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Detection of Cytosolic Phosphatidylethanolamine N-Methyltransferase in Rat Brain

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Phosphatidylethanolamine N-methyltransferase (PEMT) is known to be a membrane-associated protein. However, cytosolic PEMT was detected when sufficient amounts of exogenous phospholipids were added in the incubation media. The methylation of phospholipids was measured by the incorporation of the [^{3}H]-methyl group from S-adenosylmethionine and the methylated phospholipids were analyzed by thin-layer chromatography. The essence of the assay condition for the cytosolic enzyme was the inclusion of 200 μg of each substrate, phosphatidylethanolamine (PE), phosphatidyl N-monomethylethanolamine (PME) and phosphatidyl N,N-dimethylethanolamine (PDE), in the reaction mixture of 100 μl. The subcellular fractionation of brain PEMT activities revealed that approximately 38.1% for PME, 39.5% for PDE, and 22.4% for PC formation was present in the cytosolic fraction. The general properties of cytosolic PEMT were characterized and compared with those of neuronal nuclei PEMT.

Keywords: Cytosolic, Phosphatidylethanolamine N-Methyltransferase, Translocation.

Introduction

Phosphatidylethanolamine N-methyltransferase (EC 2.1.1.17, PEMT) is an enzyme which catalyzes the sequential methylation of phosphatidylethanolamine (PE) to phosphatidylcholine (PC) using S-adenosylmethionine (SAM) as a methyl donor. The presence and characteristics of this enzyme have been reported in the membrane fraction of various animal sources such as rat brain (Blusztajn *et al.*, 1985; Chung *et al.*, 1990; Park *et al.*, 1991), rat heart (Panagia *et al.*, 1985), rat liver (Ridgway and Vance, 1988), bovine mammary gland (Yang *et al.*, 1991), and bovine retina (Roque and Giusto, 1995). However, our previous study implicated a significant amount of enzyme activity in the cytosolic fraction when the assay condition was altered by saturation with exogenous phospholipids (Park *et al.*, 1991).

PEMT has been known to consist of two methyltransferase proteins, PEMT I and PEMT II (Pajares. *et al.*, 1986; Yang *et al.*, 1991). The first enzyme (PEMT I) methylates PE to form phosphatidyl N-monomethylethanolamine (PME) and has a high affinity for the methyl donor SAM. The second enzyme (PEMT II) methylates PME to form PC by way of phosphatidyl N,N-dimethylethanolamine (PDE) and has a low affinity for SAM. However, several reports have indicated that a single protein could exert all three methylation activities (Ridgway and Vance, 1987). Recently a novel PEMT was cloned from rat liver (Cui *et al.*, 1993).

The physiological roles of PEMT have been speculated, e.g., that the enzyme could play a role related to signal transduction (Hirata and Axelord, 1980). The sequential methylation reactions increase membrane fluidity (Hirata and Axelord, 1978), which aids the coupling of the receptor, transducer, and amplifier. Other examples included Ca^{2+} influx (Hirata and Axelord, 1980), histamine release (Ozawa and Segawa, 1988), and activation of protein kinase C (Villalba *et al.*, 1987). The expression of PEMT in rat hepatomal cells has been shown to suppress cell growth (Cui *et al.*, 1994; Vance *et al.*, 1996; Houweling *et al.*, 1997).

In this study, the cytosolic activity of PEMT previously observed in brain was optimized further and characterized after addition of sufficient amounts of substrate phospholipids in the incubation media. The general properties of cytosolic PEMT, such as effects of temperature, pH, SAM concentration, phospholipid concentration, and Mg^{2+} concentration were examined and compared with those of neuronal nuclei PEMT.

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Materials and Methods

Materials Phosphatidyl N-monomethylethanolamine (dipalmitoyl), phosphatidyl N,N- dimethylethanolamine (dipalmitoyl), egg phosphatidylcholine (PC), S-adenosylmethionine, and S-adenosyl homocysteine were obtained from Sigma Chemical Co. (St. Louis, USA). TLC plastic sheets of silica gel 60 F_{254} was obtained from Merck AG (Darmstadt, FRG). Phosphatidylethanolamine was prepared by transphosphatidylation of egg PC in the presence of ethanolamine and cabbage phospholipase D (Eibl and Kovatchev, 1981). Radioactive S-adenosyl-L-[methyl-^{3}H]-methionine was prepared by the reaction of rat liver methionine adenosyltransferase (Rhim *et al.*, 1991) using L-[methyl-^{3}H]-methionine (specific activity, 72.2 Ci/mmol) which was purchased from NEN (Boston, MA). All other chemicals were reagent grade commercially available. Rats were supplied by the Animal Breeding Laboratory of Seoul National University (Seoul, Korea).

Preparation of the cytosolic fraction Wistar rats (3–4 weeks old) were killed by decapitation and the brains were homogenized by a glass-teflon tissue homogenizer driven by a motor at 850 rpm, with four to five up-and-down strokes in 10 volumes of 0.32 M sucrose. The homogenate was centrifuged at 100,000 × *g* for 1 h and the supernatant was used as the enzyme source.

Assay of cytosolic PEMT The methylation of phospholipids was measured by the incorporation of radioactive methyl groups from SAM (Park *et al.*, 1991; Park *et al.*, 1992). In the standard assay condition, the incubation medium consisted of 100 μl solution containing 10 mM carbonate buffer (pH 10.0), 10 mM $MgCl_2$, 200 μg of PE, 200 μg of PME, 200 μg of PDE, 0.1–0.2 mg protein of the cytosolic fraction, and [^{3}H-methyl]-SAM (specific activity, 600 μCi/mmol). After 2 h incubation at 37°C, the reaction was quenched by adding 3 μl of chloroform/methanol/HCl (2:1:0.02, v/v/v). After vigorous shaking, the organic layer was pipetted and washed twice with 2 ml of 0.1 M KCl in 50% methanol. The extract was dried under a stream of N_2 gas, and the residue was redissolved in 20 μl of chloroform/methanol mixture (2:1, v/v) and applied to a silica gel plate. Phospholipid standards were spotted together and the plate was developed employing a solvent system of chloroform/methanol/n-propanol/water (2:2:3:1, v/v/v/v). The phospholipids were visualized by exposing the air-dried plate to iodine vapor. The spots corresponding to the authentic phospholipid standards were scraped and transferred to a scintillation vial. The scraped samples were counted using LKB 1219 Rackbeta scintillation counter with Omniflour scintillation cocktail. The counting efficiency of 60% was determined by the quenching curve which was obtained by LKB internal standard isotope capsules and adding CCl_4. The enzyme activity was expressed in pmol/mg protein/h and was determined from at least two separate measurements with duplicate samples.

DEAE cellulose chromatography The brain was homogenized with 5 volumes of buffer A (0.32 M sucrose, 0.1 mM PMSF, 5 mM mercaptoethanol, 25 mM Tris-HCl, pH 8.5) and centrifuged at 100,000 × *g* for 1 h. The supernatant was loaded onto a column of DEAE cellulose (5 ml bed vol) previously equilibrated with buffer A. The column was then eluted with buffer A at a flow rate of 70 ml/h. The next step was elution with a linear gradient using buffer A and buffer A plus 1 M NaCl at a flow rate of 60 ml/h.

Assay of ethanolamine N-methyltransferase The methylation of ethanolamine was measured by the incorporation of radioactive methyl groups from SAM (Andriamampandry *et al.*, 1989). The incubation medium consisted of 150 μl of solution containing 10 mM CAPS buffer (pH 10.0), 5 mM $MgCl_2$, 2 mM ethanolamine, 150 μM [^{3}H-methyl]-SAM (2 μCi in a test tube), and protein of the cytosolic fraction. After 2 h incubation at 37°C, the reaction was quenched by adding 500 μl of chloroform/methanol/HCl/water (0.1:1:0.01:1, v/v/v/v). After vigorous shaking, the aqueous layer was washed with 1 ml of $CHCl_3$/methanol (2:1, v/v) and then twice with 1 ml of 0.1 M KCl in 50% methanol. The water phase was pooled and dried under a stream of N_2 gas. The residue was redissolved in 100 μl of 0.5 M HCl and applied to a silica gel plate. Ethanolamine and choline standards were also spotted and the plate was developed employing a solvent system of butanol/methanol/HCl/water (10:10:1:1, v/v/v/v). The spots were visualized by exposing the air-dried plate to iodine vapor. The spots corresponding to the authentic standards were scraped and transferred to scintillation vials. The scraped samples were counted using a scintillation cocktail of toluene/Triton X-100 (2:1, v/v). The enzyme activity was expressed in nmol/mg protein/h.

Results and Discussion

Detection of cytosolic PEMT activities in rat brain In order to detect PEMT activity in the cytosolic fraction (S3), the lipid environment of the cytosolic fraction was maintained uniformly as much as possible to the pellet fraction by saturating with exogenous phospholipid substrates such as PE, PME, and PDE. Under this condition it could be assumed that the activities of PEMT in the cytosolic fraction were fully activated by eliminating the uncertainty of the PEMT activities due to the different lipid environments. With a sufficient amount of phospholipids to drive the reaction, the cytosolic PEMT activities for formation of PME, PDE, and PC were 38.1%, 39.5%, and 22.4%, respectively, of activity presented in the whole homogenate (Table 1). Among the cytosolic PEMT activities, the PME synthesizing activity showed considerably high specific activity compared to the other synthesizing activities and those of the pellet fraction. A typical TLC pattern of methylated phospholipids produced by PEMT in the cytosolic fraction is presented in Fig. 1. About 90% of the recovered radioactivity was detected in the spots of PME (Rf: 0.7), PDE (Rf: 0.5–0.6), and PC (Rf: 0.4). This pattern was reproducible within 10% deviation.

Effects of exogenous phospholipids To examine the effects of exogenous phospholipids on cytosolic PEMT activities, each of the PE, PME, and PDE suspensions were sonicated for 3–5min at 25–30°C and added to the assay media. When the additions of PE without PME and PDE,

Table 1. Detection of PEMT activities in the cytosolic fraction of rat brain

Fraction	Protein[a] (mg)	PME formation	PDE formation	PC formation
Whole Homogenate	170.5	3015.3[b] (100%)[c] 17.69 ± 2.27[d]	1532.3 (100%) 8.99 ± 1.42	1563.1 (100%) 9.17 ± 2.07
100,000 × *g* Pellet	110.5	1845.4 (61.2%) 16.70 ± 1.93	924.9 (60.3%) 8.37 ± 2.73	1182.4 (75.6%) 10.7 ± 2.21
100,000 × *g* Supernatant	50.9	1151.3 (38.1%) 22.62 ± 4.23	605.9 (39.5%) 11.90 ± 1.38	351.2 (22.4%) 6.90 ± 2.78

[a] Total amount of protein from rat brain of 1.5 g.
[b] Total activity (CH_3-incorporation, pmol/h).
[c] Percent activity, proportion to the activity of whole homogenate.
[d] Means of specific activity (CH_3-incorporation, pmol/mg protein/h). Values represent the mean ± S.E. for the activity obtained from four observation in two independent experiments.

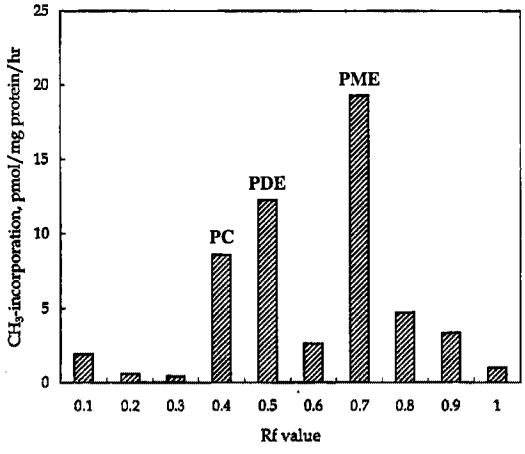

Fig. 1. A typical TLC pattern of methylated phospholipids produced by PEMT in the cytosolic fraction of rat brain.

PME without PE and PDE, and PDE without PE and PME were tested, each activity for PME, PDE, and PC showed sigmoidal curves (data not shown). This observation again suggested that certain amounts of phospholipids are necessary for detection of cytosolic PEMT activity. Alteration of the PE concentration in the presence of PME (200 μg) and PDE (200 μg) caused the activation of PME synthesizing activity but made no noticeable change in the other two activities (Fig. 2A). This PME activation contrasted to the N1 fraction where PE had no effect on the PME activity (Park *et al.*, 1992). The K_m values and maximum velocities of PME synthesizing activity for PE were estimated to be 2.04 mM and 29.83 pmol/mg protein/h, respectively. Similarly, addition of PME vesicles increased PDE synthesizing activity (Fig. 2B) and addition of PDE vesicles increased PC synthesizing activity (Fig. 2C) leaving the other two activities unchanged. The kinetic parameters of the PDE synthesizing activity calculated from PME were 2.21 mM for K_m and

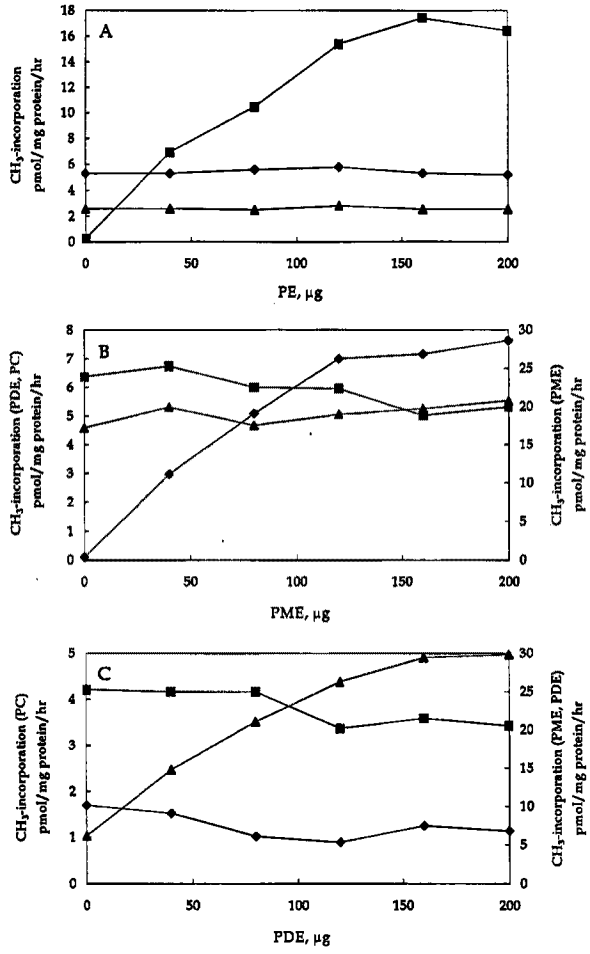

Fig. 2. Effects of exogenous phospholipids on cytosolic PEMT activities in rat brain. A, effect of PE; B, effect of PME; C, effect of PDE. PME, —■—; PDE, —◆—; PC, —▲—.

14.69 pmol/mg protein/h for V_{max} and those corresponding values for PC obtained from PDE were 1.00 mM and 6.89 pmol/mg protein/h, respectively. The kinetic

Table 2. Kinetic parameters of cytosolic (S3) and membrane (N1) PEMT in rat brain.

	Cytosolic (S3) PEMT			Membrane (N1) PEMT[a]		
	PME	PDE	PC	PME	PDE	PC
Phospholipids						
K_m (mM)[b]	2.04	2.21	1.00	ND[c]	0.27	0.22
V_{max} (pmol/mg protein/h)	29.83	14.69	6.89	ND	21.98	12.96
SAM						
K_m (μM)	30.70	14.24	-22.95	15.1	14.9	49.0
V_{max} (pmol/mg protein/h)	21.84	11.93	8.86	7.7	21.1	18.0

[a] The data of membrane PEMT in neuronal nuclear fraction (N1) was recalculated from Park *et al.* (1992).
[b] The K_m values for phospholipid substrates were calculated from synthesizing activities of PME from PE, of PDE from PME, and of PC from PDE.
[c] No dependency.

parameters of cytosolic and membrane (N1) PEMT activities for phospholipid substrate are summarized in Table 2. The V_{max} of PME synthesizing activity shows the highest value among the three activities. However, the V_{max} values of the neuronal nuclear fraction was the highest for PDE synthesizing activity (Park *et al.*, 1991)

Effects of SAM concentration The enzyme activity patterns affected by the variable concentration of SAM are shown in Fig. 3. The PDE and PC synthesizing activities appeared to be saturated by SAM at low concentrations but the PME synthesizing activity was saturated by SAM at a far higher concentration. The kinetic parameters were estimated by a Lineweaver-Burk plot and summarized in Table 2. The K_m value (30.7 μM) and V_{max} (21.84 pmol/mg/h) for PME synthesis was the highest, while K_m for PDE was the lowest value at 14.24 μM and likewise V_{max} for PC was the lowest at 8.86 pmol/mg/h. Comparing with the PEMT activities of the membrane fraction, the PC synthesizing activity had the highest K_m value (49.0 μM) and that of PDE synthesis had the highest V_{max} value (21.1 pmol/mg/h). Alternatively, PDE had the lowest K_m value (14.9 μM) whereas PME had the lowest V_{max} (7.7 pmol/mg/h).

Other general properties The activities of PEMT increased linearly according to the increased amount of cytosolic protein in the incubation media in the range of 0.05–0.2 mg (data not shown). The linearity of incubation time on cytosolic PEMT activities was observed up to 3 h for all PME, PDE, and PC synthesizing activities. The activities seemed to decrease beyond 3 h incubation (data not shown). On examining the effect of temperature on the activities, the maximum activities were observed at 37°C for PDE and PC synthesizing activities, but at 45°C for PME synthesizing activity (Fig. 4A). The pH optima of the activities were pH 10 for all three (Fig. 4B). The cytosolic PEMT activities for the formation of PDE and PC were

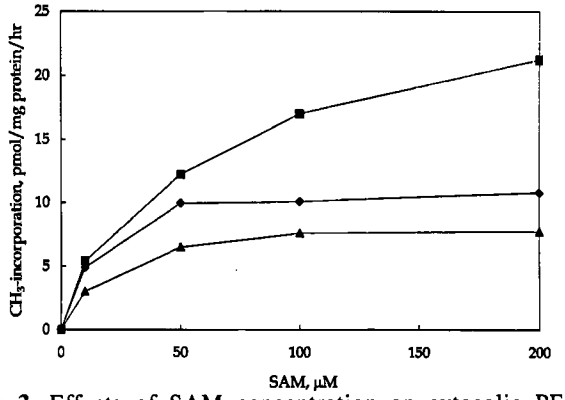

Fig. 3. Effects of SAM concentration on cytosolic PEMT activities in rat brain. PME, —■—; PDE, —◆—; PC, —▲—.

significantly dependent on Mg^{2+} (3–5 fold activation), but that for synthesizing PME was not affected (Fig. 4C). These effects of temperature, pH, and Mg^{2+} were similar to those of the PEMT activities of the neuronal nuclear fraction (Park *et al.*, 1992).

Possibility of translocation of cytosolic PEMT In order to search for a possibility of translocation of cytosolic PEMT activity to the membrane fraction, the effect of EGTA and EDTA in homogenizing medium was investigated (Table 3). In an isotonic solution of 0.32 M sucrose, the proportion of (100,000 × *g*) supernatant to pellet activity was about 40% for PME and PDE synthesizing activity and 22% for PC synthesizing activity. However, with divalent-cation chelators, the cytosolic proportion was shifted to 12–15% for all PEMT activities. Thus the activities of PEMT appeared to be translocated from the cytosolic fraction to the membrane fraction in the presence of EGTA and EDTA. The specific activities of cytosolic PEMT under chelator conditions decreased to about 22% for PME formation, 16% for PDE, and 43% for PC as compared to the isotonic condition. Although the chelators somehow inhibited all PEMT activities upto

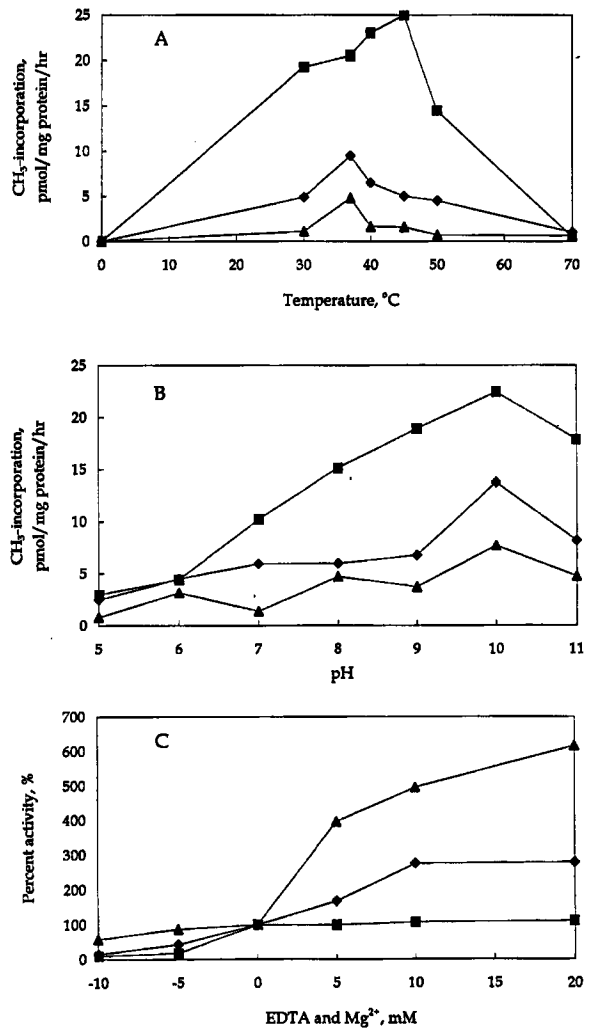

Fig. 4. Effects of incubation temperature (A), pH (B), and Mg^{2+} (C) on the cytosolic PEMT activities. PME, —■—; PDE, —◆—; PC, —▲—.

10–20%, the present data apparently relate chelation of divalent cations to the translocation of cytosolic PEMT to membrane fraction.

DEAE cellulose chromatography of cytosolic PEMT Figure 5A shows the DEAE profile of cytosolic PEMT activities. The cytosolic PEMT activities separated to two activities — one bound and the other unbound. The unbound peak had the activity of PME formation and bound peaks had activities of PDE and PC formation. This result thus indicates that the cytosolic PEMTs consist most likely of two kinds of proteins. This two-enzymes system is in agreement with the report of liver microsomes (Tanaka *et al.*, 1990). Furthermore, it is desirable to compare the cytosolic PEMT with another type of cytosolic methyltransferase which has been known to exist for methylation of water-soluble materials such as ethanolamine or phosphoryl ethanolamine (Andriamampandry *et al.*, 1991). Therefore, we carried out a measurement of the ethanolamine N-methyltransferase (EMT) activity in DEAE cellulose chromatography of the cytosolic fraction (Fig. 5B). Most of the EMT activity was found to be unbound to DEAE cellulose column. The absence of a bound fraction of EMT in DEAE cellulose suggests that the EMT seems to consist of one enzyme only. Therefore, the two DEAE profiles of cytosolic PEMT and EMT could not be superimposed. These observations thus suggest that the enzymes involved in cytosolic PEMT activities could be different from that of cytosolic EMT.

In summary, the present study suggests an existence of cytosolic PEMT in brain. The detection was possible through a consequence of reoptimization of the assay condition by saturating with exogenous phospholipids. The effects of exogenous phospholipids thus indicate that the cytosolic PEMTs need certain amounts of phospholipids to

Table 3. Effect of divalent-cation chelator on brain PEMT activities in the cytosolic fraction.

Medium of Homogenization	PME formation		PDE formation		PC formation	
	Percent activity	Specific activity[a]	Percent activity	Specific activity	Percent activity	Specific activity
0.32 M sucrose						
Homogenate	100%	17.69	100%	8.99	100%	9.17
100,000 × *g* pellet	61.2%	16.70	60.3%	8.37	75.6%	10.70
100,000 × *g* supernatant	38.1%	22.62	39.5%	11.90	22.4%	6.90
0.32 M sucrose with chelator[b]						
Homogenate	100%	16.2	100%	7.54	100%	7.22
100,000 × *g* pellet	86.3%	20.71	88.3%	10.83	82.1%	11.81
100,000 × *g* supernatant	12.0%	4.96 (22%)[c]	14.4%	1.93 (16%)	15.3%	2.99 (43%)

[a] Specific activity (CH_3 – incorporation, pmol/mg protein/h)
[b] 10 mM EGTA + 5 mM EDTA
[c] Percent of recovered specific activity in chelated condition compared to 0.32 M sucrose only condition.

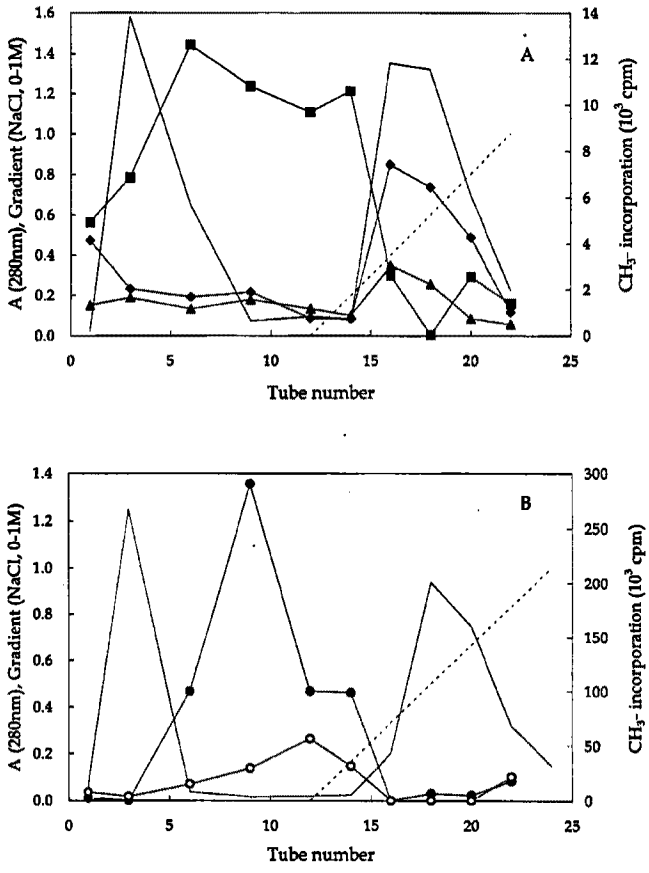

Fig. 5. DEAE cellulose chromatography of cytosolic PEMT (A), and EMT (B) in rat brain. A, _____; gradient, – – –; PME, —■—; PDE, —◆—; PC, —▲—; DME, —●—; MME, —○—.

exert their full activities. The presence of cytosolic PEMT turned out to be nearly 40% of total activity in the whole homogenate. However, in the presence of chelators, the cytosolic PEMT appeared to translocate to the membrane fraction. The general properties of the cytosolic PEMT, except for some kinetic data, are similar to N1 PEMT. Certainly, further studies of the enzymological aspects of the cytosolic PEMT would be very interesting to define the physiological role of the enzyme.

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