring pyridoxal phosphate[2,3,6–8] and free thiol groups[3,6] for its maximal activity. It has been shown that various animal tissues[9] contain cysteine sulfinate decarboxylase activity, the highest

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enzyme activity being found in dog and rat livers. All preparations of rat liver cysteine sulfinate decarboxylase which were available prior to this report[3,6,7,10–12] also catalyze the decarboxylation of cysteate (2-amino-3-sulfonopropionate). Furthermore, although various investigators have obtained partially purified rat liver cysteine sulfinate decarboxylase by fractionation of the particle free supernatant fraction between 0–50% (ref. 3), 25–50% (ref. 6), and 30–50% (ref. 8) saturation with $(NH_4)_2SO_4$ none has attempted to further purify and characterize this enzyme. Therefore, the problem of purification and characterization of rat liver cysteine sulfinate decarboxylase was undertaken. This paper reports a method for the preparation of cysteine sulfinate decarboxylase from rat liver to a high state of purity and some properties of the purified enzyme.

MATERIALS AND METHODS

Materials

Animals and tissues

Male Wistar rats (200–300 g) were obtained from the Huntingdon Farms, West Conshohocken, Pa. The liver of male Wistar rats was also purchased from Rockland, Gilbertsville, Pa.

Chemicals and reagents

Thioglycolic acid was obtained from Evans Chemetics, New York. Imidazole was purchased from K and K Laboratories, Jamaica, N.Y. L-Cysteinesulfinic acid, L-cysteic acid, glutathione, pyridoxal phosphate, 2-mercaptoethanol, dithiothreitol (Cleland's reagent), Tris (Sigma 121), bovine serum albumin (crystalline), ovalbumin, α-cyhmotrypsinogen A, cytochrome *c*, *p*-hydroxymercuribenzoate, iodoacetic acid, iodoacetamide and *N*-ethylmaleimide, were purchased from the Sigma Chemical Co., St. Louis, Mo. Sephadex G-100 and Blue Dextran 2000 were purchased from Pharmacia Fine Chemicals, Piscataway, N.J. DEAE-cellulose, DE-52, was a product of W. and R. Balston, Ltd. England. Cyanogum-41 was supplied by E. C. Apparatus Corp., Philadelphia, Pa. Mann Research Laboratories, New York, supplied 2-mercaptoethanolamine hydrochloride (cysteamine·HCl), and $(NH_4)_2SO_4$ granular, enzyme grade. All other chemicals were of analytical reagent grade.

Methods

Preparation of crude cysteine sulfinate decarboxylase

Unless otherwise noted, all operations were performed at 4° in the cold room. The method used to prepare the crude cysteine sulfinate decarboxylase (supernatant fraction) was essentially the same as that described by SÖRBO AND HEYMAN[12] for the preparation of dog liver cysteine sulfinate decarboxylase with the exception that the rat liver was homogenized in 0.25 M sucrose. The fraction which was precipitated at 35–45% $(NH_4)_2SO_4$ was dissolved in 0.02 M phosphate buffer (pH 6.8) containing 1 mM 2-mercaptoethanol (to be further referred to as the buffer solution) to give a solution containing 40 mg protein/ml.

Column chromatography

Gel filtration. A column (4 cm × 90 cm) was prepared with Sephadex G-100, and equilibrated with the buffer solution. Filtration of the 35–45% ammonium

sulphate fraction through the gel bed was carried out with the same buffer solution. The effluent was collected in 10-ml fractions with a flow rate of 30 ml/h. Ultraviolet absorption of the fraction was determined manually in a Hitachi Model 139 spectrophotometer at 280 nm. The active fractions were pooled and concentrated by the use of Amicon Diaflow system with ultrafiltration cell Model 202 and UM-10 filter.

DEAE-cellulose. A column (0.9 cm × 20 cm) of DEAE-cellulose, DE-52, was prepared and the buffer solution was run through until equilibrium was reached as indicated by conductivity.

The clear enzyme solution from the gel filtration step was introduced into the column which was washed with the buffer solution until an inert peak had emerged. The column was then eluted with a linear gradient of 100 ml of the buffer solution and 100 ml of 1 M NaCl. The flow rate was held constant by means of a Buchler peristaltic pump. The effluent was collected in 1.5-ml fractions with a flow rate of about 40 ml/h. The elution pattern was monitored in the same way as that described for gel filtration. To the pooled fractions containing the decarboxylase activity, solid $(NH_4)_2SO_4$ was added to 70% saturation. This was kept at 4° until used.

Analytical procedures

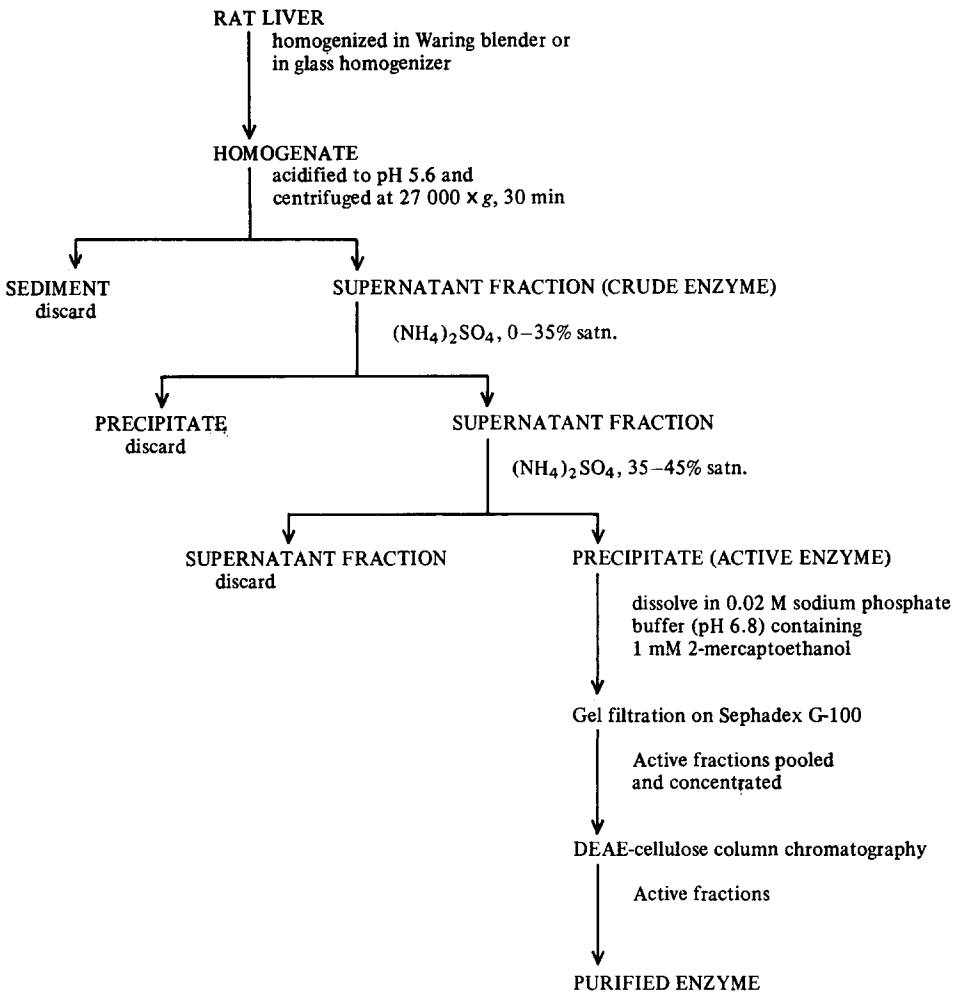
Protein estimation. Protein was determined by the method of LOWRY *et al.*[13] with crystalline bovine serum albumin as the standard.

Enzyme assay. The decarboxylase activity was determined manometrically in a standard Warburg apparatus. The main compartment of each Warburg flask contained 0.2–1.0 ml of a suitably diluted enzyme solution, 200 μmoles of sodium phosphate buffer (pH 6.8), 0.6 μmoles of pyridoxal phosphate, 6.0 μmoles of glutathione or 2-mercaptoethanol, and water to 2.7 ml. The side arm contained 0.1 ml of 0.3 M sodium cysteine sulfinate, and the center well contained 0.2 ml of 2.5 M H_2SO_4. The incubation was carried out at 37.5° with air as the gas phase. The reaction was started by tipping in the substrate from the side arm after temperature equilibration of 10 min. At the end of the incubation period the acid was tipped in to liberate the absorbed CO_2. The total CO_2 production was calculated from the manometer readings from which the reading of a blank flask lacking the enzyme preparation was subtracted. 1 unit of enzyme activity is defined as that amount which will liberate 1.0 μmole of CO_2 per min under the standard assay conditions. Specific activity is expressed as munits per mg protein.

Discontinuous slab polyacrylamide-gel electrophoresis. Analytical polyacrylamide-gel electrophoresis of enzyme preparations at various stages of the purification was performed with the E-C vertical gel electrophoresis cell (E-C Apparatus Corp., Philadelphia, Pa.) according to the procedure of SULD AND HERBUT[14].

Molecular weight determination by gel filtration on Sephadex G-100. The molecular weight was estimated according to the procedure of WHITAKER[15]. The protein elution profile was monitored by a Uvicord II ultraviolet absorption monitor at 280 nm attached to an LKB recording system.

Ultracentrifugation. Sedimentation velocity was performed in a Spinco Model E analytical ultracentrifuge at 56 000 rev./min at 20°, using a single sector cell with 12-mm aluminium center piece and schlieren optics. The pictures were taken at 16 min intervals with a phase plate angle of 60°. The movement of the schlieren peaks were measured by a Nikon Shadowgraph Model 6 C microcomparator. The sedimentation coefficient was calculated by using a least squares procedure.



Scheme 1. Preparation of cysteine sulfinate decarboxylase.

RESULTS AND DISCUSSION

Purification of rat liver cysteine sulfinate decarboxylase

The procedure shown in Scheme 1 for the preparation of cysteine sulfinate decarboxylase provides a relatively simple and rapid means for purifying this enzyme. A 33% rat liver homogenate was made in a Waring Blender. After the homogenate was acidified to pH 5.6, the supernatant fraction was obtained by centrifugation in a Sorvall SS-34 rotor for 30 min at 8000 rev./min. A semipurified cysteine sulfinate decarboxylase preparation was obtained from this supernatant fraction by precipitation between 35–45% saturation with $(NH_4)_2SO_4$, based on the results obtained from preliminary $(NH_4)_2SO_4$ fractionation experiments. When the supernatant fraction was made stepwise to 25, 35, 45, and 55% saturation with $(NH_4)_2SO_4$, 85% of the total activity was recovered in the 35–45% $(NH_4)_2SO_4$ fraction with a 3-fold

increase in specific activity. This is a much narrower $(NH_4)_2SO_4$ fraction containing cysteine sulfinate decarboxylase activity than those that were previously reported[3,6,8].

Gel filtration on Sephadex G-100

A portion of the 35–45% $(NH_4)_2SO_4$ fraction was applied to a Sephadex G-100 column and elution was carried out as described in *Methods*. A typical profile is presented in Fig. 1. It is seen that the protein was eluted as one broad peak with some trailing. The activity was invariably found in three peaks (designated I, II, and III) in the descending edge of the protein profile. Peak II, eluted at 518–598 ml as indicated by the arrows, contained about 70% of the total decarboxylase activity applied on the column. The portion of the effluent in the major activity peak, which emerged at 500–620 ml of effluent and contained 260–300 mg of protein, was pooled and concentrated for the next and final step of purification.

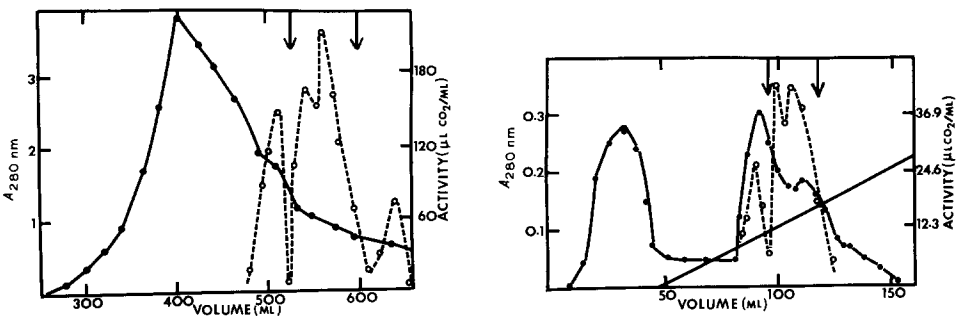


Fig. 1. Chromatography of rat liver cysteinesulfinate decarboxylase on Sephadex G-100. Thirty ml of the 35–45% $(NH_4)_2SO_4$ fraction containing 1200 mg of protein were applied to the column (4 cm × 90 cm). Elution was performed with 0.02 M phosphate buffer (pH 6.8) containing 1 mM 2-mercaptoethanol; 10-ml fractions were collected. ●—●, absorbance at 280 nm; ○---○, enzyme activity. The arrows denote the Sephadex G-100-purified enzyme.

Fig. 2. Chromatography of rat liver cysteine sulfinate decarboxylase on DEAE-cellulose. 20 ml of Sephadex G-100-purified enzyme preparation containing 70 mg of protein were applied to the column (0.9 cm × 20 cm). Elution was performed with a linear gradient of 0.02 M phosphate buffer (pH 6.8), containing 1 mM 2-mercaptoethanol to 1 M NaCl. Fractions of 1.5 ml were collected. ●—●, absorbance at 280 nm; ○---○, enzyme activity; ———, gradient. The arrows denote the DEAE-cellulose-purified enzyme.

DEAE-cellulose chromatography

An aliquot of the Sephadex G-100 purified enzyme solution was chromatographed on a DEAE-cellulose column according to the procedure described in *Methods*. Representative data of the elution profile and the enzyme activity are shown in Fig. 2. The enzyme activity was eluted from the column in three peaks at 25–60 ml of the gradient solution. All the fractions containing cysteine sulfinate decarboxylase activity were pooled and brought to 70% $(NH_4)_2SO_4$ saturation until used for further studies.

Stability

The cysteine sulfinate decarboxylase (supernatant fraction from acid precipitated homogenate and 35–45% $(NH_4)_2SO_4$ fraction) was stable for at least 3 months

with little loss of activity when stored at −20°. This was in agreement with the findings of JACOBSEN *et al.*[6].

The Sephadex G-100 purified enzyme as eluted from the column was less stable. All activity was lost after 1 month of storage at −20°. However, the enzyme was stable for at least 1 month if stored at 4° in $(NH_4)_2SO_4$ at 70% saturation.

The DEAE-cellulose-purified enzyme as eluted from the column was stable for only 4 days under refrigeration. The loss of activity was rapid so that the enzyme retained only 28% of the initial activity after 10 days of storage. This loss of activity was prevented if the DEAE-cellulose-purified enzyme was stored at 4° in $(NH_4)_2SO_4$ at 70% saturation. The desalting of the enzyme preparation through a small column

TABLE I

PURIFICATION OF RAT LIVER CYSTEINE SULFINATE DECARBOXYLASE

The enzyme preparation from three rat livers was carried out according to the procedure presented in the text and *Methods*.

Step	*Total protein (mg)*	*Specific activity (munits/mg protein)*	*Total activity (units)*	*Recovery (%)*	*Purification*
Whole homogenate	24 600	2.3	56.6	100	4.6
Supernatant Fraction (from acid-precipitated homogenate)	3 220	10.4	33.7	60	4.6
$(NH_4)_2SO_4$ fraction (35–45% satn.)	510	47.5	24.2	43.2	20.9
Sephadex G-100	116	191.5	22.2	39.6	84.0
DEAE-cellulose	25.6	678.4	17.4	31.0	297.6

of Sephadex G-25 resulted in no loss of activity. Recovery of enzyme activity from such a column was usually 85–95%. The various steps of a typical purification, with the enzyme activity and yields are summarized in Table I. The overall purification is 298-fold with 30% recovery of the total activity found in the whole homogenate. The active enzyme protein isolated was 0.1% of the total liver protein.

Some properties of purified cysteine sulfinate decarboxylase

Catalytic properties

Enzyme activity. Although phosphate has been the buffer of choice for various investigators in their studies on the properties of the crude cysteine sulfinate decarboxylase, the reported optimum pH varied between 6.5 to 7.4 (refs. 2, 4 and 6). In the present study, the maximal activity was obtained at pH 6.8. Using the standard assay conditions as described in the method section, the rate of CO_2 production was linear for at least 30 min.

Effect of substrate concentration. The double reciprocal plot of Lineweaver and Burk revealed that the apparent concentration of the substrate (cysteine sulfinate) required for one-half maximal rate of reaction was approx. 0.20 mM. This K_m value is in essential agreement with the K_m of 0.14 mM for semi-purified cysteine sulfinate decarboxylase reported by JACOBSEN *et al.*[6].

Effect of thiol compounds as activators. To determine whether sulfhydryl com-

TABLE II

EFFECT OF VARIOUS THIOL COMPOUNDS AS ACTIVATORS OF CYSTEINE SULFINATE DECARBOXYLASE ACTIVITY

DEAE-cellulose purified cysteinesulfinate decarboxylase (123.5 μg of protein) was used for each incubation. The assay conditions are described in the text. The concentration of the thiol compounds was 2 mM.

Thiol compound	*Relative activity (%)*
Glutathione	100
2-Mercaptoethanol	110.3
2-Mercaptoethanolamine	19.9
Thioglycolic acid	59.3
Dithiothreitol	106.9

pounds other than glutathione may also activate cysteine sulfinate decarboxylase; 2-mercaptoethanol, 2-mercaptoethanolamine, Cleland's reagent (dithiothreitol), and thioglycolic acid were tested. As seen in Table II, it is evident that 2-mercaptoethanol and dithiothreitol are as effective as glutathione; while, thioglycolic acid and 2-mercaptoethanolamine are much less effective. The former gives about 60% and the latter only 20% of the activity obtained with glutathione. The degree of effectiveness of the five sulfhydryl activators could not have been due to difference in their sizes, but might possibly be related to the difference in their charges at pH 6.8. In all subsequent experiments, 2-mercaptoethanol was used, because of its ease in handling.

Effect of different buffers and divalent metal ions. To find a buffer system other than phosphate for measuring the cysteine sulfinate decarboxylase activity, Tris–maleate and imidazole buffers at pH 6.8 were tested. It was found that the production of CO_2 in Tris–maleate buffer was 88% and in imidazole buffer was 67% of that in phosphate. Therefore, the buffer used in all experiments was phosphate with the exception that Tris–maleate was used in the study on the effect of metal ions.

The effect of a number of divalent cations on cysteine sulfinate decarboxylase activity was studied. The cations were either in sulfate or chloride form. Parallel incubations were made to show that SO_4^{2-} and Cl^- exerted no effect on the enzyme activity. At a final concentration of 1 mM, Cu^{2+} and Hg^{2+} inhibited the enzyme activity 58 and 27%, respectively, while Fe^{2+} and Cd^{2+} increased the enzyme activity 15 and 10%, respectively. Other ions tested Mn^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+}, however, did not have any significant effect.

Effect of thiol group reagents. *p*-Hydroxymercuribenzoate and iodoacetate had been shown to inhibit the activity of semi-purified cysteine sulfinate decarboxylase by DAVIDSON[4]. Therefore, the effect of these inhibitors and two others, iodoacetamide and *N*-ethylmaleimide was examined on the catalytic activity of the DEAE-cellulose purified cysteine sulfinate decarboxylase. To test the inhibition, 0.2 mM 2-mercaptoethanol (1/10 the optimal concentration used routinely) was added to the incubation mixture. Under these conditions, a final concentration of 2 mM iodoacetate, iodoacetamide, and *N*-ethylmaleimide inhibited the enzyme activity about 65%, whereas 2 mM *p*-hydroxymercuribenzoate inhibited the enzyme activity 40%. These results are consistent with earlier observations[3,6] that cysteine sulfinate decarboxylase requires free sulfhydryl groups for maximal activity.

TABLE III

DECARBOXYLASE ACTIVITIES AT DIFFERENT STAGES OF PURIFICATION

The assay conditions used for both substrates are identical and described in the text.

Stage	*Specific activity (munits/mg protein)*		
	Cysteine sulfinate (A)	*Cysteate (B)*	*A/B ratio*
Whole homogenate	2.5	1.04	2.4
Supernatant Fraction from acid-precipitated homogenate	14.0	5.3	2.6
$(NH_4)_2SO_4$ fraction (35–45% satn.)	22.1	9.7	2.3
Sephadex G-100 (pooled)	55.3	5.9	9.4
DEAE-cellulose (pooled)	444.3	0	

Substrate specificity. To clarify the question of substrate specificity, the activity of our enzyme preparation with respect to cysteine sulfinate and cysteate as substrates was compared during the different stages of purification.

It is evident from the data presented in Table III that the purification by Sephadex G-100 column increased the ability of the enzyme to decarboxylate cysteine sulfinate to a much greater extent than that of cysteate. The DEAE-cellulose-purified cysteine sulfinate decarboxylase shows no measurable activity toward cysteate. This observation was taken as a strong indication that the two compounds are decarboxylated by different enzymes. This finding is contrary to the previous reports that rat liver cysteine sulfinate decarboxylase can also catalyze the decarboxylation of cysteate. However, all previous work on rat liver cysteine sulfinate decarboxylase was done with relatively impure preparations which were not purified beyond a rather broad $(NH_4)_2SO_4$ fraction. However, the possibility can not be excluded that the assay condition used in the present study was not optimal for the decarboxylation of cysteate. The presence of two distinct decarboxylases could best be established by the separation of two protein fractions, each acting primarily or only on its specific substrate. However, no attempt to isolate a purified cysteate decarboxylase was made at this time.

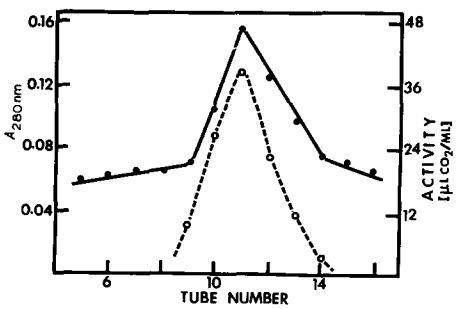


Fig. 3. Determination of the homogeneity of cysteine sulfinate decarboxylase by gel filtration on Sephadex G-100. Two ml of pooled Sephadex G-100-purified cysteine sulfinate decarboxylase (11.44 mg protein) were rechromatographed on a Sephadex G-100 column (1.5 cm × 88 cm). Temperature was 26°. Fractions of 5.6 ml were collected at a flow rate of 10 ml/h and monitored by Uvicord 11 at 280 nm. The absorbance value was calculated from the recording of each tube at mid-point. ●—●, protein elution profile; ○--○, enzyme activity.

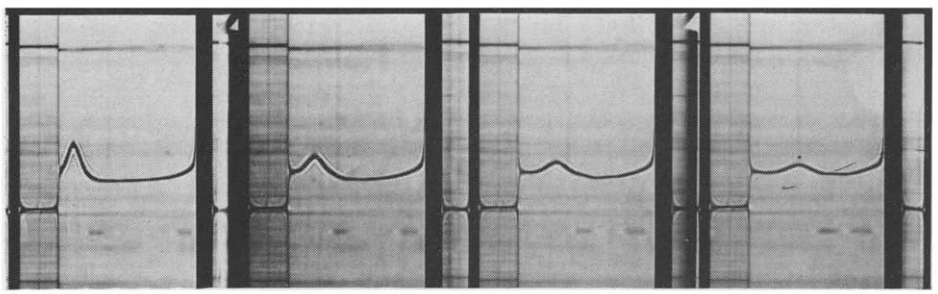


Fig. 4. Schlieren pattern of the DEAE-cellulose-purified rat liver cysteinesulfinate decarboxylase. The solution contained 5 mg of protein/ml in 0.1 M sodium phosphate buffer (pH 7.0). Photographs from left to right, were taken at 16, 32, 48, and 64 min after full speed (56 000 rev./min) had been attained; temperature, 20°; phase angle, 60°.

Physical properties

Polyacrylamide-gel electrophoresis. The electrophoretic patterns on polyacrylamide gels showed that both faster and slower moving protein bands were removed from the enzyme preparation by Sephadex G-100 column chromatography. The DEAE-cellulose purified cysteine sulfinate decarboxylase showed only one protein band.

Gel filtration on Sephadex G-100. The protein elution profile and the activity curve of cysteine sulfinate decarboxylase from gel filtration on Sephadex G-100 column are shown in Fig. 3. The activity peak corresponded well with the almost symmetrical protein peak. Furthermore, the specific activity of the three peak tubes was similar, suggesting a fairly homogeneous distribution of the enzyme in this region of the protein profile.

Sedimentation velocity. The results of a sedimentation velocity run are presented in Fig. 4. The schlieren patterns showed a single peak of 4.76 S. The last picture taken at 64 min revealed the presence of a very small smount of a component with higher sedimentation rate. The sedimentation coefficient of this minor peak (less than 5%

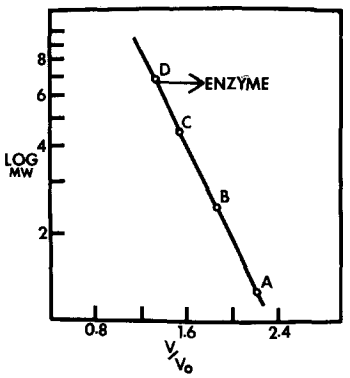


Fig. 5. Molecular weight estimation of cysteinesulfinate decarboxylase by gel filtration on Sephadex G-100. The Sephadex column (1.5 cm × 88 cm) was calibrated with a solution in 0.5 ml of 0.02 M phosphate buffer (pH 6.8), containing 5 mg each of cytochrome *c* (A), *α*-chymotrypsinogen A (B), ovalbumin (C), bovine serum albumin (D), and 1 mg of blue dextran-2000.

of the total area under the curve) could not be determined because it did not appear until 64 min of running time.

Molecular weight estimation. The molecular weight of cysteine sulfinate decarboxylase was estimated by the use of a standardized Sephadex G-100 column according to the method of WHITAKER[15]. A plot of the elution volume/void volume ratio (V/V_0) of the four known proteins against the logarithm of their molecular weight is given in Fig. 5. The molecular weight of the enzyme was 66 000. Since the standards used were globular proteins, this molecular weight is based upon the assumption that cysteine sulfinate decarboxylase is a globular protein. The $s_{20,w}$ value of 4.41 for molecular weight of 63 650 (rat serum albumin), 4.7 for molecular weight of 67 360 (sheep-follicle stimulating hormone), and 4.88 for molecular weight of 73 050 (horse liver alcohol dehydrogenase) have been reported[16]. Therefore, the estimated molecular weight of 66 000 for cysteine sulfinate decarboxylase is reasonable on the basis of its $s_{20,w}$ value of 4.76 S. This is the first time the molecular weight of this enzyme has been determined.

ACKNOWLEDGMENTS

This study was supported in part by Grant AM-00740-14 from the National Institutes of Health, U.S. Public Health Service. The authors are grateful to Dr. Bernard Schepartz for his helpful advice and discussions.

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