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Purification and Characterization of *S*-Adenosyl-L-methionine Decarboxylase from Mouse Mammary Gland and Liver†

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ABSTRACT: *S*-Adenosyl-L-methionine decarboxylase, a key enzyme in polyamine biosynthesis, has been purified to apparent homogeneity from mouse mammary gland and liver by a combination of ammonium sulfate fractionation, DEAE-cellulose and methylglyoxal bis(guanylhydrazone)–Sepharose 4B affinity chromatographies, and gel filtration. Crucial factors for good yield of the pure enzyme include the use of putrescine, pyridoxal phosphate, a detergent (0.01% deoxycholate), and high salts as stabilizing agents at various stages of purification. The purified enzyme from both tissues has essentially identical specific activities and other properties examined and is completely free of propylamine transferase activity (spermidine or spermine synthase). The apparent molecular weight of native enzyme as determined by gel filtration and by sucrose density centrifugation was approximately 68 000 and 74 000, respectively, whereas the subunit molecular weight of 32 000 was obtained with sodium dodecyl sulfate–polyacrylamide gel electrophoresis, suggesting that the enzyme is a dimer. The enzyme has an isoelectric point of 5.9 and a pH optimum of 7.5. The activity of purified enzyme is stimulated by physiological concentrations of putrescine (K_a = 0.5 μM), which decreases the apparent K_m for the substrate from 1.1×10^{-4} to 2×10^{-5} M and also prevents inactivation of the enzyme. Another related polyamine, spermine, but not spermidine, inhibits the enzyme activity by reducing the V_{max} of the enzyme reaction at physiological concentrations (K_i = 0.5 mM). By use of the purified liver *S*-adenosyl-L-methionine decarboxylase, a monospecific mouse antibody to this enzyme has been raised in rabbits. The antibody cross-reacts with the enzyme from mammary gland, inactivates the enzyme, and forms a single precipitation line with *S*-adenosine-L-methionine decarboxylase from both tissues as shown by an Ouchterlony double-diffusion test. Immunoproduct analysis by sodium dodecyl sulfate gel electrophoresis of the antigen synthesized by mouse mammary explants in vitro demonstrated the presence of a sharp band which comigrated with the subunit of authentic (pure) enzyme. The antibody was used in measuring levels of antigen in the mammary gland of mice treated with methylglyoxal bis(guanylhydrazone), and the results indicated that the increased accumulation of the enzyme activity was primarily due to alterations in synthesis and degradation of enzyme molecules.

S-Adenosyl-L-methionine decarboxylase (*S*-Ado-Met decarboxylase)[1] serves an essential role for polyamine biosynthesis by catalyzing the formation of *S*-methyladenosyl-L-homocysteamine (decarboxylated *S*-Ado-Met), the sole donor of the propylamine moiety for the biosynthesis of spermidine

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[1] Abbreviations used: MGBG, methyl glyoxal bis(guanylhydrazone); *S*-Ado-Met, *S*-adenosyl-L-methionine; $NaDodSO_4$, sodium dodecyl sulfate; BSA, bovine serum albumin; ADH, alcohol dehydrogenase; LDH, lactate dehydrogenase.

and spermine (Pegg & Williams-Ashman, 1969; Williams-Ashman et al., 1972; Tabor & Tabor, 1972). Earlier studies on *S*-Ado-Met decarboxylase in mammary gland have shown that the enzyme activity increases in parallel with the rise in the intracellular concentration of spermidine during the development of mammary epithelium that occurs in lactating rats (Russell & McVicker, 1972) and mice (Oka et al., 1978) as well as during the hormonally induced development of mouse mammary gland in culture (Oka & Perry, 1974; Sakai et al., 1978). Further studies on the organ culture system with an inhibitor of *S*-Ado-Met decarboxylase, methylglyoxal bis(guanylhydrazone) (MGBG), have indicated (Oka & Perry, 1974; Rillema, 1976; Sakai et al., 1978) that *S*-Ado-Met decarboxylase may be a rate-limiting enzyme for the biosynthesis of spermidine, which plays a key role for the hormone-dependent development of mouse mammary epithelium.

For facilitation of our previous attempts to elucidate the cellular mechanism involved in the regulation of *S*-Ado-Met decarboxylase in the development of mouse mammary epithelium, a specific antibody probe of the enzyme would be a valuable tool for direct quantitative analysis. Thus, we have undertaken the purification of mouse *S*-Ado-Met decarboxylase and report here the isolation of the homogeneous enzyme, its characterization, and the preparation of antibody against the protein.

Materials and Methods

Materials

Materials were purchased as follows: [*carboxy*-^{14}C]-*S*-adenosyl-L-methionine (54.6 mCi/mmol), [1,4-^{14}C]putrescine dihydrochloride (51.8 mCi/mmol), [^{14}C]spermidine trihydrochloride (10.0 mCi/mmol), and [3,4,5-^{3}H$_3$]leucine from New England Nuclear; *S*-adenosyl-L-methionine, dithiothreitol, ammonium sulfate, and sucrose from Schwartz/Mann; putrescine dihydrochloride, pyridoxal phosphate, cadaverine, and L-ornithine from Calbiochem; spermidine, spermine, *N*-ethylmaleimide, and *p*-(chloromercuri)benzoate from Sigma Chemical Co.; MGBG from Aldrich Chemical Co.; DEAE-cellulose (DE-52) from Whatman, Inc. Marker proteins for molecular weight determination, such as aldolase, bovine serum albumin, ovalbumin, chymotrypsinogen, cytochrome *c*, and ferritin, were purchased from Boehringer Mannheim Corp. and Pharmacia Fine Chemicals, Inc. CH-Sepharose 4B and Sephacryl S-200 were obtained from Pharmacia Fine Chemicals, Inc. Medium 199 and phosphate-buffered saline were obtained from GIBCO.

Female C3H/HeN mice in the fifth to tenth day of lactation were obtained from the National Institutes of Health.

Decarboxylated *S*-adenosyl-L-methionine was synthesized enzymatically by the method of Tabor (1962) with partially purified adenosylmethionine decarboxylase from *Escherichia coli*. The product was isolated from the reaction mixture by a Dowex 50 column and purified to homogeneity by preparative paper electrophoresis. Preparation of CH-Sepharose 4B containing covalently bound MGBG was carried out by using carbodiimide (Sigma) according to the method of Pegg (1974).

Methods

Purification of S-Ado-Met Decarboxylase from Mouse Mammary Gland and Liver. All mice used were given an intraperitoneal injection of 2 mg of MGBG in 0.2 mL of 0.15 M NaCl. This treatment was given to increase the level of the enzyme in mouse liver and mammary gland by 10–20-fold, as reported in other species (Pegg, 1974; Heby et al., 1973; Pösö et al., 1975). Twenty-four hours after the injection, the animals were killed by cervical dislocation, and the liver and mammary gland were removed. The tissues were minced, washed in 0.15 M NaCl, and homogenized with a Potter–Elvehjem homogenizer in 4 volumes of ice-cold 25 mM sodium phosphate buffer, pH 7.6, which contained 1 mM dithiothreitol, 0.1 mM EDTA, 0.5 mM putrescine, and 5 μM pyridoxal phosphate (buffer A), unless stated otherwise. All operations were carried out at 0–4 °C. The homogenate was centrifuged at 24000*g* for 60 min, and the supernatant was saved. The precipitate was rehomogenized with 10–15 mL of buffer A and centrifuged again in the same manner. A portion of the combined supernatant fluids was removed, dialyzed against buffer A to remove MGBG, and assayed for enzyme activity. The remaining supernatant fluid was fractionated by stepwise addition of solid ammonium sulfate. Proteins precipitating between 35 and 65% saturation were collected by centrifugation at 25000*g* for 1 h and then dissolved in 10–15 mL of buffer A and dialyzed against 100 volumes of the same buffer for 12 h. The dialyzed enzyme solution was applied to a DEAE-52 cellulose column (2.5 × 20 cm) which had been previously equilibrated with buffer A. After the column was washed with 200 mL of buffer A, *S*-Ado-Met decarboxylase was eluted with a linear gradient of 0–0.4 M NaCl in buffer A. The total volume of the gradient solution was 800 mL. All fractions containing *S*-Ado-Met decarboxylase were pooled and reprecipitated with 65% ammonium sulfate. The precipitate was dissolved in 10 mM Tris-HCl buffer, pH 7.6 (buffer B), containing all the substances present in buffer A except sodium phosphate and dialyzed against 1000 volumes of the same buffer. This change of the buffer system was necessary since the phosphate buffer formed insoluble materials with MGBG which was used in the subsequent affinity chromatography step. The dialyzed enzyme solution was applied to a column (1 × 10 cm) of Sepharose 4B to which MGBG was covalently bound and which had been previously equilibrated with buffer B. The column was washed with 100 mL of buffer B and then with 100 mL of 0.3 M NaCl in the same buffer, and *S*-Ado-Met decarboxylase activity was eluted from the column with buffer B containing 0.3 M NaCl and 1 mM MGBG. During the elution process, each fraction was collected in a tube containing one-tenth volume of 0.1% deoxycholate, which was found to be necessary to prevent the loss of the enzyme activity. In the absence of the detergent, recovery of the enzyme activity was reduced to less than 10% of the activity applied to the column. Each 1-mL fraction was dialyzed against 1000 volumes of buffer B containing 0.01% deoxycholate with three buffer changes over a 24-h period. Fractions containing *S*-Ado-Met decarboxylase activity were pooled and concentrated by an Amicon Centriflo membrane cone (Amicon F25) to ~0.5 mL. The enzyme solution was applied to a column (0.7 × 55 cm) of Sephacryl S-200 which had been previously equilibrated with buffer B containing 0.5 M NaCl and 0.01% deoxycholate. Following elution with the same buffer, each fraction was assayed for the enzyme activity and the active fractions were pooled and stored at 4 °C without appreciable loss of activity within a 1-month period.

Molecular Weight Determinations. The molecular weight of the native enzyme was determined under nondenaturing conditions by sucrose density centrifugation (Martin & Ames, 1961) and by Sephacryl S-200 gel filtration. An analytical column of Sephacryl S-200 (1.1 × 44 cm) was calibrated with proteins of known molecular weights.

Analytical Methods. The activity of *S*-Ado-Met decarboxylase was assayed by measuring $^{14}CO_2$ release from

Table I: Summary of Purification Procedure for *S*-Ado-Met Decarboxylase from Mouse Mammary Gland and Liver[a]

step	*S*-Ado-Met decarboxylase					spermidine synthase,
	total protein (mg)	total act. (nmol/h)	sp act. [nmol/(mg h)]	recovery (%)	purifn (*x*-fold)	sp act. [pmol/(mg h)]
	Mammary Gland					
(1) 25000*g* supernatant	1653	1607	0.972	100	1	240
(2) ammonium sulfate ppt (35–65%)	524	1389	2.65	86	2.73	820
(3) DEAE column chromatography	168	1089	6.48	68	6.67	1490
(4) MGBG affinity column chromatography	0.0514	251	4892	16	5033	0
(5) Sephacryl gel filtration	0.0327	220	6724	14	6918	
	Liver					
(1) 25000*g* supernatant	19266	1720	0.0893	100	1	290
(2) ammonium sulfate ppt (35–65%)	5800	1233	0.213	72	2.39	960
(3) DEAE column chromatography	342	1284	3756	75	42.1	2410
(4) MGBG affinity column chromatography	0.0540	267	4944	16	55363	<20
(5) Sephacryl gel filtration	0.0369	226	6120	13	68533	0

[a] Summarized from 10 to 12 preparations of mammary gland (27–35 g) and liver (80–120 g) of 47–55 lactating mice treated with MGBG.

carboxy-labeled substrate as described previously (Oka & Perry, 1974). The enzyme reaction was carried out in a total volume of 2 mL consisting of enzyme sample and 0.25 mM sodium phosphate buffer, pH 7.6, containing 0.5 mM putrescine, 5 μM pyridoxal phosphate, 1 mM dithiothreitol, 0.1 mM EDTA, and adenosyl[*carboxy*-^{14}C]methionine (0.05 μCi/mL). In separate experiments, the stoichiometry of CO_2 production and the formation of the decarboxylated product (*S*-methyladenosylhomocysteamine) were established by using ^{14}COOH- and $^{14}CH_3$-labeled *S*-adenosylmethionine as substrate and a pure preparation of the enzyme to ascertain that the release of CO_2 is a valid measurement of the activity of *S*-Ado-Met decarboxylase. The activity of spermidine synthase was assayed in 25 mM sodium phosphate buffer, pH 7.6, 0.1 mM dithiothreitol, 0.1 mM unlabeled decarboxylated *S*-Ado-Met, and 1 mM [1,4-^{14}C]putrescine as described (Oka et al., 1977) by a method of Jänne & Williams-Ashman (1971). The activity of spermine synthase was assayed by a similar method except that [1,4-^{14}C]putrescine was replaced by 1 mM [^{14}C]spermidine.

Sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis was performed either according to Weber & Osborn (1969), using a 10% gel, or by the method described by Laemmli (1970), using slab gels (14 × 10 × 0.5 cm). Following electrophoresis, the gels were stained in 0.2% Coomassie blue in 10% acetic acid and 40% methanol or in 0.2% Brilliant Blue R in 10% acetic acid and 50% methanol. The gels were destained in a solution containing 5% acetic acid and 25% methanol for 1 to 2 days.

Isoelectric focusing was performed with an LKB 8100 Ampholine (LKB Instruments, Inc.) equipped with a double cooling jacket and using 1% ampholine (pH 3–10) in a linear sucrose gradient from 5 to 50%.

Protein was measured by the method of Lowry et al. (1951) with crystalline bovine serum albumin as a standard.

Immunological Procedures. Purified mouse liver *S*-Ado-Met decarboxylase (200 μg/0.5 mL of buffer B) was emulsified in an equal volume of complete Freund's adjuvant, and this emulsion (1 mL) was injected intradermally to a New Zealand male rabbit (3-kg body weight) at 10–12 different areas of the back. Two weeks later booster injections, using 100 μg of enzyme in complete adjuvant, were given subcutaneously. Three weeks after the booster injections the rabbit was bled from the ear vein and the serum was collected. Control (preimmunized) serum was obtained from the animal prior to injections of the antigen preparation.

Plates for double-immunodiffusion assays were prepared according to Ouchterlony (1949). The precipitin lines appeared after 48–72 h at room temperature, and the agar plates were then washed with 0.15 M NaCl for 3 days and water for 2 days. Finally, they were stained and destained as described for acrylamide gels.

The immunoprecipitation reaction was carried out in a small polyethylene tube (Beckman) in a total volume of 0.2 mL consisting of enzyme samples and antiserum, which had been diluted appropriately with phosphate-buffered saline, pH 7.4. This mixture was incubated at 37 °C for 60 min and at 4 °C overnight. The precipitates were collected by centrifugation and washed twice with ice-cold phosphate-buffered saline. When preimmunized serum was used, there was no precipitate. The supernatant solutions were measured for the remaining *S*-Ado-Met decarboxylase activity.

For the analysis of immunoprecipitated materials, radioisotope-labeled *S*-Ado-Met decarboxylase was prepared by incubating mammary explants from virgin mice in Medium 199 containing insulin (~5 μg/mL) for 18 h as described previously (Topper et al., 1975) and then by pulse labeling the explants with [^{3}H]leucine (50–100 μCi/mL) for 3 h. The enzyme extracts were prepared by homogenization of the explants with buffer A and centrifugation of the homogenates at 105000*g* for 45 min. The resultant supernatant containing [^{3}H]leucine-labeled enzyme was mixed with the antiserum and incubated at 37 °C for 60 min and at 4 °C overnight. The antigen–antibody complex was completely precipitated by the addition of goat antirabbit serum. The immunoprecipitate was collected by centrifugation, washed twice with phosphate-buffered saline containing 0.5% Triton X-100, and finally dissolved by boiling for 20 min in the presence of 1% $NaDodSO_4$ and 2% 2-mercaptoethanol. The resultant solution was analyzed by $NaDodSO_4$ gel electrophoresis for the distribution of radioactivity among various protein species.

Results

Purification of S-Ado-Met Decarboxylase. Table I illustrates a typical experiment for the complete purification of the enzyme from mouse mammary gland and liver. The data show that the enzyme was purified approximately 69 000-fold in liver and 6900-fold in mammary gland with an overall recovery of 13 to 14%. The final enzyme preparations from the two tissues showed essentially the same specific activity, although at the initial step of purification (crude supernatant) the specific activity of the enzyme in mammary gland was more than 10 times higher than that of the liver enzyme. In 10–12 other preparations the value of the final specific activity

of the enzyme was found to vary from 6000 to 7000 nmol/(mg h), primarily because of the inherent variation in protein determination at the low concentration of protein.

Both putrescine and pyridoxal phosphate were required during the DEAE column chromatography to prevent the loss of *S*-Ado-Met decarboxylase; omission of either putrescine or pyridoxal phosphate or both resulted in a marked loss (75–90%) of enzyme activity (data not shown). The eluates containing the decarboxylase activity were contaminated with spermidine synthase activity, which amounted to over 50% of the synthase activity present in step 2. Complete separation of *S*-Ado-Met decarboxylase from spermidine synthase was achieved by passage of DEAE chromatography fractions through the MGBG affinity chromatography column. At this step, spermidine synthase was readily eluted by the 0.3 M NaCl solution, whereas elution of *S*-Ado-Met decarboxylase required the presence of 1 mM MGBG and 0.3 M NaCl in buffer B. Following affinity chromatography, it was found necessary to add a detergent such as deoxycholic acid, Triton X-100, or Tween 80, each at a concentration of 0.01%, to the enzyme solution in order to prevent the rapid loss of enzyme activity. In the absence of a detergent, the activity of the purified enzyme decreased by ~70–80% within 1 h during subsequent procedures such as transfer, dialysis, assay, concentration, and storage. The addition of bovine serum albumin (2.5% final concentration) was also effective in preventing the loss of activity, although it was not suitable for the purpose of enzyme purification. Use of such a stabilizing agent was required only for the enzyme preparation after affinity chromatography, while the enzyme preparation up to step 3 was quite stable. Such results indicate that the unstable nature of the purified enzyme may be partly due to denaturation resulting from a very low protein concentration.

Analysis of the enzyme preparation at step 4 from both liver and mammary gland by polyacrylamide gel electrophoresis indicated that the enzyme was still contaminated by a protein(s) of molecular weight 80 000–100 000, which comprised ~25% of the protein of the enzyme preparation (data not shown). Such a contaminant could not be removed by repeating the affinity chromatography but was readily separated from the decarboxylase by Sephacryl S-200 gel filtration. $NaDodSO_4$ gel electrophoresis of the active fractions from Sephacryl gel filtration indicated that one protein band was present in the final enzyme preparation from both mammary gland and liver. The molecular weight of the *S*-Ado-Met decarboxylase, as estimated by its electrophoretic mobility ratio with those of molecular weight standards, was 32 000.

The apparent molecular weight of native, enzymatically active *S*-Ado-Met decarboxylase from liver and mammary gland was determined both by sucrose gradient centrifugation and by Sephacryl S-200 gel filtration, which gave an estimated value of 68 000 and 74 000, respectively. Thus, it appears that native enzyme from both tissues is composed of the two subunits of M_r 32 000.

The activity of *S*-Ado-Met decarboxylase after the final step is very labile unless high salts, such as 0.4 M NaCl or bovine serum albumin (0.1 mg/mL), are present in buffer B containing 0.01% deoxycholate. As shown in Figure 1, the activity of the enzyme decreases by more than 50% in 1 h at 37 °C in the absence of 0.4 M NaCl. The pure enzyme can be stored for at least a month without loss of activity at 4 °C in the presence of 0.4 M NaCl and 0.01% deoxycholate in buffer B.

While our studies were in progress, it was reported by Suresh & Adiga (1977) that the putrescine-dependent activity of *S*-Ado-Met decarboxylase in the plant system was an artifact

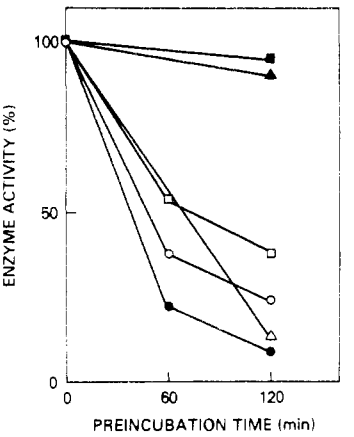

FIGURE 1: Stability of *S*-Ado-Met decarboxylase. Homogeneous enzyme preparation (step 5) from mammary gland was divided into two portions; one was exhaustively dialyzed against 2000 volumes of buffer A containing 0.01% deoxycholate without putrescine, and the other was exhaustively dialyzed against the same buffer with 0.4 mM putrescine. The enzyme preparations were then incubated at 37 °C for the indicated time period in buffer A containing no addition (○), 0.4 M NaCl (□), 0.1 mg/mL BSA (△), 0.5 mM putrescine (●), 0.5 mM putrescine plus 0.4 M NaCl (■), or 0.5 mM putrescine plus 0.1 mg/mL BSA (▲). After the incubation the activity was assayed under the standard assay conditions. Data were expressed as the relative percent of activity retained by assigning the enzyme activity at the beginning of incubation as 100%.

due to the H_2O_2 generated by contaminating diamine oxidase and that activity was, in fact, inhibited by catalase. Our studies of the purified enzyme preparation with respect to the catalase sensitivity did not reveal such an artifactual putrescine-dependent decarboxylase activity.

Various Properties of S-Ado-Met Decarboxylase. The characterization of *S*-Ado-Met decarboxylase was made by using the enzyme preparation from liver and mammary gland at various purification steps. The pH optimum for the enzyme was about pH 7.6 in phosphate buffer (buffer B). In Tris-HCl buffer (buffer A) the enzyme activity was reduced to about half of that in the phosphate buffer system.

Isoelectric focusing of the mammary decarboxylase showed that the enzyme has a single isoelectric point of 5.9.

The activity of mouse *S*-Ado-Met decarboxylase, like the enzyme from other mammalian tissues and yeast, was markedly stimulated by putrescine. As shown in Figure 2, the effective concentration of putrescine was dependent on the degree of the purity of the enzyme preparation; only 5 μM putrescine was required for maximal stimulation of the enzyme preparation at step 4 in contrast to 0.3–0.5 mM putrescine for the crude or the partially purified DEAE fractions (step 3). The enhancement of enzyme activity by putrescine is due, at least in part, to a decrease in the apparent K_m of the enzyme substrate *S*-Ado-Met from 1.1×10^{-4} to 2×10^{-5} M, as determined by kinetic analysis.

The effect of two other naturally occurring polyamines, spermidine and spermine, on the decarboxylase reaction was also studied (Figure 3). Spermine markedly inhibited the decarboxylase activity with a K_i of 5×10^{-4} M by lowering the V_{max} (Figure 3, insert). Spermine also inhibited the "base line" activity of *S*-Ado-Met decarboxylase that was measured in the absence of putrescine. In contrast, spermidine, which inhibited the partially purified DEAE fractions, had a small stimulatory effect on the purified enzyme at 10^{-3} M. These opposite effects of spermidine are explained by the observed contamination of crude preparations of the decarboxylase by spermine synthase which formed spermine from spermidine in the enzyme reaction. Thus, when the decarboxylase, free

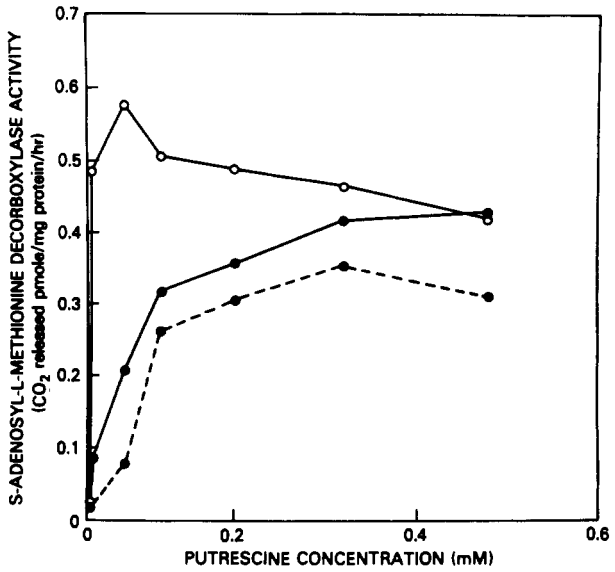

FIGURE 2: Effect of putrescine on *S*-Ado-Met decarboxylase activity at various stages of purification. The liver enzyme preparations from the 2500*g* supernatant (120 μg of protein, ●---●), DE-52 column chromatography (75 μg of protein, ●—●), and MGBG affinity chromatography (2 μg of protein, O—O) were incubated in the presence of various concentrations of putrescine, and the enzyme activity was determined as described under Methods, except that bovine serum albumin was added at a concentration of 1 mg/mL to maintain the overall protein concentration constant among the samples.

of spermine synthase (preparation at step 4), was incubated with spermidine, no inhibitory effect of spermidine was observed.

With the pure enzyme preparation, it was observed that neither pyridoxal phosphate nor divalent cations, such as Mg^{2+}, affected enzyme activity and that the enzyme catalyzed no detectable level of decarboxylation of other amino acids such as ornithine, lysine, methionine, and glutamic acid when each amino acid was tested at concentrations of 1 μM–10 mM (data not shown).

Antibody to S-Ado-Met Decarboxylase. The isolation of pure *S*-Ado-Met decarboxylase permitted the preparation of specific antibody to this enzyme. Ouchterlony double-diffusion analysis revealed a single precipitin band without spur formation following the incubation of rabbit antimouse liver *S*-Ado-Met decarboxylase serum with the purified enzymes from liver and mammary gland, respectively (Figure 4). Control serum did not show any cross-reactivity with the decarboxylases from either tissue. These results indicate that the enzyme preparation used as the antigen was pure and that the enzyme molecules derived from the two types of tissues were immunochemically indistinguishable.

As shown in Figure 5, rabbit antiserum raised against liver *S*-Ado-Met decarboxylase precipitates quantitatively the activity of purified enzyme from both liver and mammary gland, confirming the immunological identity of the enzyme from both tissues. Furthermore, the antibody preparation precipitated the *S*-Ado-Met decarboxylase in the crude preparation from both liver and mammary gland (data not shown). As determined from Figure 5, 150 μL (300 μg of protein) of the antiserum can precipitate 0.05 μg of the enzyme protein (0.21 nmol/h enzyme activity) at the equivalence point. Control serum, up to 200 μL, did not affect the enzyme activity.

For further examination of the specificity of the anti-*S*-Ado-Met decarboxylase serum, the immunoprecipitates derived from an incubation of antiserum with the mammary cytosol enzyme preparation labeled with ^3H-labeled amino acids were analyzed by $NaDodSO_4$ disc gel electrophoresis. As shown in Figure 6, immunoprecipitates contained a major protein peak with a molecular weight of 34 000, which corresponds to the subunit size of the *S*-Ado-Met decarboxylase. Thus, these data are in accord with the above contention that the antiserum preparation is monospecific and that *S*-Ado-Met decarboxylase may consist of two subunits with molecular weights of ~34 000.

The ability of anti-*S*-Ado-Met decarboxylase serum to inactivate enzyme activity can be used to assess the amount of antigen in a given enzyme preparation. The Ab_{50}, defined as the amount of monospecific antibody required to inactivate 50% of the enzyme activity in samples containing various amounts of the decarboxylase activity, has been determined (Figure 7). When these values are plotted against the amount

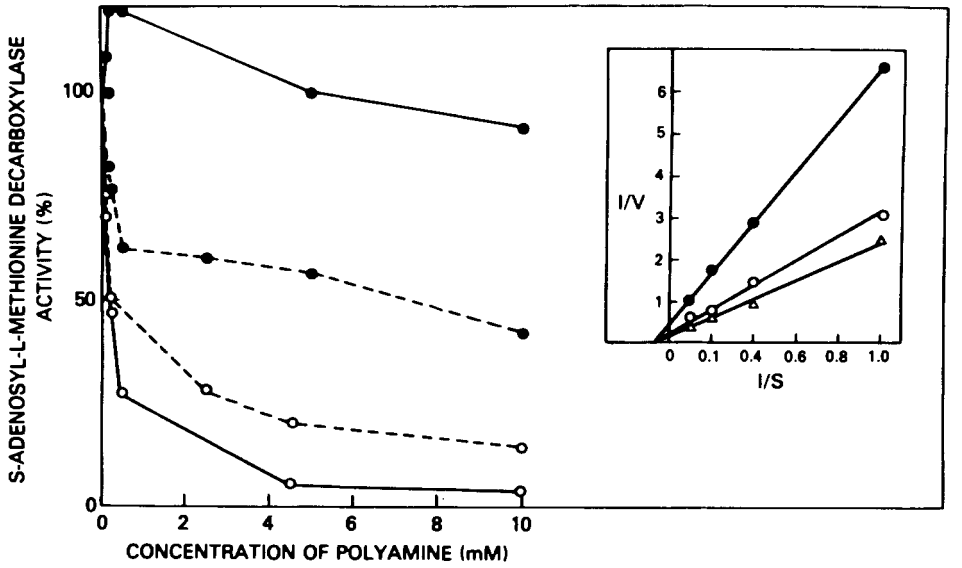

FIGURE 3: Effect of spermidine and spermine on *S*-Ado-Met decarboxylase activity at various stages of purification. The liver enzyme preparations from DE-52 column chromatography and from MGBG affinity chromatography were assayed in the presence of various concentrations of spermidine (●) and spermine (O) as described under Methods. Enzyme from DE-52 column (---); from MGBG column (—). Data were expressed as the relative percent of control which was assayed in the standard assay system without spermidine and spermine. Insert: effect of spermine on the kinetics of *S*-Ado-Met decarboxylase activity. The enzyme activity was assayed as described under Methods by varying the concentration of the substrate and in the presence of 1 (●), 0.1 (O), and 0 (△) mM spermine.

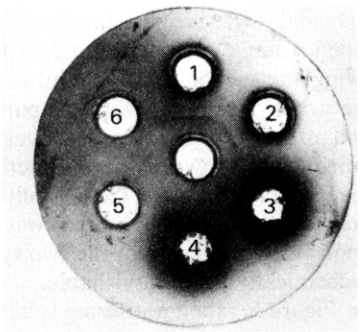

FIGURE 4: Ouchterlony double-diffusion analysis of *S*-Ado-Met decarboxylase. The agar gel (1%) contained 20 mM potassium phosphate buffer, pH 7.5, 0.15 M NaCl, and 0.02% sodium azide. Wells 1 and 2 contained 1 μg of the partially purified (step 2) and pure (step 5) enzyme from mammary gland, respectively; wells 3 and 4 contained 1 μg of the partially purified (step 2) and pure (step 5) enzyme from liver. The center well contained 150 μL of antiserum against liver antigen diluted 8 times with 0.15 M NaCl, and wells 5 and 6 contained 150 μL of preimmunized serum.

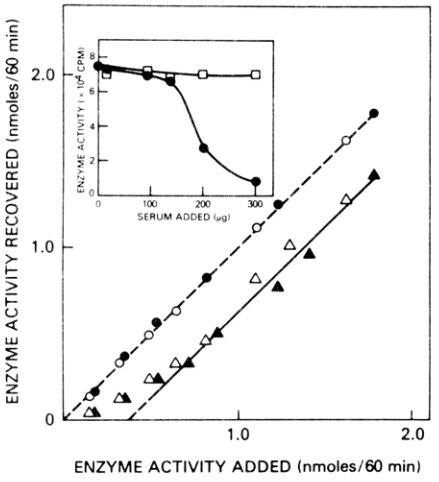

FIGURE 5: Immunotitration of *S*-Ado-Met decarboxylase with rabbit anti-*S*-Ado-Met decarboxylase serum. The antibody precipitation of *S*-Ado-Met decarboxylase was assessed by incubating overnight at 4 °C varying amounts of enzyme with 100 μL (140 μg) of control or anti-*S*-Ado-Met decarboxylase serum diluted 50-fold with standard buffer in a final volume of 0.2 mL. After incubation, the mixtures were centrifuged and the supernatants were assayed for enzymatic activity. Control serum incubated with (●) liver enzyme or with (○) mammary gland enzyme; anti-*S*-Ado-Met decarboxylase serum incubated with (△) liver enzyme or with (▲) mammary gland enzyme. Each enzyme preparation was from step 4 (see Table I). Insert: increasing amounts of control (□) or anti-*S*-Ado-Met decarboxylase serum (●) were added to the enzyme preparation from liver.

of enzyme initially present in the samples, a straight line is obtained, indicating that the assay for immunologically reactive protein is proportional to the amount of enzyme activity present (Figure 7, insert). Additional studies showed that the measured Ab_{50} value is not affected by the presence of varying amounts of other proteins in enzyme preparations. Thus, an Ab_{50} determination can be used to examine whether the increase in enzyme activity is accompanied by a concomitant increase in immunologically reactive protein.

It has been shown previously (Fillingame & Morris, 1972; Hölttä et al., 1973; Pegg et al., 1973; Heby et al., 1973; Sakai et al., 1978) that administration of MGBG, a specific inhibitor of *S*-Ado-Met decarboxylase, results in a marked accumulation of *S*-Ado-Met decarboxylase and putrescine, an activator of mammalian *S*-Ado-Met decarboxylase. The data in Table II demonstrate that MGBG treatment caused a 28-fold increase in the activity of *S*-Ado-Met decarboxylase in lactating mouse

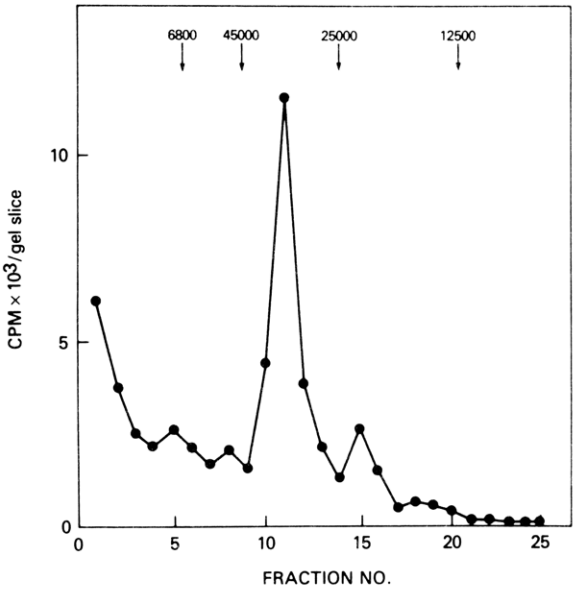

FIGURE 6: $NaDodSO_4$–polyacrylamide gel electrophoresis of immunoprecipitates of *S*-Ado-Met-decarboxylase synthesized by mouse mammary explants in culture. Mammary explants from six mature virgin mice were cultured in the presence of insulin for 24 h, and 3 h before termination of culture [^{3}H]leucine (500 μCi/mL) was added. About 1.3-g tissue explants were homogenized in 2.5 mL of buffer B, and the homogenates were centrifuged at 105000*g* for 60 min. The supernatant solution was divided into two aliquots, and one was incubated with control serum (1 μL) and the other with anti-*S*-Ado-Met decarboxylase serum (1 μL). After incubation at 25 °C for 30 min and then for 2 nights at 4 °C, the mixtures were treated with 10 μL of goat antirabbit serum at 25 °C for 1 h and 4 °C overnight. The immunoprecipitate was collected and washed 3 times as described under Materials and Methods. The immunoprecipitate was then boiled for 15 min in the presence of 1% $NaDodSO_4$ and 2% mercaptoethanol and applied to $NaDodSO_4$ electrophoresis gel (10% gel). After electrophoresis, gels were sliced (each 2 mm), and each slice was counted for radioactivity in a Triton–toluene base counting mixture as described under Materials and Methods.

Table II: Effect of MGBG on the Activity and the Amount of *S*-Ado-Met Decarboxylase in Mouse Mammary Gland[a]

treatment	sp act. [pmol/(mg h)]	increase (*x*-fold)	Ab_{50} (μg/mg)	increase (*x*-fold)
saline	17.8		92.6	
MGBG	49.8	27.9	1990	21.5

[a] Mice were injected with MGBG (2 mg/100 g of body weight, intraperitoneally) and killed 24 h thereafter. The mammary glands were homogenized, and the 105000*g* supernatant fractions were prepared as described under Materials and Methods. Enzyme activity was measured as described under Materials and Methods, and Ab_{50} was determined as described in the caption to Figure 7.

mammary gland and that this increase is accompanied by about the same degree of change in the Ab_{50} value. Thus, the observed increase in enzyme activity appears to be due to an increase in the amount of enzyme and not to activation of preexisting inactive enzyme by putrescine.

Discussion

In the present studies, *S*-Ado-Met decarboxylase in mouse mammary gland and liver has been purified to apparent homogeneity with an overall yield of 13 to 14%. The purification procedure, which differs from a previously published method for rat liver (Pegg, 1974; Demetriou et al., 1977) and yeast enzyme (Pösö et al., 1975; Cohn et al., 1977), requires additional gel filtration at the final step and the use of various stabilizing agents, including putrescine, pyridoxal phosphate, a detergent (0.01% deoxycholate), and high salts (0.4 mM

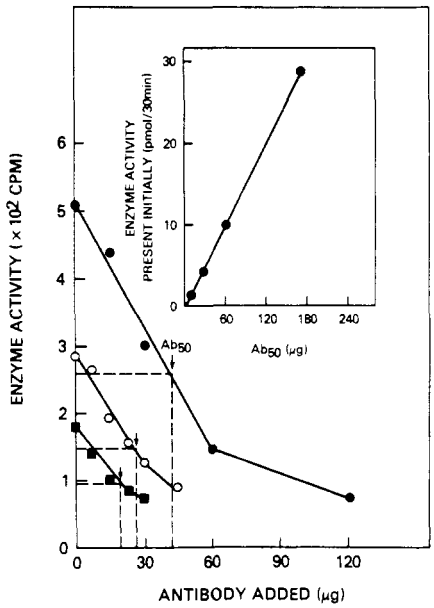

FIGURE 7: Determination of Ab_{50}. *S*-Ado-Met decarboxylase preparations containing various amounts of enzyme activities were prepared from mouse liver and mammary gland and were incubated with varying amounts of diluted anti-*S*-Ado-Met decarboxylase serum or control serum in a final volume of 0.2 mL. The mixtures were incubated at 27 °C for 30 min and then at 4 °C overnight. The mixtures were centrifuged, and the supernatant fractions were assayed for enzyme activity remaining. Ab_{50} is determined graphically as the amount of antibody preparation necessary to inactivate 50% of the enzyme activity. Insert: a separate experiment indicating that Ab_{50} is linear as a function of the amount of enzyme activity initially present.

NaCl), at appropriate steps of purification. Calculations based on the specific activities of crude and purified preparations indicate that *S*-Ado-Met decarboxylase constitutes about 0.0014 and 0.014% of the total soluble protein of MGBG-treated mouse liver and mammary gland, respectively.

The purified enzymes from the two tissues are identical with respect to various enzymatic, physicochemical, and immunological properties. The molecular weight determined under denaturing conditions either with the purified enzyme or with immunoprecipitated enzyme was ~32 000, indicating that the native protein appears to be a dimer. Some of these characteristics of mouse *S*-Ado-Met decarboxylase are similar to those reported earlier for enzyme from rat liver (Pegg, 1974, 1977; Hannonen, 1975; Demetriou et al., 1977), prostate gland (Pegg & Williams-Ashman, 1969), and yeast (Pösö et al., 1975; Cohn et al., 1977). The present data are also in accord with the recent findings by Pegg (1974), Pösö et al. (1975), Cohn et al. (1977), Demetriou et al. (1977), and Oka et al. (1977) that *S*-Ado-Met decarboxylase neither possesses spermidine synthase activity nor requires pyridoxal 5′-phosphate as the enzyme cofactor. More recently, several groups of investigators have demonstrated the presence of covalently bound pyruvate, in place of pyridoxal 5′-phosphate, in the enzymes from yeast (Cohn et al., 1977) and rat liver (Pegg, 1977; Demetriou et al., 1977), which provides a catalytically essential carbonyl group at the catalytic center, as was shown earlier with bacterial decarboxylase (Wicker et al., 1970).

One of the unique features of eucaryotic *S*-Ado-Met decarboxylase is that the enzyme activity is stimulated markedly by putrescine (Pegg & Williams-Ashman, 1969; Coppoc et al., 1971). The present studies with a purified enzyme preparation showed that maximal stimulation by putrescine can be effected at a concentration as low as 5×10^{-7} M, which is far below the value obtained with crude preparations (Pegg & Williams-Ashman, 1969; Manen & Russell, 1974; Pösö et al., 1975) and is in the range of physiological concentrations (Oka & Perry, 1974; Sakai et al., 1978). Stimulation by putrescine is effected partly by the change in the apparent K_m for the enzyme substrate and partly by stabilization of the enzyme. On the other hand, another polyamine, spermine, has been shown to inhibit enzyme activity at physiological concentrations (Oka & Perry, 1974; Sakai et al., 1978) by decreasing the maximum velocity of the enzyme reaction. Inhibition by spermine could be competitively reversed by the concomitant presence of putrescine (unpublished experiments). However, once the enzyme is inhibited, putrescine does not reactivate it. Although the molecular mechanism for these actions of putrescine and spermine remains unknown, the present data suggest that the precursor (putrescine) and the end product (spermine) of polyamine biosynthesis may act as positive and negative regulatory factors, respectively, in cellular control of polyamine biosynthesis via their action on *S*-Ado-Met decarboxylase, a key target enzyme.

The treatment of mammalian cells with MGBG, a potent inhibitor of *S*-Ado-Met decarboxylase (Williams-Ashman & Schenone, 1972) has been shown to block polyamine biosynthesis with the resultant accumulation of both putrescine and *S*-Ado-Met decarboxylase (Fillingame & Morris, 1972; Hölttä et al., 1973; Pegg et al., 1973; Sakai et al., 1978). The present studies indicate that increased accumulation of *S*-Ado-Met decarboxylase activity in the mammary gland of mice treated with MGBG is primarily due to alteration in synthesis and degradation of enzyme molecules rather than activation of preexisting inactive enzyme. Similar results have recently been reported by Pegg in the studies of rat liver enzyme using similar immunological methods (Pegg, 1979).

Rapid turnover of mammalian *S*-Ado-Met decarboxylase has been suggested by earlier studies with cycloheximide (Russell & Taylor, 1971). The present studies have shown that putrescine exerts a stabilizing effect on the activity of *S*-Ado-Met decarboxylase. Furthermore, it has been shown in various systems (Fillingame & Morris, 1972; Heby et al., 1973; Heby & Russell, 1974; Sakai et al., 1978) that the intracellular level of putrescine increases markedly following treatment with MGBG. Thus, it is conceivable that MGBG-induced accumulation of *S*-Ado-Met decarboxylase may be primarily effected through stabilization of the enzyme by putrescine. It is also possible, however, that MGBG itself may also protect the enzyme against degradation by altering the conformation of enzyme through its high binding characteristics, as postulated previously (Fillingame & Morris, 1973; Pegg et al., 1973; Hölttä et al., 1973). Further studies are in progress to assess immunochemically the role of synthesis and degradation in the regulation of *S*-Ado-Met decarboxylase in the development of mouse mammary gland.

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Stereochemical Courses of Nucleotidyltransferase and Phosphotransferase Action. Uridine Diphosphate Glucose Pyrophosphorylase, Galactose-1-phosphate Uridylyltransferase, Adenylate Kinase, and Nucleoside Diphosphate Kinase†

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ABSTRACT: Reactions catalyzed by UDP-glucose pyrophosphorylase, galactose-1-P uridylyltransferase, and nucleoside diphosphate kinase can be analyzed for their stereochemical courses, by using nuclear magnetic resonance and mass spectroscopic techniques with either sulfur or sulfur and ^{18}O-substituted nucleotides as substrates. The UDP-glucose pyrophosphorylase reaction proceeds with inversion of configuration at P_α of uridine-5′-(1-thiodiphospho)glucose, and the galactose-1-P uridylyltransferase reaction proceeds with retention. Therefore, the bond cleavage/formation mechanisms for these chemically matched reactions cannot be identical. The simplest explanation for the stereochemical results is that the former reaction involves a single displacement at P_α and the latter involves a double-displacement mechanism. Thiophosphoryl group transfer by nucleoside diphosphate kinase acting on the [^{18}O]phosphorothioate substrate analogue of ATP chirally substituted at P_γ proceeds with retention of configuration, in contrast to the inversion of configuration observed earlier in the adenylate kinase reaction [Richard, J. P., & Frey, P. A. (1978) *J. Am. Chem. Soc. 100*, 7757]. As in the uridylyltransferases, these chemically matched reactions occur by different mechanisms, the former by a double and the latter by a single displacement at P_γ of ATP. UDP-glucose pyrophosphorylase accepts uridine 5′-(1-thiophosphate) having the *R* configuration at P_α and uridine 5′-(1-thiodiphospho)-glucose with the *S* configuration at P_α as substrates, while galactose-1-P uridylyltransferase accepts only the *R* configuration at P_α of uridine 5′-(1-thiodiphospho)glucose or -galactose. By use of these stereochemical preferences P_α epimers of uridine 5′-(1-thiotriphosphate) and uridine 5′-(1-thiodiphosphate)glucose can be prepared in pure form from synthetic mixtures.

Phosphotransferase and nucleotidyltransferases are large and interesting classes of enzymes whose mechanisms have been extensively studied. In enzymatic group transfer reactions many questions arise concerning the precise functional role of the enzyme. One question is whether the enzyme catalyzes the direct transfer of the group between acceptors bound at the active site or whether the enzyme mediates the transfer by providing a functional group to which the group being transferred is covalently bonded as a transient intermediate.

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