Guengerich, F. P., Wang, P., & Davidson, N. K. (1982b) *Biochemistry 21*, 1698–1706.
Gustafsson, J. A., & Stenberg, A. (1974) *J. Biol. Chem. 249*, 711–718.
Gustafsson, J. A., Mode, A., Norstedt, G., & Skett, P. (1983) *Annu. Rev. Physiol. 45*, 51–60.
Heuman, D. M., Gallagher, E. J., Barwick, J. L., Elshourbagy, N. A., & Guzelian, P. S. (1982) *Mol. Pharmacol. 21*, 750–760.
Imai, Y. (1979) *J. Biochem. (Tokyo) 86*, 1697–1707.
Jacobson, M., & Kuntzman, R. (1969) *Steroids 13*, 327–341.
Kamataki, T., Ando, M., Yamazoe, Y., Ishii, K., & Kato, R. (1980) *Biochem. Pharmacol. 29*, 1015–1022.
Kamataki, T., Maeda, K., Yamazoe, Y., Nagai, T., & Kato, R. (1983) *Arch. Biochem. Biophys. 225*, 758–770.
Kaminsky, L. S., Fasco, M. J., & Guengerich, F. P. (1981) *Methods Enzymol. 74*, 262–272.
Kato, R. (1974) *Drug Metab. Rev. 3*, 1–32.
Kuwahar, S., Harada, N., Yoshioka, H., Miyata, T., & Omura, T. (1984) *J. Biochem. (Tokyo) 95*, 703–714.
Levin, W., Ryan, D., Kuntzman, R., & Conney, A. H. (1975) *Mol. Pharmacol. 11*, 190–200.
MacDonald, M. G., Fasco, M. J., & Kaminsky, L. S. (1981) *Dev. Pharmacol. Ther. 3*, 1–11.
Maeda, K., Kamataki, T., Nagi, T., & Kato, R. (1984) *Biochem. Pharmacol. 33*, 509–512.
Miwa, G. T., West, S. B., & Lu, A. Y. H. (1978) *J. Biol. Chem. 253*, 1921–1929.
Nerland, D. E., & Mannering, G. J. (1978) *Drug Metab. Dispos. 6*, 150–153.
Pasleau, F., Kolodzici, C., Kremers, P., & Gielen, J. E. (1981) *Eur. J. Biochem. 120*, 213–220.
Pfeiffer, C. A. (1936) *Am. J. Anat. 58*, 195–221.
Ryan, D. E., Thomas, P. E., Reik, L. M., & Levin, W. (1982) *Xenobiotica 12*, 727–744.
Ryan, D. E., Iida, S., Wood, A. W., Thomas, P. E., Lieber, C. S., & Levin, W. (1984) *J. Biol. Chem. 259*, 1239–1250.
Shiverick, K. T., & Neims, A. H. (1979) *Drug Metab. Dispos. 7*, 290–295.
Strobel, H. W., & Dignam, J. D. (1978) *Methods Enzymol. 52*, 89–96.
Thomas, P. E., Korzeniowski, D., Ryan, D., & Levin, W. (1979) *Arch. Biochem. Biophys. 192*, 524–532.
van der Hoeven, T. A., & Coon, M. J. (1974) *J. Biol. Chem. 249*, 6302–6310.
Waxman, D. J. (1984) *J. Biol. Chem. 259*, 15481–15490.
Waxman, D. J., & Walsh, C. (1982) *J. Biol. Chem. 257*, 10446–10457.
Waxman, D. J., & Walsh, C. (1983) *Biochemistry 22*, 4846–4855.
Waxman, D. J., Ko, A., & Walsh, C. (1983) *J. Biol. Chem. 258*, 11937–11947.
West, S. B., Huang, M.-T., Miwa, G. T., & Lu, A. Y. H. (1979) *Arch. Biochem. Biophys. 193*, 42–50.
Wood, A. W., Ryan, D. E., Thomas, P. E., & Levin, W. (1983) *J. Biol. Chem. 258*, 8839–8847.

Quantitation of *S*-Adenosylmethionine Decarboxylase Protein†

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ABSTRACT: A method for the specific labeling of the active site of *S*-adenosylmethionine decarboxylase was developed. The method consisted of incubating cell extracts with ^{3}H-decarboxylated *S*-adenosylmethionine and sodium cyanoborohydride in the presence of a spermidine synthase inhibitor. Under these conditions, *S*-adenosylmethionine decarboxylase was labeled specifically and stoichiometrically. This procedure was used (a) to establish that the subunit molecular weight of *S*-adenosylmethionine decarboxylase from rat liver, prostate, and psoas and from mouse SV-3T3 cells was 32 000, (b) to titrate the number of active molecules of *S*-adenosylmethionine decarboxylase in various cell extracts, and (c) to provide a high specific activity labeled preparation of *S*-adenosylmethionine decarboxylase for use in radioimmunoassay of this enzyme. Competitive radioimmunoassays using this labeled antigen had a sensitivity such that 3 fmol (0.1 ng) of enzyme protein could be quantitated. The rapid loss of *S*-adenosylmethionine decarboxylase which occurred when SV-3T3 cells were exposed to exogenous polyamines was shown to be due to a rapid decline in the amount of enzyme protein measured both by titration of the active site and by radioimmunoassay.

The biosynthesis and interconversion of polyamines in mammalian cells are highly regulated processes, and several of the enzymes involved in this pathway exhibit remarkable fluctuations in activity under a wide range of physiological conditions (Jänne et al., 1978; Pegg & McCann, 1982; Tabor & Tabor, 1984a). These enzymes [ornithine decarboxylase, spermidine/spermine N^1-acetyltransferase, and *S*-adenosylmethionine decarboxylase (AdoMetDC)] are present in very small amounts even after maximal induction (Pegg et al., 1982). Therefore, methods for the quantitative estimation of the amount of protein, which are needed to investigate the mechanism by which changes in enzyme activity are brought about, have been difficult to develop. Recently, advances in the understanding of the regulation of ornithine decarboxylase have been made by the use of a technique in which the enzyme is labeled stoichiometrically by reaction with radioactive α-(difluoromethyl)ornithine (DFMO) (Pritchard et al., 1981). Such labeling has been used to titrate the number of enzyme molecules present in cell extracts, to identify the protein after

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electrophoretic separation, and to provide a labeled antigen for competitive radioimmunoassay (Seely et al., 1982a–c; Seely & Pegg, 1983). The objective of the present experiments was to develop a comparable method for the study of AdoMetDC.

AdoMetDC, which has been the subject of several recent reviews (Williams-Ashman & Pegg, 1981; Tabor & Tabor, 1984b; Pegg, 1984a), is a key enzyme of polyamine biosynthesis, providing decarboxylated AdoMet which is the aminopropyl donor substrate for the spermidine and spermine synthase reactions. AdoMetDC isolated from mammalian cells is activated by putrescine whereas AdoMetDC from *Escherichia coli* requires Mg^{2+}. Also, the mammalian and bacterial enzymes differ substantially in size and subunit composition. However, all forms of AdoMetDC so far examined contain a covalently bound pyruvate which serves as a prosthetic group (Tabor & Tabor, 1984a,b; Pegg, 1984a). Pankaskie & Abdel-Monem (1980a,b) reported that AdoMetDC can be inactivated by the addition of sodium cyanoborohydride in the presence of the AdoMet substrate or decarboxylated AdoMet product. Such inhibition presumably results from the reduction of the azomethine bond between the substrate (or product) and the pyruvate moiety. Covalent binding of radioactive AdoMet (or decarboxylated AdoMet) to protein during such inactivation was described by Pankaskie & Abdel-Monem (1980b) for the rat liver enzyme and by Markham et al. (1982) for the *E. coli* AdoMetDC. In the present paper, we describe conditions under which this procedure can be used to label and quantitate AdoMetDC in crude tissue extracts. The labeling procedure was also used to investigate the purity of AdoMetDC purified from various rat tissues, to set up a radioimmunoassay for AdoMetDC protein, and to investigate the molecular weight of the enzyme subunit since there are substantial discrepancies in the values reported in the literature (Pegg, 1974, 1977; Demetriou et al., 1978; Sakai et al., 1979; Pösö & Pegg, 1982; Seyfried et al., 1982; Shain et al., 1983). The titration procedure and radioimmunoassay were then used to investigate the mechanism by which spermidine regulates the activity of AdoMetDC in SV-3T3 cells.

Materials and Methods

Materials. DFMO was a generous gift from Merrell Dow Research Institute, Cincinnati, OH. *S*-Adenosyl-1,8-diamino-3-thiooctane was kindly provided by Dr. J. Coward, Rensselaer Polytechnic Institute, Troy, NY. Radiochemicals were obtained from NEN, Boston, MA. Methylglyoxal bis(guanylhydrazone) was purchased from Aldrich, Milwaukee, WI. Biochemical reagents were purchased from Sigma Chemical Co., St. Louis, MO. *E. coli* strain HT383/pSPD1, which overproduces AdoMetDC (Markham et al., 1982), was kindly provided by Drs. C. W. Tabor and H. Tabor, National Institutes of Health, Bethesda, MD.

Assay of AdoMetDC Activity. AdoMetDC was assayed by measuring the release of $^{14}CO_2$ from [carboxyl-^{14}C]AdoMet as previously described (Pösö & Pegg, 1982; Pegg & Jacobs, 1983). One unit of enzyme activity was defined as the amount of enzyme which releases 1 nmol of CO_2 per minute under the standard assay conditions. Protein was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Preparation of AdoMetDC. AdoMetDC from *E. coli* was purified by a slight modification of the method of Markham et al. (1982) in which high-pressure liquid chromatography (HPLC) separation on DEAE-cellulose replaced the final conventional DEAE-cellulose step. The enzyme eluted from methylglyoxal bis(guanylhydrazone) (MGBG)–Sepharose was concentrated by ultrafiltration and 0.5 mL applied to a Waters DEAE-SPW (7.5 mm × 7.5 cm) column which was eluted with a gradient of 200–800 mM potassium phosphate, pH 7.4, at a flow rate of 1.0 mL/min. The peak corresponding to AdoMetDC activity which was coincident with the major UV-absorbing peak was collected and used as a source of AdoMetDC. This preparation gave a single band on analysis by polyacrylamide gel electrophoresis and represented 143-fold purification of the original extract in 25% yield. AdoMetDC from rat tissues was isolated essentially as described by Pösö & Pegg (1982). The rats were treated with 80 mg/kg MGBG 23 h prior to death in order to increase the amount of AdoMetDC, and the major step in the purification involved affinity chromatography on MGBG–Sepharose (Pegg, 1974). Crude tissue extracts for the assay of AdoMetDC activity or the estimation of AdoMetDC protein were prepared by homogenization of the tissue in 2 volumes of 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.5, 2.5 mM putrescine dihydrochloride, 2.5 mM dithiothreitol, and 0.1 mM ethylenediaminetetraacetic acid (EDTA) (buffer A) at 0–2 °C. The homogenates were centrifuged at 105000*g* for 30 min, and the supernatant was removed and dialyzed for 48 h against two changes of 100 volumes of buffer A before use. Tissues were removed from male Sprague-Dawley strain rats treated with 80 mg of MGBG/kg body weight 23 h prior to death.

Preparation of ^{3}H-Decarboxylated AdoMet. Radioactive decarboxylated AdoMet was synthesized enzymatically by using *E. coli* AdoMetDC. The enzyme (13 units) was incubated with 0.46 mCi of [*methyl*-^{3}H]AdoMet (80 Ci/mmol), 0.2 M Tris-HCl, pH 7.5, 0.1 M $MgCl_2$, and 5 mM dithiothreitol at 37 °C for 1 h in a total volume of 5 mL. The reaction was terminated by the addition of 1.25 mL of 50% (w/v) trichloroacetic acid. After removal of the precipitate by centrifugation at 4000*g* for 10 min, the supernatant was applied to a column (0.8 cm × 10 cm) of Dowex 50x2 (H^+ form). The column was washed with 0.5 N HCl and the column was then eluted with 6 N HCl. The eluate was evaporated to dryness under reduced pressure and the residue dissolved in 0.2 mL of 0.3 M ammonium formate, pH 4.0, and separated by HPLC on a Partisil-SCX column (4.6 mm × 100 mm) at 50 °C at a flow rate of 1 mL/min using isocratic elution with 0.3 M ammonium formate, pH 4.0. The peak corresponding to the decarboxylated AdoMet was collected and desalted by use of the Dowex 50 column as described above. About 0.16 mCi of *methyl*-^{3}H-decarboxylated AdoMet was obtained.

Titration of AdoMetDC. The standard procedure used for crude tissue extracts consisted of incubation with 12–15 nM (100000–125000 dpm) methyl-^{3}H-decarboxylated AdoMet, 150 mM sodium cyanoborohydride, 8 μM *S*-adenosyl-1,8-diamino-3-thiooctane (AdoDATO), 2.5 mM dithiothreitol, 2.5 mM putrescine, 0.1 mM EDTA, 50 mM sodium phosphate, pH 7.5, and 20 mM methionine at 37 °C in a total volume of 0.05 mL. After 90 min, 1 mL of 1 M perchloric acid was added followed by 0.03 mL of 5% (w/v) bovine serum albumin. After 5 min at 37 °C, the samples were sonicated in a Branson B-3 ultrasonic cleaning bath, and the pellet was collected by centrifugation at 15600*g* for 5 min and washed twice by resuspension in 1 mL of 1 M perchloric acid followed by sonication and centrifugation. The pellet was dissolved in 0.3 mL of NCS tissue solubilizer (Amersham-Searle), mixed with 10 mL of formula 949 liquid scintillation fluid (New England Nuclear), and counted for radioactivity at an efficiency of 38–43%. The standard titration assay procedure contained 20 mM methionine since this reduced the blank

value for nonspecific incorporation without affecting the specific binding. The remaining nonspecific incorporation was determined in a control incubation which contained 1 mM MGBG. Specific incorporation was obtained by subtracting the radioactivity bound in this reaction from that found in the standard assay. In some experiments, the time of incubation and concentration of reagents were varied, and purified preparations of AdoMetDC were used as indicated in the figure legends. Also, in some experiments, aliquots were withdrawn at various times and used to determine the amount of AdoMetDC activity remaining.

Radioimmunoassay (RIA) of AdoMetDC Protein. AdoMetDC was purified from rat liver as previously described (Pösö & Pegg, 1982), and about 40 μg of the protein in 0.12 mL of 10 mM Tris-HCl, pH 7.5, 2.5 mM putrescine, 2.5 mM dithiothreitol, 0.1 mM EDTA, and 0.02% Brij 35 (buffer B) was emulsified with an equal volume of Freund's complete adjuvant and used to immunize a Balb/c female mouse by hypodermal injection. The mouse received two further injections of 40 μg of protein in incomplete adjuvant, the first by hypodermal injection 3 weeks later and the second by intraperitoneal injection 5 weeks later. After a further 3 weeks, the mouse received 40 μg of protein in buffer B containing 0.15 M NaCl by intravenous injection. Three days later, the mouse was sacrificed, and the spleen was used for production of monoclonal antibodies to AdoMetDC. The blood was collected and the serum used as a source of antiserum for RIA. The labeled antigen was prepared by incubating a total volume of 1 mL containing 34 units of the purified rat liver AdoMetDC with 0.125 μM methyl-^{3}H-decarboxylated AdoMet (80 Ci/mmol), 150 mM sodium cyanoborohydride, 100 mM sodium phosphate (pH 7.5), 150 mM NaCl, 2.5 mM putrescine, 2.5 mM dithiothreitol, and 0.1 mM EDTA at 37 °C for 90 min. The unbound labeled decarboxylated AdoMet was removed by dialysis twice against 200 volumes of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2.5 mM putrescine, 2.5 mM dithiothreitol, and 0.1 mM EDTA for 24 h at 4 °C. Approximately 2.9 μCi of labeled AdoMetDC was obtained. The RIA procedure, dilution of antiserum, ^{3}H-labeled AdoMetDC, and protein A bacterial adsorbent were carried out in buffer C which consists of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2.5 mM putrescine, 2.5 mM dithiotheitol, 0.1 mM EDTA, and 0.1% bovine serum albumin. The sample (40 μL) was mixed with 100 μL of antiserum (diluted 250-fold) and incubated for 3 h at room temperature (20 °C). Labeled AdoMetDC (50 μL; 8000 dpm) was then added, and after being thoroughly mixed, the sample was incubated for 16–18 h at 4 °C. After the incubation, 25 μL of 10% protein A suspension was added and mixed, and the mixture was incubated for 90 min at 4 °C. The pellet was collected by centrifugation at 15600*g* for 1 min, washed once by resuspension in 0.2 mL of buffer C, dissolved in 0.3 mL of NCS tissue solubilizer, mixed with 10 mL of formula 949 liquid scintillation fluid, and counted for radioactivity. Calibration curves were constructed by using preparations of purified AdoMetDC from rat liver in which the amount of enzyme present was calculated by the titration method described above. Dilutions of the purified AdoMetDC were made in the buffer used for preparation of cell extracts containing 0.1% bovine serum albumin. The amount of antiserum used was such that about 30% of the added tracer labeled ligand was precipitated in the absence of any unlabeled AdoMetDC.

Polyacrylamide Gel Electrophoresis and Autoradiography. Aliquots of the samples from the titration were diluted with 4 volumes of distilled water and mixed with an equal volume of 5% (w/v) sodium dodecyl sulfate, 5% 2-mercaptoethanol, 10% glycerol, and 62.5 mM Tris-HCl (pH 6.8). The mixture was heated at 100 °C for 2 min and subjected to electrophoresis in 0.1% sodium dodecyl sulfate–15% acrylamide gels (Laemmli, 1970). After electrophoresis, the protein was fixed with 30% methanol, 10% trichloroacetic acid, and 10% acetic acid, treated with Enhance (New England Nuclear), dried, and placed in contact with Kodak XAR-5 X-ray film in the dark at −70 °C for 3–7 days before developing.

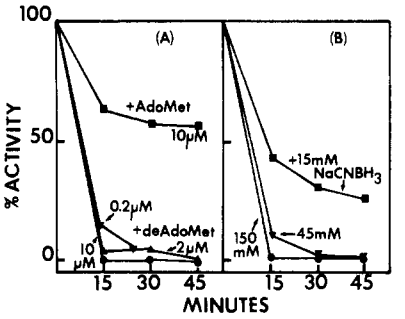

FIGURE 1: Inactivation of AdoMetDC. Partially purified rat liver AdoMetDC was incubated with either AdoMet or decarboxylated AdoMet (deAdoMet) and $NaCNBH_3$ at 37 °C for various times. Duplicate aliquots (10 μL) were removed at the times shown, diluted with 90 μL of 50 mM phosphate buffer, pH 7.5, and assayed for residual AdoMetDC activity. In panel A, results are shown for 150 mM $NaCNBH_3$ with 10 μM AdoMet (■) or with 10 (●), 2 (▲), or 0.2 μM (▼) decarboxylated AdoMet. In panel B, 2 μM decarboxylated AdoMet was used with 150 (●), 45 (▼), or 15 mM (■) $NaCNBH_3$.

Preparation of Extracts from SV-3T3 Cells. Cells were grown in the presence of 5 mM α-(difluoromethyl)ornithine (DFMO) and polyamines added as described by Pegg (1984b). After removal of the medium, the cells were washed 3 times at 4 °C with phosphate-buffered saline and lysed by freeze–thawing in 0.5 mL of 25 mM Tris-HCl, pH 7.5, 2.5 mM putrescine, 2.5 mM dithiothreitol, and 0.1 mM EDTA. The resultant extract was centrifuged at 17000*g* for 30 min and the supernatant removed and used for assays of AdoMetDC. Samples from SV-3T3 cells used for autoradiographic localization of labeled AdoMetDC were concentrated after labeling by precipitation with 1 M perchloric acid and redissolved in the buffer described above and neutralized with 1 N NaOH.

RESULTS

When partially purified rat liver AdoMetDC was incubated in the presence of AdoMet or decarboxylated AdoMet and sodium cyanoborohydride, there was an irreversible loss of enzyme activity (Figure 1). Decarboxylated AdoMet was considerably more effective than AdoMet itself (Figure 1A), and 0.2 μM decarboxylated AdoMet was sufficient to produce complete loss of activity in 30 min. The inactivation rate was increased at higher concentrations of cyanoborohydride (Figure 1B), and 150 mM was used as a standard concentration in further experiments. When radioactive decarboxylated AdoMet was used in a similar procedure, there was incorporation of the labeled nucleoside into protein (Figure 2). The incorporation was temperature dependent (Figure 2A), proceeded slightly more rapidly at pH 7–8 than at pH 6.5 (Figure 2B), and was dependent on the cyanoborohydride concentration (Figure 2C) and the decarboxylated AdoMet concentration (Figure 2D). Incorporation was saturable at a value dependent on the amount of enzyme added, and the same saturation value was reached in all cases even when the decarboxylated AdoMet concentration was reduced to 0.02 μM, although in this case complete reaction required 90 min.

As shown in Figure 3, the inactivation and labeling of mammalian AdoMetDC were completely prevented by the

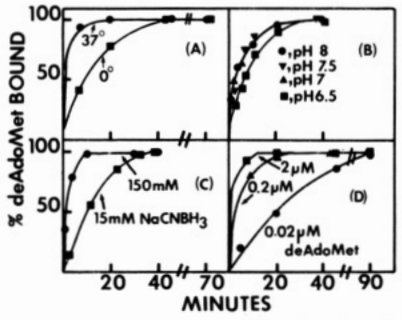

FIGURE 2: Labeling of AdoMetDC with ^{3}H-decarboxylated AdoMet. Partially purified rat liver AdoMetDC was incubated with ^{3}H-decarboxylated AdoMet for various times, and incorporation of label into protein was measured as described under Materials and Methods. The results are expressed as a percentage of the maximal labeling achieved after 90-min incubation at pH 7.5, 37 °C, with 150 mM $NaCNBH_3$ and 2 μM decarboxylated AdoMet (deAdoMet). Panel A shows effects of temperature [37 (●) or 0 °C (■)] with 150 mM $NaCNBH_3$ and 2 μM decarboxylated AdoMet. Panel B shows effects of pH [8 (●), 7.5 (▼), 7 (▲), or 6.5 (■)] with 150 mM $NaCNBH_3$ and 0.2 μM decarboxylated AdoMet. Panel C shows effects of 150 (●) and 15 mM (■) $NaCNBH_3$ with 0.2 μM decarboxylated AdoMet. Panel D shows effects of 0.02 (●), 0.2 (▲), or 2 μM (■) decarboxylated AdoMet with 150 mM $NaCNBH_3$.

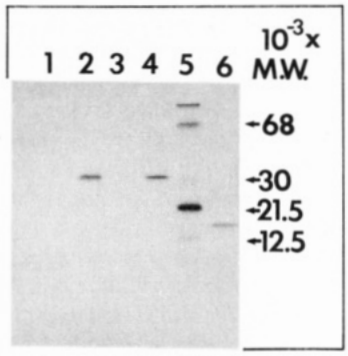

FIGURE 3: Autoradiograph of AdoMetDC labeled in the presence and absence of MGBG. Protein was labeled by reaction with ^{3}H-decarboxylated AdoMet as in Figure 2 for 90 min and subjected to polyacrylamide gel electrophoresis as described under Materials and Methods using partially purified rat liver AdoMetDC (lanes 1–4) or purified *E. coli* AdoMetDC (lane 6). Results in lanes 1 and 3 are from incubations containing 1 mM MGBG; results in lanes 1 and 2 are from assays also containing crude 105000*g* supernatant from rat liver homogenates. Lane 5 shows molecular weight markers. Those corresponding to M_r 68 000, 30 000, 21 500, and 12 500 are indicated by arrows.

presence of 1 mM methylglyoxal bis(guanylhydrazone) (MGBG), which is known to be a potent competitive inhibitor (Williams-Ashman & Pegg, 1981; Porter et al., 1981). When rat liver AdoMetDC preparations were labeled by this procedure, the radioactivity was incorporated into a protein of M_r 32 000 whereas when *E. coli* AdoMetDC was used the protein labeled had a molecular weight of about 16 000 (Figure 3). These values are in reasonable agreement with published values for the subunit molecular weight of AdoMetDC from these sources (Pegg, 1984a; Tabor & Tabor, 1984b; see Discussion). The specificity of labeling was demonstrated by the experiment shown in Figure 4 in which both crude and purified AdoMetDC preparations from rat liver, prostate, and psoas were labeled and subjected to polyacrylamide gel electrophoresis and autoradiography. In all cases, only the M_r 32 000 protein subunit of AdoMetDC was labeled. Similarly, when crude extracts from SV-3T3 cells were used as a source of AdoMetDC, only a M_r 32 000 protein was labeled (results not shown).

A faint band of lower molecular weight (approximately 26 000) was seen when some purified liver AdoMetDC preparations were analyzed by this method (Figure 4, lane 1). This band was seen only with preparations which had been stored for several weeks at −20 °C and was not found in freshly purified preparations from liver or other tissues. It is, therefore, likely that this band represents a partial degradation product.

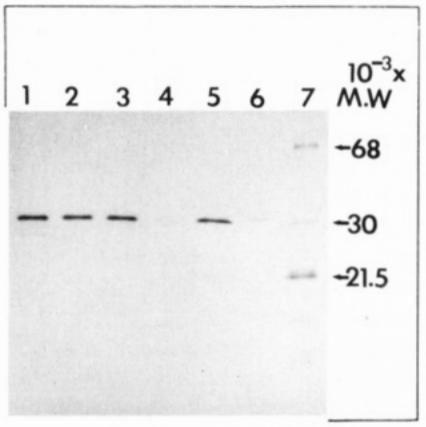

FIGURE 4: Autoradiographs of labeled AdoMetDC from rat tissues. Protein was labeled and analyzed as in Figure 3. The following results are shown: (lane 1) purified liver AdoMetDC (48 000 cpm incorporated as determined by counting the protein precipitated by perchloric acid after the labeling procedure); (lane 2) purified prostate AdoMetDC (49 000 cpm); (lane 3) psoas AdoMetDC (50 000 cpm); (lane 4) 105000*g* supernatant of liver homogenates (1000 cpm); (lane 5) 105000*g* supernatant of prostate homogenate (28 000 cpm); (lane 6) 35–65% $(NH_4)_2SO_4$-precipitated fraction from psoas 105000*g* supernatant (about 1500 cpm); (lane 7) molecular weight markers as in Figure 3.

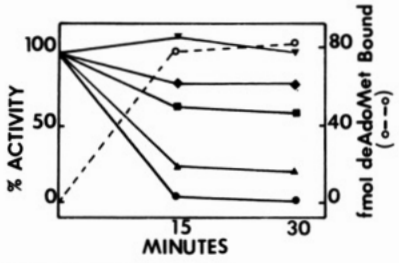

FIGURE 5: Effect of spermidine synthase inhibitors on inactivation and labeling of AdoMetDC in unpurified liver extracts. A 35–65% saturated $(NH_4)_2SO_4$ precipitate of a liver homogenate was used as a source of AdoMetDC and incubated with 150 mM $NaCNBH_3$ and 0.2 μM ^{3}H-decarboxylated AdoMet at 37 °C for the times shown. Duplicate 15-μL aliquots were removed and diluted with 85 μL of 50 mM phosphate buffer, pH 7.5, and residual AdoMetDC activity and labeling of protein were measured. Results are shown for loss of activity in the absence of additional compounds (♦), in the presence of 100 μM dicyclohexylammonium sulfate (■), 1 μM AdoDATO (▲), or 8 μM AdoDATO (●), in the presence of 8 μM AdoDATO, and in the absence of ^{3}H-decarboxylated AdoMet (▼) and for binding of ^{3}H-decarboxylated AdoMet in the presence of 8 μM AdoDATO (○).

In order to maintain a sufficiently high specific radioactivity in decarboxylated AdoMet for labeling of the small amount of AdoMetDC in unfractionated cell extracts, it was necessary to use only a very low concentration of the nucleoside. When crude rat liver extracts were incubated with 0.2 μM decarboxylated AdoMet and 150 mM sodium cyanoborohydride, only a small loss of AdoMetDC activity occurred (Figure 5). Such extracts contain spermidine synthase which in the presence of putrescine can degrade decarboxylated AdoMet by using it as a substrate for spermidine synthesis. This is very likely to be the reason for the lack of AdoMetDC inhibition since the addition of either dicyclohexylamine or *S*-adenosyl-1,8-diaminothiooctane (AdoDATO), which are

Table I: Titration of AdoMetDC from Various Sources[a]

source of enzyme	ratio of initial act. to ^{3}H-decarboxylated AdoMet bound (units/pmol)
purified rat liver (10–240 units/mg)	0.107
crude rat liver (0.004–0.065 unit/mg)	0.210
purified rat prostate (2700–3500 units/mg)	0.121
crude rat prostate (0.06–1.7 units/mg)	0.212
purified rat psoas (50 units/mg)	0.115
crude rat psoas (0.6 unit/mg)	0.183
crude SV-3T3 cells (0.005–0.40 unit/mg)	0.209
E. coli (260 units/mg)	0.060

[a] Results shown are the mean values for estimations on a number of preparations which had the specific activities shown.

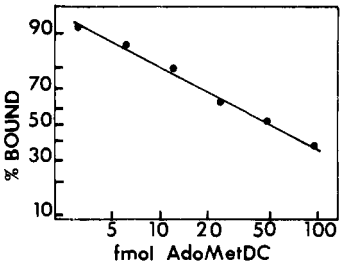

FIGURE 6: Calibration of RIA for AdoMetDC. RIA was carried out as described under Materials and Methods. Results are expressed as the percentage of the tracer ligand bound in the absence of AdoMetDC, and the logit of this value is plotted against the log of AdoMetDC protein added.

known inhibitors of spermidine synthase (Hibasami et al., 1980; Tang et al., 1980; Pegg et al., 1983), increased the loss of AdoMetDC activity (Figure 5). AdoDATO was much more effective in this respect, and 8 μM was sufficient to allow complete inactivation of the AdoMetDC (Figure 5). Under these conditions, there was an excellent correspondence between loss of AdoMetDC activity and incorporation of radioactive decarboxylated AdoMet into protein (Figure 5). Complete labeling could be achieved within 1-h incubation with a decarboxylated AdoMet concentration as low as 15–20 nM (results not shown).

This procedure was used to titrate AdoMetDC preparations from various sources (Table I). Crude cell extracts from various rat tissues and from mouse SV-3T3 cells had activities of about 0.2 unit/pmol (Table I). Highly purified preparations had slightly lower values of about 0.1–0.12 unit/pmol. This loss of catalytic activity during purification may be due to changes in the protein structure which slightly reduce the enzyme turnover number. AdoMetDC from *E. coli* had a titration value of about 0.06 unit/pmol. Assuming that the mammalian enzyme has two subunits (Pösö & Pegg, 1982) of subunit M_r 32 000 and the bacterial enzyme has six subunits of M_r 17 000 (Markham et al., 1982), the titration values indicate that homogeneous AdoMetDC preparations from these sources should have specific activities of 3000 and 3500 units/mg, respectively.

Antiserum was obtained from mice immunized with AdoMetDC purified from rat liver. The antiserum bound the AdoMetDC which had been labeled by titration with radioactive decarboxylated AdoMet, and this protein was used as a ligand for a competitive radioimmunoassay. This radioimmunoassay was sensitive enough for quantitation of 3 fmol of rat or mouse AdoMetDC (Figure 6). As shown in Figure 7, there was no apparent difference between AdoMetDC preparations from various rat tissues in detection by the antiserum. *E. coli* AdoMetDC was not recognized by the antiserum (results not shown).

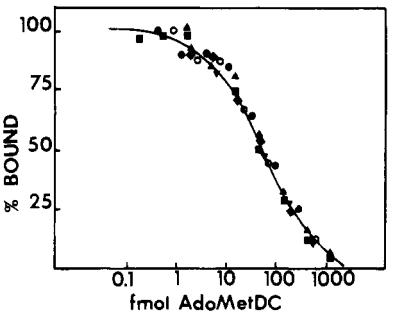

FIGURE 7: RIA of AdoMetDC. RIA was carried out as described under Materials and Methods with the exception that sample, tracer ligand, and antiserum were mixed simultaneously and incubated at 4 °C for 33 h. Results are expressed as the percentage of the tracer ligand bound in the absence of any AdoMetDC against the log of the added AdoMetDC. Results are shown for AdoMetDC preparations purified from liver (○), prostate (▲), or psoas (■) and for 105000*g* supernatant fractions from liver (●) and 35–65% saturated $(NH_4)_2SO_4$ precipitates of supernatant fractions from prostate (♦) or psoas (▼). The amount of AdoMetDC added was determined by titration with ^{3}H-decarboxylated AdoMet.

AdoMetDC activity in SV-3T3 cells depleted of polyamines by DFMO treatment was increased about 10-fold (Pegg, 1984b). When exogenous polyamines were added, there was a rapid reduction in activity (Table II). Activity was completely reduced by spermine within 18 h. Spermidine produced a more rapid fall, but a stable residual activity of about 3% of the starting value was reached (Table II). The decline in AdoMetDC activity was brought about by a loss of AdoMetDC protein measured either by titration or by radioimmunoassay (Table II). Also, there was no change in the molecular weight of the AdoMetDC subunit (which was detected by radioautography as in Figure 4).

Discussion

When carried out under the conditions described in this paper in the presence of spermidine synthase inhibitors, the covalent attachment of decarboxylated AdoMet in the presence of sodium cyanoborohydride provides a highly specific and sensitive method for labeling AdoMetDC. The sensitivity of this procedure could probably be improved further by using decarboxylated AdoMet labeled with ^{35}S. The use of MGBG to prevent binding at the active site of AdoMetDC provides a control for nonspecific binding, but even with extracts containing quite low amounts of AdoMetDC, the nonspecific binding was only a small fraction of the binding to the enzyme. In addition to the obvious use for the method for labeling the active site of AdoMetDC and for titrating the number of molecules of the enzyme, it can also be used to assess the purity of preparations of AdoMetDC. The data in Table I suggest that homogeneous preparations of AdoMetDC should have specific activities of about 3000 units/mg which is quite close to those reported for the best preparations (Markham et al., 1982; Pösö & Pegg, 1982; Seyfried et al., 1982; Shain et al., 1983). The apparent loss of activity during purification which is indicated by the results in Table I may be related to the formation of less active aggregates which happens very readily with purified preparations (Pösö & Pegg, 1982; Pegg, 1984a). It is unlikely to result from partial proteolytic degradation since no change in molecular weight was seen when freshly prepared purified and crude labeled AdoMetDC preparations were compared (Figure 4).

The labeling technique provides a particularly valuable method to assess the molecular weight of AdoMetDC and to provide a marker for autoradiography. In all experiments, rat and mouse AdoMetDC from a variety of cell types had an

Table II: Effect of Polyamines on AdoMetDC in SV-3T3 Cells Treated with α-(Difluoromethyl)ornithine

treatment of cells[a]	AdoMetDC act. (milli-units/mg)	AdoMetDC protein by titration (pmol/mg)	AdoMetDC protein by RIA (pmol/mg)
control	399 ± 49	2.33 ± 0.21	5.04 ± 0.55
10 μM spermine, 4 h	272 ± 24	1.31 ± 0.18	1.93 ± 0.30
10 μM spermine, 8 h	13 ± 4	0.16 ± 0.04	0.39 ± 0.11
10 μM spermine, 18 h	<3	<0.02	<0.14
10 μM spermidine, 4 h	45 ± 3	0.23 ± 0.01	0.69 ± 0.06
10 μM spermidine, 8 h	11 ± 1	0.07 ± 0.01	0.27 ± 0.04
10 μM spermidine, 18 h	14 ± 1	0.06 ± 0.01	0.18
10 μM *sym*-homo-spermidine, 24 h	26 ± 1	0.09 ± 0.01	0.20

[a] Cells were grown in DFMO for 3 days, and the polyamine shown was added. Cells were harvested at the time indicated, and AdoMetDC activity was measured. There was no significant change in the AdoMetDC content of the control cells to which no polyamine was added over the 24-h period.

apparent subunit molecular weight of 32 000 (Figures 4–6). This is in excellent agreement with previous reports from our laboratory (Pegg, 1977; Pösö & Pegg, 1982) and with values for mouse AdoMetDC (Sakai et al., 1979) and calf AdoMetDC (Seyfried et al., 1982). It does not agree with the values reported by Demetriou et al. (1978) of 42 000 for rat liver AdoMetDC or 26 000 reported by Shain et al. (1983) for rat prostate AdoMetDC. The latter preparation could possibly have been slightly degraded (see Figure 4, lane 1), but we have no explanation for the discrepancy between our results and those of Demetriou et al. (1978) since even crude liver extracts show only a single band of M_r 32 000.

Since the labeling procedure with decarboxylated AdoMet and sodium cyanoborohydride is highly specific for AdoMetDC, the labeled protein is particularly valuable as the labeled antigen in competitive radioimmunoassay procedures. Such specific labeling eliminates problems which arise when the antigen is labeled by nonspecific iodination since nonreactive protein fragments can be generated and minor impurities to which antibodies may also have been formed will also be labeled. The radioimmunoassay procedure is much more sensitive and more quantitative than previous procedures for assay of AdoMetDC by immunotitration of activity (Pegg, 1979; Sakai et al., 1979). It is also much more convenient for studies of the regulation of AdoMetDC since the protein can be measured irrespective of its enzymatic activity. However, it should be noted that AdoMetDC from rat psoas muscle is clearly distinguishable from AdoMetDC from rat liver by several criteria including electrophoretic mobility under nondenaturing conditions (Pösö & Pegg, 1982). No difference in the immunoreactivity of AdoMetDC from these sources was seen (Figure 7), indicating that changes in structure responsible for these differences in properties do not affect the antigenic sites. At present, the molecular basis of the difference between the two forms of rat AdoMetDC remains unknown.

The combination of radioimmunoassay to quantitate total AdoMetDC protein and titration with decarboxylated AdoMet to determine the number of molecules of the enzyme with active catalytic centers provides a powerful technique for studying the regulation of AdoMetDC. Several groups have provided evidence that AdoMetDC activity is increased in response to polyamine depletion and is decreased by exposure to exogenous polyamines (Alhonen-Hongisto, 1980; Hopkins & Manchester, 1980; Mamont et al., 1981; Pösö & Pegg, 1981; Pegg, 1984b). The experiment shown in Table II indicates that the rapid reduction in AdoMetDC activity which is brought about by exposure of transformed mouse fibroblasts to polyamines is due to a loss of the enzyme protein. The loss of almost 90% of the activity and protein within 4-h exposure to spermidine confirms the very rapid turnover of AdoMetDC protein. Further studies are needed to investigate the mechanism of degradation and the extent to which polyamines shut off the synthesis of the enzyme.

Registry No. AdoMetDC, 9036-20-8; methyl-^{3}H-decarboxylated AdoMet, 96999-43-8; [*methyl*-^{3}H]AdoMet, 77314-12-6.

References

Alhonen-Hongisto, L. (1980) *Biochem. J. 190*, 747–754.

Bradford, M. M. (1980) *Anal. Biochem. 72*, 248–254.

Demetriou, A. A., Cohn, M. S., Tabor, C. W., & Tabor, H. (1978) *J. Biol. Chem. 253*, 1684–1686.

Hibasami, H., Tanaka, M., Nagani, J., & Ikeda, T. (1980) *FEBS Lett. 116*, 99–101.

Hopkins, D., & Manchester, K. L. (1980) *FEBS Lett. 109*, 299–301.

Jänne, J., Pösö, H., & Raina, A. (1978) *Biochim. Biophys. Acta 473*, 241–293.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Mamont, P. S., Joder-Olenbusch, A.-M., Nussli, M., & Grove, J. (1981) *Biochem. J. 196*, 411–422.

Markham, G. D., Tabor, C. W., & Tabor, H. (1982) *J. Biol. Chem. 257*, 12063–12068.

Pankaskie, M., & Abdel-Monem, M. M. (1980a) *J. Pharm. Sci. 69*, 1000–1006.

Pankaskie, M., & Abdel-Monem, M. M. (1980b) *J. Med. Chem. 23*, 121–127.

Pegg, A. E. (1974) *Biochem. J. 141*, 581–583.

Pegg, A. E. (1977) *FEBS Lett. 84*, 33–36.

Pegg, A. E. (1979) *J. Biol. Chem. 254*, 3249–3253.

Pegg, A. E. (1984a) *Cell Biochem. Funct. 2*, 11–15.

Pegg, A. E. (1984b) *Biochem. J. 224*, 29–38.

Pegg, A. E., & McCann, P. P. (1982) *Am. J. Physiol. 243*, C212–C221.

Pegg, A. E., & Jacobs, G. (1983) *Biochem. J. 213*, 495–502.

Pegg, A. E., Seely, J. E., Pösö, H., Della Ragione, F., & Zagon, I. S. (1982) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 41*, 3065–3072.

Pegg, A. E., Bitonti, A. J., McCann, P. P., & Coward, J. K. (1983) *FEBS Lett. 155*, 192–196.

Porter, C. W., Dave, C., & Mihich, E. (1981) in *Polyamines in Biology and Medicine* (Morris, D. R., & Marton, L. J., Eds.) pp 407–436, Marcel Dekker, New York.

Pösö, H., & Pegg, A. E. (1981) *Biochem. J. 200*, 629–637.

Pösö, H., & Pegg, A. E. (1982) *Biochemistry 21*, 3116–3122.

Pritchard, M. L., Seely, J. E., Pösö, H., Jefferson, L. S., & Pegg, A. E. (1981) *Biochem. Biophys. Res. Commun. 100*, 1597–1603.

Sakai, T., Hori, C., Kano, K., & Oka, T. (1979) *Biochemistry 18*, 5541–5548.

Seely, J. E., & Pegg, A. E. (1983) *J. Biol. Chem. 258*, 2496–2500.

Seely, J. E., Pösö, H., & Pegg, A. E. (1982a) *Biochemistry 21*, 3394–3399.

Seely, J. E., Pösö, H., & Pegg, A. E. (1982b) *Biochem. J. 206*, 311–318.

Seely, J. E., Pösö, H., & Pegg, A. E. (1982c) *J. Biol. Chem. 257*, 7549–7553.

Seyfried, C. E., Oleinik, O. E., Degen, J. L., Resing, K., &

Morris, D. R. (1982) *Biochim. Biophys. Acta 716*, 169–177.
Shain, S. A., Hilliard, J. K., & de Leon, C. (1983) *Endocrinology (Baltimore) 113*, 1292–1297.
Tabor, C. W., & Tabor, H. (1984a) *Annu. Rev. Biochem. 536*, 749–790.
Tabor, C. W., & Tabor, H. (1984b) *Adv. Enzymol. Relat. Areas Mol. Biol. 56*, 251–282.
Tang, K.-C., Pegg, A. E., & Coward, J. K. (1980) *Biochem. Biophys. Res. Commun. 96*, 1371–1377.
Williams-Ashman, H. G., & Pegg, A. E. (1981) *Polyamines in Biology and Medicine* (Morris, D. R., & Marton, L. J., Eds.) pp 43–73, Marcel Dekker, New York.

Kinetics of P_i–P_i Exchange in Rat Liver Mitochondria. Rapid Filtration Experiments in the Millisecond Time Range†

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ABSTRACT: Phosphate–phosphate exchange through the inorganic phosphate (P_i) carrier of rat liver mitochondria was investigated by a new rapid filtration technique, which does not require the use of transport inhibitors to stop the reaction and offers high time resolution (starting from 10 ms), thus allowing kinetic measurements on a fine time scale even at room temperature. At approximately 22 °C, isotopic equilibrium of [^{32}P]P_i is achieved within 0.8–2.5 s—depending on the P_i concentration—and an initial linear phase, lasting for 400–500 ms, is observed. Complete inhibition of P_i exchange by an excess (33 nmol/mg) of mersalyl, a well-known organomercurial inhibitor, required 200 ms, pointing to the insufficiency of this reagent for effective inhibitor stop. On the other hand, investigation of the effect of mersalyl (allowed to react with mitochondria for at least 20 s) on the initial rate of P_i exchange supports earlier observations on the protective effect of this inhibitor; i.e., up to 3 nmol of mersalyl/mg of protein does not decrease the transport rate whereas these low concentrations protect approximately 50% of the transport capacity from irreversible inactivation by *N*-ethylmaleimide. In nonrespiring mitochondria, at pH 7.3, P_i exchange exhibited a K_m of 1.6 mM and a V_{max} of 3.0 μmol min^{-1} (mg of mitochondrial protein)$^{-1}$. The increase of the membrane potential without any concomitant change of ΔpH had no significant influence on the kinetic parameters. The maximal velocity of P_i transport is significantly higher than the maximal velocity of all the other components of oxidative phosphorylation at comparable temperatures. The possible physiological significance of this excess capacity is discussed.

The mitochondrial phosphate carrier brings about both P_i–P_i exchange and net movement of the anion across the inner membrane, the latter process being accompanied by the cotransport of H^+ (or the countertransport of OH^-) [for a review, see Fonyó (1979)]. The carrier protein has been partially purified (Kolbe et al., 1982, 1984), and its kinetic properties have been investigated in reconstituted proteoliposomes (Wohlrab & Flowers, 1982; Wohlrab et al., 1984). The exceptional activity of this transport protein explains the scarcity of comparable data obtained in intact mitochondria. In fact, the only published study (Coty & Pedersen, 1974) was carried out at 0 °C with pCMB,[1] an organomercurial SH reagent in an "inhibitor-stop" assay.

In this paper we report a new approach to kinetic investigations that does not require the use of inhibitor substances to stop the transport process and exhibits significantly higher time resolution than previous techniques. The new method has allowed us to determine kinetic parameters at room temperature and under different metabolic conditions.

Experimental Procedures

Materials. For filtration 1.2-μm pore size cellulose nitrate filters (Millipore RAWP) were used. Carrier-free [^{32}P]-orthophosphoric acid, [^{14}C]methylamine, and [^{14}C]acetate were produced in Saclay, France. The supplied [^{32}P]P_i source was purified by filtration through Millex-HA sterile 0.45-μm filters (Millipore). All the other reagents were of the highest purity commercially available.

Theoretical Aspects of Rapid Filtration. The essential idea of the rapid filtration technique resides in the fact that the transport process is stopped by the cessation of substrate supply and not by the addition of a specific inhibitor. In this way there is no complication due to the evaluation of the time of mixing and action of the blocking agent. The scheme of Figure 1 shows the principle of the rapid filtration device. By means of a specifically designed filter holder, mitochondria are im-

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[1] Abbreviations: NADH, reduced nicotinamide adenine dinucleotide; NEM, *N*-ethylmaleimide; pCMB, *p*-(chloromercuri)benzoic acid; POPOP, 1,4-bis[5-phenyl-2-oxazolyl]benzene; PPO, 2,5-diphenyloxazole; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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