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Purification and Characterization of *S*-Adenosyl-L-methionine Decarboxylase from Rat Liver†

M. J. Feldman, C. C. Levy, and D. H. Russell*

ABSTRACT: *S*-Adenosyl-L-methionine decarboxylase has been purified more than 350-fold from rat liver by a new procedure. Certain characteristics of the decarboxylase are described. The enzyme is inhibited by known inhibitors of pyridoxal phosphate dependent enzymes, and at later stages of purification, by sulfhydryl group reagents. No metal cations were required by this enzyme and certain metals were inhibitory. The DEAE-cellulose fraction exhibited maximal activity only when pyridoxal phosphate was added to the assay. The molecular weight of the enzyme determined at various stages of purification in three different rat tissues, *i.e.*, liver, uterus, and ventral prostate, was approximately 50,000. Higher stages of purification failed to uncouple the decarboxylation of *S*-adenosyl-L-methionine from spermidine formation.

The polyamine, spermidine, is a naturally occurring polycation which occurs ubiquitously in animal and plant tissues and in microorganisms. Spermidine concentration and synthesis are increased in a number of systems that are characterized by rapid growth, such as neoplasms (Russell and Snyder, 1968), regenerating liver (Jänne *et al.*, 1964; Dykstra and Herbst, 1965; Russell and Snyder, 1968; Jänne and Raina, 1968; Russell and Lombardini, 1971), and embryonic tissues (Calderara *et al.*, 1965; Russell, 1970, 1971).

In bacteria, spermidine is synthesized by two enzymes: *S*-adenosyl-L-methionine is first decarboxylated by *S*-adenosyl-L-methionine decarboxylase and the decarboxylated *S*-adenosyl-L-methionine then serves as a propylamine donor for putrescine. A propylamine transferase then completes spermidine synthesis by transferring propylamine from decarboxylated *S*-adenosyl-L-methionine to putrescine (Tabor and Tabor, 1964). The bacterial decarboxylase requires magnesium, has a pH optimum of 7.4, and contains covalently bound pyruvate as the prosthetic group (Wickner *et al.*, 1970). The propylamine transferase, on the other hand, has no known cofactor requirements and exhibits a broad pH optimum that centers around 8.3 (Tabor, 1962).

In mammalian systems spermidine formation appears to be

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TABLE I: Purification of *S*-Adenosyl-L-methionine Decarboxylase from Rat Liver.[a]

Fraction	Vol (ml)	Protein[a] (mg)	Sp Act. (Units/mg of Protein)	Purification	Recov (%)
I. Ultracentrifuged supernatant	9	270	0.05		
II. Sephadex gel	32	96	0.21	4.2	130
III. DEAE-cellulose	22	2.5	6.19	123.0	100
IV. Calcium hydroxylapatite	8	0.22	18.6	372.0	60

[a] Protein was determined by the method of Lowry *et al.* (1951).

catalyzed by a somewhat different system than that found in bacteria. Working with preparations obtained from the ventral prostate and liver of the rat, Pegg and Williams-Ashman (1968) described a cell-free system responsible for both the decarboxylation of *S*-adenosyl-L-methionine and for the formation of spermidine. They showed that the prostatic enzyme was more active than the liver one but that magnesium was not required for enzyme activity in either preparation. Moreover, these authors found that the decarboxylase activity could be inhibited by compounds affecting pyridoxal phosphate function and that putrescine stimulated decarboxylase activity specifically, with the stoichiometric formation of CO_2 and spermidine. Later, although a partial purification of the prostatic enzyme was achieved, the propylamine transferase enzyme could not be separated from decarboxylase activity (Pegg and Williams-Ashman, 1969). Recently, however, a considerably higher degree of purification of the prostatic system has been reported (Jänne and Williams-Ashman, 1971) and that, as a result, the enzyme responsible for propylamine transfer has been isolated (Jänne *et al.*, 1971a,b).

In contrast to the prostatic enzyme, the *S*-adenosyl-L-methionine decarboxylase in liver has not been studied as extensively. Russell and Lombardini (1971) using a crude preparation (100,000*g* supernatant preparation) obtained from the livers of 2-month-old rats, found that the decarboxylase activity after partial hepatectomy increased some threefold within 48 hr. They showed, further, that in weanling rats there was a twofold increase in *S*-adenosyl-L-methionine decarboxylase activity within 24 hr after the injection of growth hormone. In these studies the relationship between decarboxylation and spermidine formation was found to be stoichiometric. Similar increases in decarboxylase activity and spermidine formation following partial hepatectomy were observed by Raina *et al.* (1970), using liver preparations from older animals. These authors could find no inhibition of decarboxylase activity with compounds known to affect pyridoxal phosphate.

The absence of more detailed information on the liver system may be attributed to the rather crude preparations of the liver enzyme available to date. To correct this and to compare the liver and prostatic systems we report a partial purification of *S*-adenosyl-L-methionine decarboxylase from normal rat liver and describe some of the characteristics of this enzyme.

Materials

[1,4-^{14}C]Putrescine dihydrochloride (17.7 μCi/μmole) was obtained from New England Nuclear Corp. [*carboxyl*-^{14}C]-*S*-Adenosyl-L-[*carboxyl*-^{14}C]methionine (47 μCi/μmole) was purchased from Amersham-Searle. Dithiothreitol, pyridoxal phosphate, putrescine dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride, *S*-adenosyl-L-methionine, and EDTA were purchased from either Sigma or Calbiochem. DEAE-cellulose (Whatman DE52) was prepared by the method of Peterson and Sober (1962). Calcium hydroxylapatite (Bio-Gel HT) was obtained from Bio-Rad Laboratories and was prepared by washing four times with 0.01 M phosphate buffer (pH 7.2), containing 1 mM EDTA, 0.1 mM dithiothreitol, and 3 μM pyridoxal phosphate. Smith and Nephew Research, Ltd., supplied 4-bromo-3-hydroxybenzoyloxyamine dihydrogen phosphate.

Methods

Enzyme Assay. The standard reaction mixture contained 10 μmoles of sodium phosphate buffer (pH 7.2), 0.5 μmole of putrescine, 0.085 μmole of *S*-adenosyl-L-[*carboxyl*-^{14}C]-methionine, 3.9 μmoles of pyridoxal phosphate, 0.1 mg of albumin, and 0.05–0.1 ml of enzyme preparation in a total volume of 0.25 ml. Enzyme activity was assayed by the liberation of [^{14}C]CO_2 from *S*-adenosyl-L-[*carboxyl*-^{14}C]-methionine as described by Pegg and Williams-Ashman (1969). The concentration of *S*-adenosyl-L-methionine used in these assays was nonsaturating. However, in those experiments in which a saturating level was used, the values obtained were proportionate to those reported here. The amount of spermidine synthesized was estimated as described by Feldman *et al.* (1971) after substitution of [1,4-^{14}C]putrescine for putrescine and *S*-adenosyl-L-methionine for *S*-adenosyl-L-[*carboxyl*-^{14}C]-methionine in the standard assay mixture.

The procedure of Beers and Sizer (1952) was used to measure catalase activity, whereas the spectrophotometric assay for alcohol dehydrogenase was that of Vallee and Hoch (1955).

Molecular Weight. The molecular weight of the decarboxylase was estimated both by sucrose density centrifugation (Martin and Ames, 1961) and by Sephadex gel filtration (Andrews, 1964). The commercial source and the molecular weight of the individual proteins used as standards in these techniques are as follows: bovine serum albumin, 67,000 (Phelps and Putnam, 1960); ovalbumin, 45,000 (Warner, 1954); chymotrypsinogen A (bovine pancrease), 25,000 (Denuelle, 1960); ribonuclease A (bovine pancreas), 13,700 (Hirs *et al.*, 1956); yeast alcohol dihydrogenase, 150,000 (Hayes and Velick, 1954); and catalase (bovine liver), 250,000 (Sumner and Graten, 1938) were from Sigma.

Purification of the Enzyme. The operations described below were carried out at 0–5°. The purification at each step of a typical preparation is summarized in Table I.

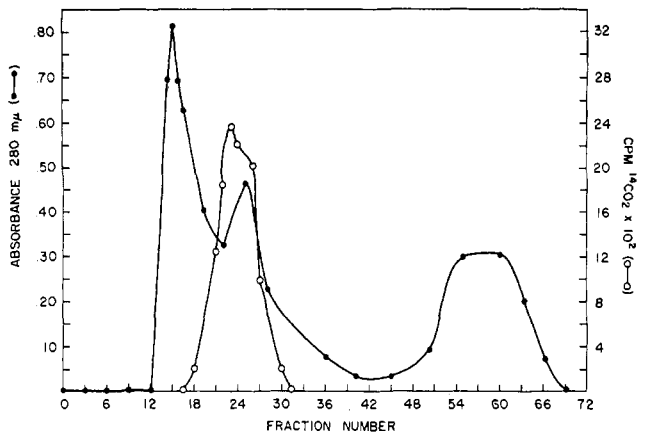

FIGURE 1: Sephadex gel filtration (G-100) of *S*-adenosyl-L-methionine decarboxylase. For details, see the section on purification of the enzyme. Protein (●) was measured by the absorption at 280 mμ. Enzyme activity (○) was assayed as described under Methods.

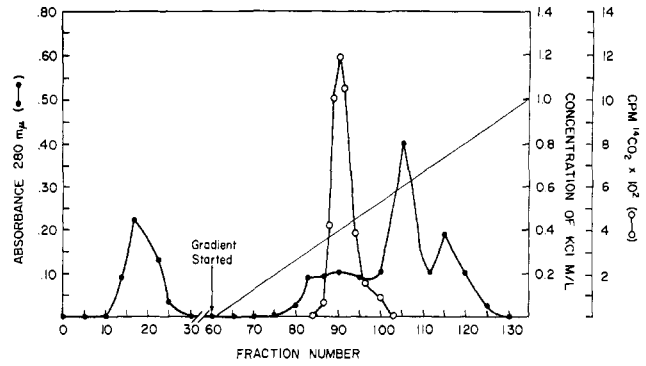

FIGURE 2: Chromatography of *S*-adenosyl-L-methionine decarboxylase on DEAE-cellulose. For details, see the section on purification of the enzyme. Enzyme activity (—○—○—) was assayed by the release of [^{14}C]CO_2 as described under Methods. Protein (●) was measured by the absorption at 280 mμ.

Crude Extract. Liver (5 g) from a Sprague-Dawley rat weighing 100–125 g was disrupted in a glass-Teflon homogenizer with four volumes of 0.05 M phosphate buffer (pH 7.2), containing 1.0 mM EDTA, 0.1 mM dithiothreitol, 3 μM pyridoxal phosphate, and 0.5 M sucrose. After removal of most of the cellular debris by centrifugation at 40,000*g* for 20 min, the crude preparation was centrifuged at 100,000*g* for an additional hour. The supernatant solution was decanted and was passed through cheesecloth to remove any suspended lipid material.

Filtration on Sephadex G-100. An aliquot (9 ml) of the enzyme preparation obtained in the previous step was applied to a Sephadex G-100 column (45 × 4.9 cm²) which had been equilibrated with 0.05 M phosphate buffer (pH 7.2), containing 1.0 mM EDTA, 0.1 mM dithiothreitol, 3 μM pyridoxal phosphate, and 0.5 M sucrose. The enzyme was eluted in 4.0-ml fractions with the equilibrating buffer under a hydrostatic pressure of 20 cm at a flow rate of 25 ml/hr. Eight of the most active fractions (20–28) were pooled (Figure 1) and used in the next step.

DEAE-cellulose Chromatography. The pooled enzyme solution was dialyzed for 1 hr against two changes of 50 volumes of 0.01 M phosphate buffer (pH 7.2), containing 1 mM EDTA, 0.1 mM dithiothreitol, 3 μM pyridoxal phosphate, and 0.5 M sucrose. Thirty milliliters of the dialyzed preparation was applied to a DEAE-cellulose column (25 cm × 4.9 cm²) which had been equilibrated with the same buffer that had been used in the dialysis. After washing the column with 90 ml of the equilibrating buffer, the enzyme was eluted with 300 ml of a linear gradient of 0–1 M KCl in the equilibrating buffer. Fractions that were 4 ml were collected, and seven of the most active fractions (88–94, Figure 2) were pooled, and dialyzed as above, with the exception that sucrose was not included in the buffer solution. When stored at −20°, enzyme activity at this stage was stable for more than 6 months.

Calcium Hydroxylapatite Chromatography. To a column of calcium hydroxylapatite (3 cm × 4.9 cm²) which had been equilibrated with the same buffer used in the last dialysis, 20 ml of the dialyzed enzyme was applied. The column was washed with 25 ml of the equilibrating buffer and then washed successively with 25 ml of 0.1 and 0.2 M phosphate buffers (pH 7.2) both solutions containing 1 mM EDTA, 0.1 mM dithiothreitol, and 3 μM pyridoxal phosphate. The enzyme was eluted in two 4-ml fractions (25–26) with the 0.2 M phosphate buffer (Figure 3). Increasing the molarity of the buffer (Figure 3) failed to release additional activity from the column.

This final fraction represented a purification of greater than 350-fold and a 60% recovery of the initial activity. At this stage of purification, the enzyme was stable at −20° for about 2 months.

Comments on the Purification. Although other authors have utilized ammonium sulfate in the purification of *S*-adenosyl-L-methionine decarboxylase from varying tissues (Jänne and Williams-Ashman, 1971), attempts at purification of the liver decarboxylase by ammonium sulfate fractionation resulted in losses as high as 80% of the original activity. Neither extensive dialysis nor the addition of various protective agents such as dithiothreitol or bovine serum albumin to the enzyme during the fractionation could reverse these losses. In the rat liver system, therefore, ammonium sulfate is not a step of choice in the purification of the decarboxylase.

Homogeneity of the Enzyme. ELECTROPHORESIS. At each step in the purification procedure about 150 μg of protein, taken from the enzyme solution, were analyzed by electrophoresis on polyacrylamide gel by the method of Ornstein and Davies (1961). The total number of protein zones as visualized with Amido-Schwarz decreased progressively until at the last stage of purification a single large zone and a faint thin one were seen.

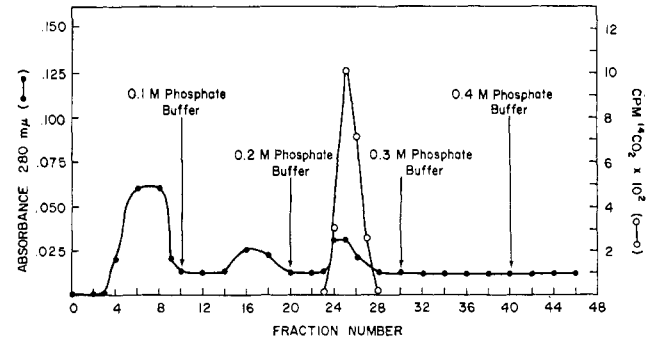

FIGURE 3: Chromatography of *S*-adenosyl-L-methionine decarboxylase on calcium hydroxylapatite. Details of the procedure are given under Purification of the Enzyme. Enzyme activity (○) was assayed as described under Methods. Protein (●) was measured by the absorption at 280 mμ.

TABLE II: Stoichiometry of the Decarboxylation of *S*-Adenosyl-L-methionine and the Formation of Spermidine.

Enzyme Fraction	Act. (nmoles/30 min per mg of Protein)	
	[^{14}C]CO_2 Production[a]	[^{14}C]-Spermidine Synthesis[a]
Ultracentrifuged supernatant	0.056	0.064
DEAE-cellulose	7.2	8.7
Calcium hydroxylapatite	17.8	18.4

[a] The assays used in these determinations are described in Methods.

TABLE III: Effect of Polyamines on *S*-Adenosyl-L-methionine Decarboxylase Activity at Various Stages of Purification.

Concn of Polyamine in Assay System	Enzyme Act. (nmoles of [^{14}C]-CO_2/30 min per mg of Protein)		
	Ultracentrifuged Supernatant Fraction[a]	DEAE-cellulose Fraction	Calcium Hydroxylapatite Fraction
None	0.021	0.091	0.333
2.5 mM putrescine	0.044	1.15	4.13
5.0 mM spermidine	0.024	0.45	1.70
5.0 mM spermidine + 0.02 mM putrescine	0.024	0.47	1.73

[a] The fractions were prepared as described in Methods.

Results

General Properties of the Enzyme. From the data summarized in Table II, 1 mole of CO_2 is released from *S*-adenosyl-L-methionine for every mole of spermidine synthesized. The stoichiometry of the reaction can be shown at any stage in the purification of the enzyme.

As indicated by the release of [^{14}C]CO_2, the pH optimum for the enzymatic decarboxylation of *S*-adenosyl-L-methionine in phosphate buffer is about 7.2 (Figure 4). A considerable degree of inhibition of the reaction is evident in Tris-HCl buffer and in addition the pH optimum has shifted to somewhat higher ranges.

The putrescine dependence of the decarboxylation reaction is demonstrated in Table III. As is apparent, considerable variation in enzyme activity was observed as a direct result of the variation of putrescine (or spermidine) concentration. Thus, at optimal concentrations of putrescine, enzyme activity increased some 6- to 10-fold. Through the use of the double-reciprocal plot, the apparent K_m for putrescine and for spermidine was determined as 3.3×10^{-4} and 2.0×10^{-3} M, respectively. These values are in good agreement with those determined for the enzyme from rat ventral prostate (Pegg and Williams-Ashman, 1969). Similarly, at saturating levels of putrescine, the K_m for *S*-adenosyl-L-methionine was determined as 3.6×10^{-5} M. This also is in good agreement with the value found for the rat ventral prostate enzyme (Pegg and Williams-Ashman, 1969).

Although either putrescine or spermidine could function as a propylamine acceptor in the transfer reaction, at saturating polyamine concentration, enzyme activity was twice as great with putrescine as with spermidine as the substrate. Moreover, the addition of putrescine to reaction mixtures containing saturating levels of spermidine did not increase enzyme activity (Table III) as reported elsewhere (Raina *et al.*, 1970).

Molecular Weight. In order to estimate the molecular weight of the decarboxylase, sucrose density centrifugation was performed (Martin and Ames, 1961) with alcohol dehydrogenase and catalase as standard proteins. These were added to a

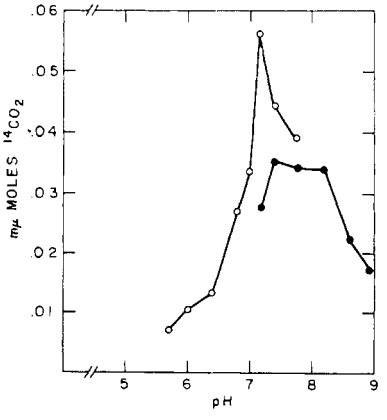

FIGURE 4: pH curve. Reaction conditions are given under Enzyme Assay except that the reaction mixture contained either 0.1 M sodium phosphate buffer (○) or 0.1 M Tris-HCl buffer (●) at the pH indicated. The extent of the reaction is shown by the amount of [^{14}C]CO_2 released over a 30-min period.

TABLE IV: Comparison of Average Molecular Weight of *S*-Adenosyl-L-methionine Decarboxylase Determined by Two Methods.

Enzyme Source	Fraction	Method	Mol Wt
Liver	Ultracentrifuged supernatant fraction	Sucrose density centrifugation[a]	52,000
		Sephadex gel filtration	50,000
	DEAE-cellulose fraction	Sephadex gel filtration	44,000
Uterus	Ultracentrifuged supernatant fraction	Sephadex gel filtration	47,000
Prostate	Ammonium sulfate fraction (40–65% saturation)	Sephadex gel filtration	52,000

[a] Alcohol dehydrogenase and catalase were used as standards in these determinations. The procedures employed are described in Methods.

TABLE V: Effect of 4-Bromo-3-hydroxybenzoyloxyamine Dihydrogen Phosphate (NSD 1055), a Known Inhibitor of Pyridoxal Phosphate Dependent Enzymes, on *S*-Adenosyl-L-methionine Decarboxylase Activity.[a]

Concn of Inhibitor (mM)	% Inhibn	Act. (nmoles of $[^{14}C]CO_2$/30 min per mg of Protein)
None		22.7
0.4	11	20.3
0.7	15	19.3
1.5	31	15.8
3.0	45	12.5
4.0	60	9.1
7.0	74	6.0
10.0	87	2.9

[a] Details of preparation of the reaction mixture and enzyme assay are described in Methods.

TABLE VI: The Effects of Sulfhydryl Inhibitors on *S*-Adenosyl-L-methionine Decarboxylase Activity at Different Stages of Purification.

	Act. (nmoles/30 min per mg of Protein)			
Agent Used[a]	Ultracentrifuged Supernatant Fraction	% Inhibn	DEAE-cellulose Fraction	% Inhibn
None	0.015		2.25	
Iodoacetamide	0.017	0	1.79	21
p-Chloromercuribenzoate	0.018	0	0	100
N-Ethylmaleimide	0.015	0	0.09	96

[a] Concentration was 2×10^{-4} M in the standard assay. The inhibitor was incubated with the enzyme for 10 min prior to addition of *S*-adenosyl-L-methionine. Details of the assay are described in Methods.

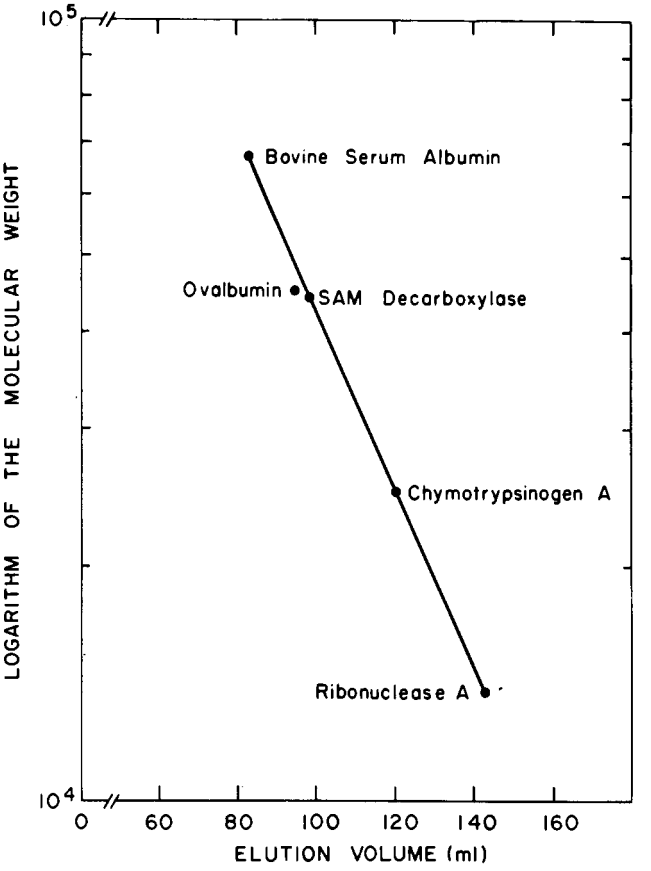

FIGURE 5: Standard curve showing the relationships between the logarithm of the molecular weights of a number of proteins and their elution volumes on a Sephadex G-100 column. The method followed was that of Andrews (1964) except that the entire procedure was carried out at 2°. The enzyme (0.4 mg, step III) was layered onto a Sephadex G-100 column (4.9 $cm^2 \times 45$) which had been equilibrated 0.05 M phosphate buffer (pH 7.2), containing 1 mM EDTA, 0.1 mM dithiothreitol, and 0.3 μM pyridoxal phosphate. Elution was carried out with this buffer and 4-ml fractions were collected at a flow rate of about 25 ml/hr. Enzyme activity was assayed as described under Methods. One milligram of each of the standard proteins was run independently of each other and as described above for *S*-adenosyl-L-methionine decarboxylase. The elution volume estimated to the nearest 1 ml was considered to be the apex of the solute peak in the elution diagram (Andrews, 1964).

preparation of the enzyme so that the final concentration of catalase, alcohol dehydrogenase and *S*-adenosyl-L-methionine decarboxylase were 0.42, 0.2, and 4.0 mg, respectively. After 0.1 ml of this solution was layered onto 4.55 ml of a 5–20% sucrose gradient, centrifugation was performed at 30,000 rpm for 20 hr in a Beckman Model L3-50 preparative ultracentrifuge with the SW-65K swinging-bucket rotor at 2°. The gradient was fractionated and the three enzymes were assayed. The distance from the meniscus to the midpoint of activity for each enzyme was measured and an estimation of the molecular weight of *S*-adenosyl-L-methionine decarboxylase was then made from the relationship between the sedimentation constant and the sedimentation constants of the standard proteins. The results of these calculations summarized in Table IV given an average value of 52,000.

Sephadex Gel Filtration. An alternate technique used to estimate the molecular weight of the enzyme was Andrews' method (1964) of gel filtration on Sephadex G-100 columns. The relationship between the logarithm of the molecular weight of a number of standard proteins and the volume of buffer (elution volume) needed to elute each protein from the column was shown by Andrews to be linear. The standard curve obtained from such a relationship can be seen in Figure 5. From the position of the elution volume of the enzyme on the standard curve (Figure 5), the molecular weight of a crude fraction of the enzyme was estimated to be 50,000 (Table IV). A somewhat lower value, 44,000, was found with a more highly purified fraction. When the enzyme was obtained from tissue organs of the rat, such as the uterus and the prostate, estimations of the molecular weight essentially were similar.

Evidence for Pyridoxal Phosphate Requirement of the Enzyme. From the work of Levine *et al.* (1965), 4-bromo-3-hydroxybenzoyloxyamine dihydrogen phosphate (NSD 1055) is known to inhibit pyridoxal phosphate requiring enzymes. As seen in Table V, this reagent at a concentration of 10^{-3} M caused almost complete inhibition of enzyme activity, suggesting that pyridoxal phosphate was indeed functioning as a cofactor in the reaction. In addition, after extensive dialysis

TABLE VII: Effect of Metals on *S*-Adenosyl-L-methionine Decarboxylase Activity.

Metal Used[a]	% Inhibn	Act. (nmoles of $[^{14}C]CO_2$/30 min per mg of Protein)
None		1.98
$MgCl_2$	19	1.62
$MnCl_2$	5	1.90
$CaCl_2$	62	0.77
$FeCl_2$	38	1.24
$ZnSO_4$	88	0.25
Co	26	1.46
Cd	88	0.25
NH_4Cl_2	22	1.56

[a] All metals were incubated with the enzyme at a concentration of 5×10^{-4} M, under standard assay conditions, 10 min prior to addition of *S*-adenosyl-L-[*carboxyl*-^{14}C]methionine.

(24 hr) of the enzyme obtained after DEAE-cellulose chromatography against 100 volumes of 0.1 M phosphate buffer (pH 7.5), enzyme activity was reduced to less than 2% of its original amount, but was restored completely by the addition of 10^{-5} M pyridoxal phosphate.

Inhibition of S-Adenosyl-L-methionine Decarboxylase Activity. Of the known sulfhydryl reagents, *p*-chloromercuribenzoate and *N*-ethylmaleimide appear to be particularly effective in causing inhibition of enzyme activity. Both compounds at concentrations of 2×10^{-4} M completely inhibited the decarboxylation reaction by the fraction obtained after DEAE-cellulose chromatography (Table VI). Of interest, however, is that the crude enzyme was insensitive to either reagent. With progressive purification, the sensitivity of the enzyme to these reagents increased.

Metal Requirements. Even in the most highly purified preparation of the enzyme, it was not possible to demonstrate any increased activity by the addition of divalent cations (Table VII). Some inhibition of enzyme activity was in fact observed after the addition of Ca^{2+}, Zn^{2+}, and Cd^{2+} (Table VII).

Discussion

The enzyme, *S*-adenosyl-L-methionine decarboxylase, lies on a pathway which may be of fundamental importance in the regulatory mechanism of the cell (Tabor and Tabor, 1964). For this reason, and to gain an understanding of its physical properties, the enzyme was purified to near homogeneity as judged by polyacrylamide gel electrophoresis.

The liver enzyme appears to differ considerably from the bacterial system. The main difference is that the bacterial system is demonstrably two enzymes, one catalyzing the decarboxylation of the substrate and the second catalyzing the transfer of a propylamine moiety from the decarboxylated substrate to an acceptor molecule such as putrescine or spermidine. At the present time, there is no evidence to indicate that the liver enzyme is similarly divisible. For even at the highest stage of purification, decarboxylation is completely putrescine dependent and is always inseparable from propylamine transfer. Although the difference between the bacterial and liver enzymes is considerable, it is nonetheless readily reconcilable. Ample precedent exists for the large variation between proteins carrying out similar reactions but isolated from different sources. Not as easily reconcilable, however, is the variation between the enzymes reported for the rat ventral prostate (Jänne and Williams-Ashman, 1971) and the liver of the same animal. Namely, that in the ventral prostate system, the decarboxylase is partially separable from the propylamine transferase enzyme after DEAE-cellulose chromatography (Jänne and Williams-Ashman, 1971). After similar treatment of the liver enzyme, as has been described above, no evidence for this type of separation could be found. Moreover, the putrescine dependence of the decarboxylation reaction as well as the stoichiometry between CO_2 released and spermidine formed, both readily demonstrable at the highest stage of purification, strongly suggest a single protein entity.

Several additional characteristics of the liver system were revealed with increasing purity of the preparation. Thus, for example, the loss of enzyme activity after exhaustive dialysis of the purified preparation could be restored completely by the addition of pyridoxal phosphate. This finding combined with the inhibition of enzyme activity after exposure to a known inhibitor of pyridoxal phosphate (4-bromo-3-hydroxybenzoyloxyamine dihydrogen phosphate) suggests that pyridoxal phosphate is indeed a cofactor of the enzyme. Similarly, the sensitivity to some sulfhydryl reagents was revealed as the purification of the enzyme progressed. Neither *N*-ethylmaleimide nor *p*-chloromercuribenzoate caused any observable effect on enzyme activity when used with crude enzyme extracts. After DEAE-cellulose chromatography the enzyme was inhibited completely (or essentially so) by either compound.

It has been reported (Raina and Hannonen, 1970) that in regenerating rat liver some separation of the two enzymes could be obtained after ammonium sulfate fractionation. We could not reproduce those results and, as stated, found that ammonium sulfate consistently caused a loss of 60–80% of the activity.

The purification procedure outlined in the text offers a relatively simple means of obtaining large amounts of stable enzyme from a readily accessible source. As such it could be a means to examine thoroughly whether the mammalian system is indeed divisible into two distinct protein entities.

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Threonine-Sensitive Aspartokinase–Homoserine Dehydrogenase Complex, Amino Acid Composition, Molecular Weight, and Subunit Composition of the Complex†

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ABSTRACT: The threonine-sensitive aspartokinase–homoserine dehydrogenase complex has been purified to apparent homogeneity from *Escherichia coli* 9723 (ATCC). The amino acid composition of the complex was determined and was found to be similar to that of a complex having similar activities previously isolated from *E. coli* K-12 except in the content of threonine. The molecular weight of the complex determined by sedimentation equilibrium and by the Svedberg method was 346,000 ± 15,000 and 334,000 ± 14,000, respectively. Sedimentation equilibrium experiments performed on the complex dissolved in 6 M guanidinium chloride showed that the molecular weight of the subunit was 80,000 ± 3000. Nonideal behavior of the solute must be taken into account when evaluating the experiments. It was concluded that the complex from *E. coli* 9723 is composed of four subunits of similar or identical molecular weight.

A complex enzyme having both aspartokinase and homoserine dehydrogenase activity and subject to inhibition by the eventual end product, L-threonine, occurs in *Escherichia coli*. The physical and catalytic properties of the complex from strain K-12 have been studied extensively and have been recently reviewed (Cohen, 1969). The native complex isolated from this strain was reported to have a molecular weight of 360,000 (Truffa-Bachi *et al.*, 1968). The molecular weight of the subunits of the complex in 6 M guanidinium chloride was reported to be 60,000, and it was concluded that the complex was composed of six subunits of identical molecular weight (Truffa-Bachi *et al.*, 1969). Recently, subcomplexes having an intermediate molecular weight of 122,000 have been reported (Wampler *et al.*, 1970). These subcomplexes, which seem to occur in certain buffer systems, appear to remain fully effective for both activities of the native complex, and both activities remain sensitive to threonine inhibition.

The threonine-sensitive aspartokinase–homoserine dehydrogenase complex from *E. coli* 9723 (ATCC) has been studied in this laboratory, particularly with respect to the control of biosynthesis of the complex and the threonine interactions with the complex (Lee *et al.*, 1966; Cunningham *et al.*, 1968). Preliminary work on the molecular weight of the complex and subunits indicated that the reported molecular weight of the complex and subunits from E. *coli* K-12 might not be adequate to explain the properties of the complex from strain 9723. Therefore, more complete studies of the physical properties of the complex from strain 9723 have been performed.

In the present work, the complex from *E. coli* 9723 has been purified to homogeneity, and the amino acid composition, the molecular weight, some general physical properties of the complex, and the molecular weight of the subunits of

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