Neurochem. Int. Vol. 10, No. 2, pp. 155–166, 1987
Printed in Great Britain.

0197-0186/87 $3.00 + 0.00

THE CHARACTERIZATION OF A MEMBRANE-BOUND PROTEIN CARBOXYLMETHYLATION SYSTEM IN BRAIN

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(*Received* 17 *June* 1986; *accepted* 8 *August* 1986)

Abstract—The membrane-bound component of the cerebral protein carboxylmethylation system, consisting of the membrane-bound enzyme protein carboxylmethyltransferase II (PCMT) and of selected membrane-bound methyl accepting proteins (MAP), is described. The cellular localization of this membrane-bound protein carboxylmethylation system is shown to include, in addition to nerve cell bodies and purified synaptosomes, astrocytes and oligodendroglia. The membrane–bound nature of the protein carboxylmethylation system was investigated and these studies revealed a tight association which exposure to several detergents could only partially solubilize. The membrane-bound PCMT could be shown to undergo activation after treatment with Na-deoxycholate and CHAPs, while after its detergent-induced solubilization PCMT activation was observed after Na-deoxycholate, Nonidet P-40 and Lubrol-P_x. Solubilization of the carboxylmethylation system in CHAPS appeared to be more effective at 0°C than at 25°C or 37°C. Detergent treatment was shown to be deleterious to the MAPs as PCMT substrates, particularly when the exposure was extended to more than 1 h. These observations prompted exposure of the brain membranes and of their Lubrol-P_x and Nonidet P-40 extracts to NH_4OH, treatment which promotes the conversion of protein asparagine residues to atypical L-isoaspartate residues, recently shown (in synthetic peptides) to be the single most effective residue recognized for carboxylmethylation by PCMT. We found up to a 400% enhancement of the carboxylmethylation of solubilized membrane MAPs by the equally solubilized PCMT (which resisted the alkaline treatment virtually unscathed) after 90 min at 37°C in 0.05 M NH_4OH. However, when brain membrane Lubrol-P_x extracts were first subjected to bis(I,I-trifluoroacetoxy)-iodobenzene, a reagent which converts the carboxyamide group of protein-bound asparagine to the corresponding primary amine, the amount of MAPs susceptible to be acted upon by 0.05 M NH_4OH became greatly reduced. Finally, acidic slab gel electrophoresis of membrane-bound MAPs, carboxyl-[^{3}H]-methylated by the membrane-bound PCMT, revealed the presence of about 12 radioactive protein bands, ranging in MW from under 20 KDa to about 90 KDa.

The carboxylmethylation of proteins is catalyzed by the enzyme protein carboxylmethyltransferase (PCMT, EC 2.1.24) (Kim, 1984; Clarke, 1985; Billingsley and Lovenberg, 1985). Although intracellular localization studies in cow and rat brain have revealed high levels of PCMT activity in the cytosol, a membrane-bound (mb) component of cerebral PCMT has also been briefly examined (Diliberto and Axelrod, 1976; Iqbal and Steenson, 1976; Clark *et al.*, 1979; Brown, 1984; Sellinger *et al.*, 1985; Johnson and Aswad, 1985a; Fischer-Bovenkerk *et al.*, 1986). Similarly, it has been reported that the endogenous substrates of brain PCMT, i.e. the methyl accepting proteins, or MAPs, may be both cytosolic and membrane-bound (Gagnon *et al.*, 1981; Aswad and Deight, 1983a, Billingsley *et al.*, 1985a, b; Johnson and Aswad, 1985a; Clarke, 1985). Most recently, a survey of MAPs serving as substrates for an isozyme of the cytosolic bovine brain PCMT (Aswad and Deight, 1983b) disclosed many to be membrane-bound (Johnson and Aswad, 1985a). This study also briefly noted that, in rat brain, some mb-MAPs may be carboxylmethylated by a mb-form of the PCMT. We have briefly reported similar observations (Sellinger *et al.*, 1985; Fischer-Bovenkerk *et al.*, 1986). A cell-specific role for brain PCMT has recently been inferred from its immunohistochemically determined localization, which revealed a predominantly neuronal distribution (Billingsley *et al.*, 1985a). Regionally, intense PCMT activity was observed in the substantia nigra, the locus coeruleus and the paraventricular nucleus (Billingsley and Balaban, 1985), with implications of a special role for the enzyme in presynaptic monoaminergic neurons and in the release and/or processing of neurohypophyseal peptides. It has been reported previously that calmodulin (Gagnon *et al.*, 1981), cAMP phosphodiesterase, Ca^{2+}-calmodulin-dependent protein kinase

and the calmodulin-activated phosphatase, calcineurin (Billingsley and Lovenberg, 1985; Billingsley *et al.*, 1985b, c; Gupta *et al.*, 1985), constitute segments of a functionally important cerebral carboxylmethylation cascade designed to modulate Ca^{2+}-calmodulin-dependent reactions. However, since in these predominantly *in vitro* studies (Clarke, 1985), highly purified, cytosolic PCMT of both neural and non-neural origin was utilized to carboxylmethylate mb- and cytosolic neural proteins, some of them in a highly purified state, it is not possible to affirm that the reported findings reflect *in situ* occurring events.

The present report documents the presence, and characterizes the properties, of a membrane-bound protein carboxylmethylation system in brain which consists of a tightly embedded membrane-bound PCMT able to act on its equally tightly membrane-bound MAPs.

EXPERIMENTAL PROCEDURES

Materials

S-adenosyl-L-methionine (di-p-toluenesulfonate salt) and S-adenosyl-L-homocysteine were gifts of Dr G. Stramentinoli, BioResearch, Liscate, Italy. Synapsin (about 60% pure) from cow brain was a gift of Dr T. Ueda, of this Institute. Crystalline ovalbumin, bovine serum albumin (BSA), calmodulin, Nonidet P-40, Lubrol-P_X, Triton X-100, Ficoll (type 4000-DL), Na-deoxycholate, 3-[(3-cholamidopropyl)-dimethylammonio]1-propanesulfonate (CHAPS), Percoll and the density marker beads, TPCK-trypsin, acetylated trypsin, soybean trypsin inhibitor and Coomassie Blue were from Sigma Co., St. Louis Mo. The MW standards for electrophoresis were from Pharmacia. Acrylamide and N,N'-bis-methylene acrylamide were from Serva, Garden City Park, New York. Bis-(I,I-trifluoroacetoxy)-iodobenzene (BTI) was from Fluka Chem. Corp., Hauppague, N.Y. En^{3}Hance was from New England Nuclear, Boston, Mass. [^{3}H]-methyl-S-adenosyl-L-methionine (1 mCi/ml, Cat. no. 24051), with a nominal specific radioactivity of 10–15 Ci/mmol was from ICN, Irvine, Calif. All other reagents were the best analytical grade available. Samples of purified, cytosolic rat brain PCMT were generously donated by Dr M. L. Billingsley, Dept of Pharmacology, Pennsylvania State University, Hershey, Pa.

Animals

Cow cerebella were from a local slaughterhouse. The organ was excised, immediately refrigerated and homogenized within 3 h. Rats were male Sprague–Dawley, 2–4 months old.

Methods

Preparation of tissue fractions

Rat cerebra and cerebella and cow cerebella were homogenized in about 10 volumes of buffer (50 mM Na-phosphate, pH 6.5 or 20 mM Tris-citrate, pH 7.4) and the homogenate centrifuged at 1000 *g*, The resulting supernatant was centrifuged at 10,000 *g* or, in later experiments, at 17,500 *g* for 30 min. The resulting pellet, washed once (pellet No. 2) or 6 times (pellet No. 6) was used in most experiments. In a few instances, specified in the appropriate Figure and Table legends the 1000 *g* step was omitted and a high-speed (100,000 *g*, 60 min) pellet and supernatant were also obtained. In one instance (Fig. 1B) the homogenizing buffer was 10 mM Na-phosphate, pH 6.8, containing 1 mM EDTA, 0.1 mM benzethonium chloride, 0.1 mM phenylmethylsulfonylchloride, 0.2% Na-azide, 0.02% bacitracin and 2 mM benzamidine-HCl (protease inhibitor cocktail).

Preparation of cells, cell components and subcellular organelles

Synaptosomes. The procedure utilizing Percoll gradients (Nagy and Delgado-Escueta, 1984) was followed. Percoll layer C (10–16% interface) contained purified synaptosomes. To obtain synaptosomal membranes, synaptosomes were collected at 20,000 *g* for 20 min in rotor SW-27 (Beckman) and the pellet was resuspended in 1 ml of 0.32 M sucrose (pH 7.5) to which 10 ml of 1 mM Na-phosphate (pH 7.5) buffer, containing 0.1 mM EDTA were added. After 30 min at 0°C, the lysed suspension was centrifuged at 10,000 *g* for 30 min. The pellet (synaptosomal membranes) was resuspended in 1 ml of 50 mM Na-phosphate buffer, pH 6.5.

Neuronal perikarya. The procedure of Sellinger *et al.* (1971) was used.

Astrocytes. The procedure of Farooq and Norton (1978) was used without significant modifications. Acetylated and TPCK-trypsin were used interchangeably with equal results.

Oligodendroglia. The procedure of Berti-Mattera *et al.* (1984) was used. It was confirmed that, for best results, it was necessary to omit Ca^{2+} and Mg^{2+} from all solutions. In a few experiments, soybean trypsin inhibitor was used to arrest trypsinization, with no apparent benefit.

Phase contrast microscopy.

The isolated cells were monitored for purity and integrity by phase contrast microscopy. The appearance of the isolated cells matched in all respects that of the cells shown in the publications describing their isolation.

Analytical

Protein was determined by the method of Peterson (1977).

Acidic slab gel electrophoresis

The procedure described by Aswad and Deight (1983a) was used. A vertical slab system (SE 400) (Hoefer Scientific Products, San Francisco, Calif.), powered by 180 V for 3.5 h at RT provided the desired separations. After electrophoresis of the radioactive proteins, the gel slabs were stained with 0.25% Coomassie Blue for 30 min, treated with En^{3}Hance and immediately dried before being placed in contact with XAR-5 X-ray film for exposure to −70°C.

The assay of PCMT activity

(a) *Acting on endogenous MAPs.* The procedure of Diliberto and Axelrod (1974, 1976) was used. Each tube (F.V.: 255 μl) contained 5 μcuries of [^{3}H]-methyl SAM and the following, at the final concentration: SAM (20 μM), EDTA (0.4 mM), dithiothreitol (1.2 mM) and up to 200 μl of tissue. Unless otherwise stated, the incubation was for 30 min at 37°C. Carboxylmethylation was arrested by the addition of 1 ml of 20% (w/v) TCA followed by 1 mg of BSA as carrier. After 15 min on ice, the tubes were centrifuged at 3400 rpm for 15 min. The pellets were sus-

pended in 0.5 ml of 0.125 M "borax" ($Na_2B_4O_7$-10 H_2O), pH 10.7 and were incubated for 5 min at 37°C. Then, 1.2 ml of a 3:2 (v/v) mixture of toluene: isoamyl alcohol were added, the tubes were thoroughly vortexed and centrifuged at 4000 rpm for 5 min. Two 0.5 ml aliquots of the upper phase, containing the released [^{3}H]-CH_3OH (Diliberto and Axelrod, 1974), were placed into 2 glass scintillation vials, one for immediate processing (total dpm) and the other for the determination of the non-volatile radioactivity (Diliberto and Axelrod, 1974, 1976; Brown, 1984). The non-volatile radioactivity did not exceed 20% of the total dpm when mb-PCMT acting on endogenous MAPs was being determined and was below 5% of the total radioactivity when PCMT acting on added MAPs was being assayed. PCMT activity is expressed as picomoles of [^{3}H]methyl transferred from [^{3}H]methyl SAM to methyl accepting proteins (MAPs) (endogenous or added).

(b) *Acting on added MAPs.* The above assay was used, unmodified, except that ovalbumin (10 μg/tube) or another purified protein (see text and Table 6 for amounts) was added to the incubation.

(c) *After preincubations and pretreatments.* Various preparations containing the PCMT-MAP system in mb- or detergent-solubilized-form were exposed to 0.05 M NH_4OH, 50% (v/v) dimethylformamide, containing 0.01 M trifluoroacetic acid ± bis-(I,I-trifluoroacetoxy)-iodobenzene (BTI), and to several other treatments (see text), including prolonged exposure to 60°C. The PCMT activity remaining after these treatments was determined and where found inactivated, the enzyme protein was replaced, for subsequent MAP determinations, by the purified, cytosolic rat brain PCMT. For further details, see legends to appropriate Tables.

RESULTS

The association of PCMT with membranes

(a) *PCMT acting on added MAPs.* Several experiments were performed to establish the membrane association of brain PCMT. Rat cerebrum homogenates were prepared in 50 mM Na phosphate buffer, pH 6.5 and cow cerebellum (Iqbal and Steenson, 1976) homogenates in 20 mM Tris-citrate buffer, pH 7.4. PCMT activity was determined in the following fractions: the homogenate, the 10,000 *g* pellets and supernatants (P_1 and S_1); the 100,000 *g* pellets and supernatants derived from S_1 (P_3 and S_3) and the 100,000 *g* pellets and supernatants derived from a suspension of P_1 in 20 ml of 0.1% Triton X-100 (P_T and S_T). After normalizing for recoveries (range 78–108%), relative to the initial homogenates (100%), the percentages of PCMT in membrane-bound form ranged from 22 to 27% in P_1 ($P_1 + S_1 = 100\%$), 44–56% in P_T ($P_T + S_T = 100\%$) and 14–19% in P_3 ($P_3 + S_3 = 100\%$). Unless stated otherwise all subsequent membrane fractions, from both rat and cow brain, were prepared in 50 mM Na-phosphate buffer, pH 6.5.

(b) *PCMT acting on endogenous MAPs.* A comparison of the specific activities of PCMT acting on endogenous MAPs vs exogenous ovalbumin in rat cerebral cortex and cerebellum is shown in Table 1. Significant increases were noted in the presence of ovalbumin in each fraction, yet the magnitude of the increase was clearly lower in the two membrane fractions than in the 100,000 *g* supernatant. Since, on the other hand, the specific activity of PCMT in fraction P_1 was invariably higher than in the high-speed pellet P_T, we chose fraction P_1 for all further work, albeit modifying the centrfugation scheme slightly by washing pellet P_1, obtained at 17,500 *g*, instead of 10,000 *g*, with 50 mM Na phosphate buffer, pH 6.5 for the number of times indicated in the appropriate table legends.

(c) *The effects of SAM and SAH.* Figure 1 shows the [SAM] dependence of the truly cytosolic PCMT [100,000 *g* for 60 min, (S_S)], the Lubrol-P_x-solubilized PCMT (S_L) and the Lubrol-P_x-resistant PCMT (P_L). Figure 1A refers to the PCMT activity acting on ovalbumin and Fig. 1B to that acting on the endogenous mb-MAPs. It may be seen that in all 3 fractions PCMT carboxylmethylated ovalbumin at a rapid rate and without approaching saturation, given the large excess of ovalbumin; conversely, PCMT appeared to reach saturation well within the range of the SAM concentrations when tested against endogenous MAP substrates, in both fractions P_L and S_L. This resulted in MAP carboxylmethylation maxima of about 3 and 10 pmol/mg in fractions (P_L) and (S_L), respectively.

Inhibition of the mb-PCMT by SAH was compared by assaying the activity acting on added MAPs in the presence of 10^{-4}–10^{-7} M SAH ± 1% Lubrol-P_x. The following % inhibitions were noted in the absence of the detergent: 23% at 10^{-7} M SAH and

Table 1. The PCMT activity acting on endogenous and added MAPs in centrifugal fractions of rat cerebrum and cerebellum

	Cerebrum			Cerebellum		
Fraction	Endogenous MAPs	Added MAPs	Added / Endogenous	Endogenous MAPs	Added MAPs	Added / Endogenous
Homogenate	8.69	21.4	2.46	7.37	21.0	2.85
10,000 *g* pellet (P_1)	7.34	13.5	1.84	7.36	14.0	1.90
100,000 *g* pellet (P_T)	5.12	7.33	1.43	4.22	6.64	1.57
100,000 *g* supernatant (S_3)	14.9	88.1	5.91	13.2	93.2	7.06

Values are in picomoles of [^{3}H]methyl/mg protein. Added MAP: Ovalbumin, 10 μg/tube.

below 40% at 10^{-4} M SAH; in the presence of the detergent, inhibition increased from 29% at 10^{-7} M to 90% at 10^{-4} M SAH. These findings strongly suggest that SAH has poor access to embedded mb-MAPs in the absence of Lubrol-P_x and a relatively unencumbered access in its presence.

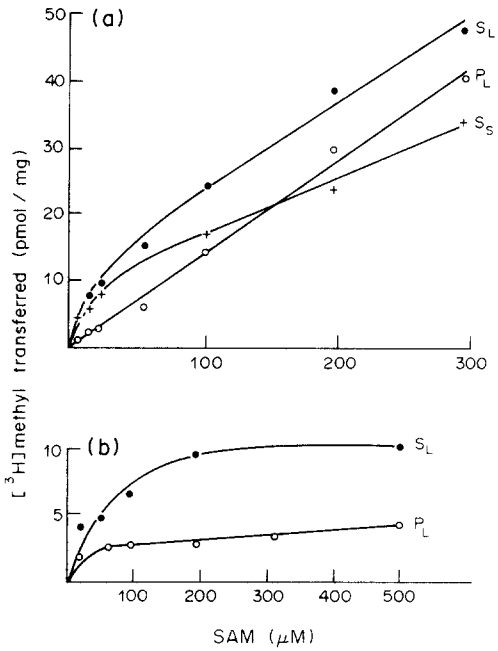

Fig. 1. The carboxyl-[^{3}H]-methylation of ovalbumin (A) and of endogenous brain membrane proteins (B) by the cytosolic (S_S), Lubrol-P_x-solubilized (S_L) and Lubrol-P_x-resistant (P_L) forms of PCMT: Effect of S-adenosyl-L-methionine (SAM). (A). Cow cerebellum (3 g) was homogenized in 40 ml of 50 mM Na-phosphate buffer pH 6.5. Nine ml of the homogenate were centrifuged at 100,000 *g* for 60 min yielding the cytosol, S_S. The remaining 31 ml were centrifuged at 1000 *g* for 10 min. The supernatant was centrifuged at 17,500 *g* for 30 min and the resulting pellet was then washed and recentrifuged 6 times. Pellet No. 6 was homogenized in 7 ml of 1% Lubrol-P_x dissolved in buffer and the suspension was kept on ice for 30 min. Centrifugation (100,000 *g*, 60 min) yielded pellet P_L, which was suspended in 5 ml of Lubrol-buffer, and supernatant S_L. All fractions were assayed for PCMT activity (see Methods) for 30 min at 37°C. S_S: each tube contained 421 μg of protein; S_L: each tube contained 169 μg of protein; P_L: each tube contained 355 μg of protein. (B) Cow cerebellum was processed as above in A, except that the buffer used up to and including the preparation of pellet No. 6 was 10 mM Na-phosphate buffer, pH 6.8, containing a protease-inhibitor cocktail, (see Methods). Pellet No. 6 was suspended in 50 mM Na-phosphate buffer, pH 6.5 and the procedure outlined in A was then followed. Incubation to determine the PCMT activity was for 30 min at 37°C (see Methods). S_L: each tube contained 203 μg of protein; P_L: each tube contained 416 μg of protein.

The mb-PCMT in neuronal perikarya, astrocytes, oligodendroglia and synaptosomes

Neuronal perikarya, astrocytes and oligodendroglia were prepared (see Methods) and their PCMT activity determined. To obtain the cytosol and membrane fractions, the cellular preparations were homogenized in hypotonic medium and centrifuged at 100,000 *g* for 60 min. PCMT acting on endogenous synaptic MAPs was determined in isotonic 0.32 M sucrose and in 1% Nonidet P-40. While the isotonic condition measured the PCMT activity associated with the outside surface of the synaptosome, total PCMT activity, including the intrasynaptosomal and the synaptic membrane-bound components, was being determined in the presence of the detergent. Table 2 reveals the ubiquitous presence of the PCMT acting on endogenous MAPs in all cellular and organelle preparations and in the cytosol and membranes prepared therefrom. As expected, the activity of the PCMT acting on added MAPs was uniformly higher. However, the increase was greater in the neuronal and astrocytic than in the oligodendroglial cytosol and smaller in the neuronal and astrocytic, than in the oligodendroglial, membranes. We also noted a greater than 7-fold increase in the PCMT activity of total synaptosomes in the presence of Nonidet P-40; This increase became greatly reduced (to *c.* 2-fold over controls) when tested in the synaptic membranes.

Solubilization of mb-PCMT

(a) *The effect of repeated washing and of detergents.* To assess the nature of the structural association of mb-PCMT, the 17,500 *g* pellet (in 50 mM $NaPO_4$ buffer, pH 6.5) was washed once, the resulting pellet No. 1 was resuspended and the suspension centrifuged at 17,500 *g* for 30 min. This was repeated 5 successive times, yielding pellet No. 6 (Table 3, A1). Mb-PCMT acting on added MAPs (ovalbumin) represented 47.8% of the total activity in pellet No. 1 and 81.9% in pellet No. 6. Three aliquots of pellet No. 6 were suspended in 1% Na-deoxycholate (D), 1% Nonidet P-40 (N) and 50 mM phosphate buffer (B). After 30 min at 0°C, centrifugation at 100,000 *g* for 30 min yielded pellets and supernatants No. 7(B), (D) and (N). As shown in A1 the recoveries, relative to the respective parent pellets, were only slightly above 100% throughout the buffer-washing procedure. However, as shown in the legend to Table 3, A2, the PCMT activity of the high-speed supernatants 7(D) and 7(N) was 3–5 times higher than the PCMT activity of supernatant 7(B), suggesting its activation upon being detergent-solubilized. Yet the

Table 2. Protein carboxylmethyltransferase (PCMT) activity in neurons, glia and synaptosomes

Cell or Cell Component	Total PCMT		Membrane-bound PCMT		Cytosolic PCMT	
	Endogenous MAPs	Added MAPs	Endogenous MAPs	Added MAPs	Endogenous MAPs	Added MAPs
Neuronal Perikarya	6.0	13.6	4.1	6.7	19.5	50.6
Astrocytes	2.6	5.8	1.9	3.6	3.4	13.2
Oligodendroglia	11.7	18.4	9.2	21.4	57.2	87.4
Synaptosomes in 0.32 M Sucrose	2.4	—	13.7	—	—	—
Synaptosomes in 1% Nonidet P-40	17.8	—	30.2	—	—	—

Values are in picomoles of [^{3}H]methyl/mg protein. The recovery of PCMT activity and of protein between the membrane-bound and the cytosolic fractions was quantitative. The values are from one representative preparation of each cell type. These values were reproduced within 25% in 4 additional isolation runs for each cell type. The synaptosomal values, averages of 2 experiments, matched within 15%.

PCMT activity in the detergent-resistant pellets 7(B), 7(D) and 7(N) was virtually identical (see legend, Table 3, A2). Table 3,B illustrates the effect of the agents listed on the PCMT activity of brain membranes in pellet No. 2. PCMT was not solubilized by 1 M KCl; it was partially solubilized by 0.2% Na-deoxycholate, yet the solubilized form did not appear activated, as was noted after 1% Na-deoxycholate and 1% Nonidet P-40 (see Table 3, A2 legend). The mb-PCMT was also effectively solubilized by 1% Lubrol-P$_x$, yet with only a moderate activation of the solubilized activity.

(b) *The effect of detergents on the specific activity of PCMT.* Five pellets No. 6 (see Table 3, A1) were suspended in buffer and to each suspension was added sufficient detergent to achieve the indicated final concentrations (Table 4). After 30 min at 0°C, the suspensions were centrifuged at 78,000 *g* for

Table 3. Effects of repeated washes, salt and detergent treatments of brain membranes on the solubilization of the membrane-bound PCMT

	Buffer* wash No.	Pellet No.	Protein carboxylmethyltransferase (PCMT) P (%)	S (%)	Recovery (%)
*A*1					
	0	1†	47.8	52.2	100
	1	2	45.9	54.1	134
	2	3	56.3	43.7	116
	3	4	70.9	29.1	127
	4	5	71.8	28.2	114
	5	6	81.9	18.1	110
*A*2					
	6	7 (B)††	65.7	34.3	100
	6	7 (D)††	40.5	59.5	178
	6	7 (N)††	29.5	70.5	242
B					
	Treatment				
	Buffer* (B)	2	55.7	44.3	100
	1 M KCl	2	55.4	44.6	102
	0.2% Na-deoxycholate	2	34.3	65.7	99
	1% Lubrol P$_x$	2	26.5	73.5	143

P: pellet; S: supernatant. PCMT assays contained ovalbumin, 10 μg/tube.

*50 mM Na-phosphate, pH 6.5.

†Represents 24–29% of the tissue PCMT and 55–69% of tissue protein.

††(B): buffer; (D): 1% Na-deoxycholate; (N): 1% Nonidet P-40.

*A*1. Cow cerebellum (1.75 g) was homogenized in 21 ml of 50 mM Na-phosphate buffer, pH 6.5 and the homogenate was centrifuged at 10,000 *g* for 20 min. The pellet (No. 1) was resuspended in 10 ml of buffer and the suspension recentrifuged for 25 min, yielding pellet No. 2. The supernatants were saved. The resuspension and recentrifugation was repeated 4 more times, except that 100,000 *g* for 30 min were employed; this yielded pellets and supernatants 3–6. The values refer to the sum of the PCMT activities of $P_n + S_n$, where $n = 1$–6. Recovery refers to the activities recovered in $P_{n+1} + S_{n+1}$, as % of the activity in P_n. The PCMT activity of pellet No. 1 transferred 0.56 pmol of [^{3}H]methyl to ovalbumin in 30 min.

*A*2. Aliquots of a pellet No. 6 suspension (2, 3 ml) (see *A*1) in buffer were incubated as such (B) or in the presence of 1% Na-deoxycholate (D) or Nonidet P-40 (N) at 0°C for 30 min. The suspensions were centrifuged at 100,000 *g* for 30 min and the pellets resuspended in buffer. PCMT activity was determined in the 3 pellet suspensions and supernatants. Recovery refers to the sum of the activity of pellet 7(B) + supernatant 7(B) = 100%. The following activities were determined (see Methods for definition of unit): P(B): 0.119; P(D): 0.131; P(N): 0.129; S(B): 0.062; S(D): 0.192 and S(N): 0.309 pmoles of [^{3}H]CH_3 transferred.

B. Cow cerebellum was homogenized as above in *A*1 and pellet and supernatant No. 1 were obtained. The pellet was resuspended in 12 ml of buffer and four 3 ml aliquots were recentrifuged in individual tubes at 10,000 *g* for 25 min, yielding 4 pellets No. 2. Each of these was resuspended in 9 ml of solution, as indicated and was recentrifuged, after 30 min at 0°C, at 100,000 *g* for 60 min. PCMT activity was determined in the resulting pellets and supernatants. Recovery refers to the % of the activity of pellet 2(B) + supernatant 2(B) = 100%.

90 min. Each pellet was suspended in the homologous detergent at the concentration which led to its generation, permitting the assay of the solubilized and the detergent-resistant PCMT in the presence of identical detergent concentrations. Of the 4 detergents tested, Nonidet P-40 and Triton X-100 caused no changes in the specific activity of the mb-PCMT, while the zwitterionic CHAPS and the ionic Na-deoxycholate produced membrane pellets highly enriched in PCMT activity.

(c) *The effect of temperature and of CHAPS.* Solubilization of membrane-bound enzymes, receptors, etc. by detergents (Hjelmeland and Chrambach, 1984), proteases (Singh *et al.*, 1985) and other suitable reagents, such as thiols (Hersh, 1985) has been carried out at temperatures ranging between 0°C and 37°C. Since in preliminary experiments using the zwitterionic detergent CHAPS at 0°C it appeared possible to partially uncouple the solubilization of the mb-PCMT from that of the mb-MAPs, it became of interest to determine what effect temperature exerted on this process.

The effects of preincubating pellet No. 6 (see Table 3, A1) at 0°, 25° and 37°C in the presence and absence of 30 mM CHAPS are shown in Table 5. CHAPS appeared to stimulate the PCMT acting on added MAPs, but not that acting on endogenous MAPs (column II vs I); this stimulation was maximal after preincubation of the membranes at 0°C. In the

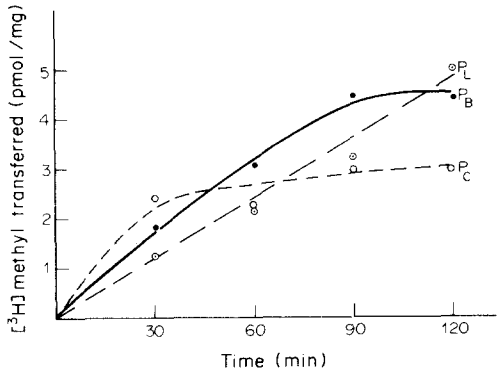

Fig. 2. The effect of exposure of cerebellar membranes to Lubrol-P_X and CHAPS on the time course of PCMT activity. Three pellets No. 6, prepared from 3 pieces of cow cerebellum, weighing 2.9 g each, as described in Methods, were suspended in 5 ml of 50 mM Na-phosphate buffer, pH 6.5 (B), 1% Lubrol-P_x (L) and 30 mM CHAPS (C) and the suspensions were kept on ice for 30 min. Following 100,000 *g* for 60 min, each pellet was resuspended in 5 ml of buffer and aliquots of each suspension (P_B, P_L and P_C) were incubated to determine the PCMT activity in the absence of ovalbumin for the times indicated.

Table 4. The effect of detergents on the specific activity of mb-PCMT

Na-deoxycholate		Nonidet P-40		Triton X-100		CHAPS	
%	s.a.*	%	s.a.	%	s.a.	mM	s.a.
0	100	0	100	0	100	0	100
0.2	189	0.2	108	0.05	117	2.5	102
0.4	245	0.4	100	0.10	112	5.0	124
0.75	304	0.8	115	0.25	97	10	155
1.0	412	1.5	116	0.50	124	30	179

*s.a. of PCMT in pellet No. 7 (see Table 3, A2), as % of the s.a. in the buffer-washed (0%) pellet, set to 100%.

Brain was homogenized in 50 mM Na-phosphate buffer, pH 6.5, except as indicated below, and pellet No. 6 was prepared as described in Methods after 4 successive washes of pellet No. 2 at 17,500 *g* for 30 min. The pellet was resuspended in 10 ml of 50 mM buffer (15 mM Na-phosphate was used in the case of Na-deoxycholate due to gelling in the 50 mM buffer at the 0.75 and 1% concentrations at 0°C), and 5 two ml aliquots were apportioned to tubes which received buffer or detergent to reach the concentrations indicated. After 30 min at 0°C, the suspensions were centrifuged at 78,000 *g* for 90 min and the pellets resuspended in buffer (0%) or detergent, as indicated, for determination of PCMT activity in the presence of 10 μg of ovalbumin.

Table 5. Effects of CHAPS and of temperature on the specific activity of PCMT

Preincubation temperature	I Control membranes (a)	(b)	II Membranes + CHAPS (a)	(b)	III CHAPS pellet (a)	(b)	IV CHAPS supernatant (a)	(b)
0°C	5.51	6.50	5.00	12.5	21.6	26.7	58.3	156.2
25°C	4.20	9.25	5.64	11.4	14.5	18.9	58.3	127.8
37°C	4.10	5.95	5.66	7.7	8.7	14.3	56.7	144.4

Values are in picomoles [³H]methyl/mg protein. (a): PCMT acting on endogenous MAPs; (b): PCMT acting on ovalbumin.

Preincubation of control membranes (I) (pellet No. 6, Table 3, A1) was in 8 ml of 0.32 M sucrose in 20 mM Na-phosphate buffer, pH 6.5. Membranes + CHAPS (II) (8 ml) contained 30 mM CHAPS and were preincubated for 30 min at the temperatures indicated. Preparations (III) and (IV) were obtained by centrifugation of preparation II (100,000 *g*, 60 min). CHAPS pellets (III) were resuspended in 30 mM CHAPS in 20 mM Na-phosphate buffer, containing 0.32 M sucrose.

CHAPS pellet (column III vs II), the s.a. of the PCMT acting on endogenous MAPs increased by 3.9- and 2.1-fold and that of the enzyme acting on added MAPs by a closely similar 4.1- and 2.4-fold at 0° and 37°C, respectively. The s.a. of PCMT in the CHAPS supernatant (column IV) appeared largely independent of the temperature of preincubation, yet its absolute values exceeded those of the s.a. of PCMT in the CHAPS pellets (column IV vs III). Conversely, although the s.a. of PCMT in the CHAPS pellet was lowest following preincubation at 37°C, its absolute values exceeded those of the enzyme in the unfractionated membranes (column III vs II), under all conditions tested.

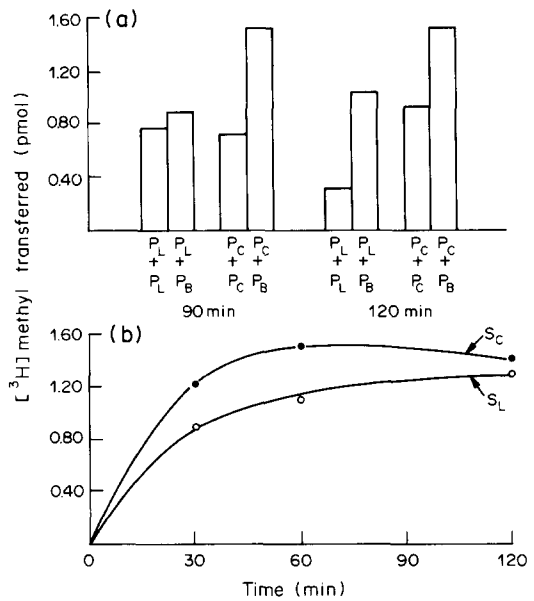

Fig. 3. (A). The effect of buffer (pH 6.5)-, Lubrol-P_x- and CHAPS-treated mb-MAPs on PCMT acting on endogenous MAPs. Three pellets No. 6 were prepared, their suspensions exposed to 50 mM Na-phosphate buffer, pH 6.5 (B), Lubrol-P_x (L) and CHAPS (C) at 0°C for 30 min and the high-speed pellets obtained, as described in the legend to Fig. 2. Unlike in the experiment depicted in Fig. 2, however, the high-speed pellets were each suspended in the solution in which they were cold-exposed, yielding suspension P_B, P_L and P_C. Aliquots (200 μl) of these suspensions were placed to incubate in the PCMT assay medium and the mb-PCMT activity determined at 30 and 60 min (not shown). To parallel tubes, the following were added at 60 min: 200 μl of P_L and 200 μl of P_C and 200 μl of P_B to tubes containing suspension P_L and 200 μl of P_B to tubes containing suspension P_C. These conditions are referred to in the figure as $P_L + P_L$, $P_L + P_B$, $P_C + P_C$ and $P_C + P_B$, respectively. Mb-PCMT activity was again determined 30 and 60 min later, i.e. 90 and 120 min after the start of the incubations: (B). The effect of Lubrol-P_x and CHAPS on the solubilized PCMT. The activity of PCMT was determined in the high-speed supernatants (S_L and S_C) obtained by centrifuging suspensions exposed to 1% Lubrol-P_x and 30 mM CHAPS for 30 min at 0°C (see legend, Fig. 2).

Factors altering the mb-MAPs

(a) *Exposure to detergents.* The following experiments were primarily designed to identify conditions which would alter the ability of the endogenous mb-MAPs to act as mb-PCMT substrates. In one experiment (Fig. 2), aliquots of a suspension of pellet No. 6 were preincubated at 0°C for 30 min in buffer, 1% Lubrol-P_x and 30 mM CHAPS, and a time course of PCMT activity was determined using high-speed pellet suspensions prepared in buffer. It may be noted that the brief exposure of the mb-MAP-PCMT system to the two detergents at 0°C resulted in significant differences between the P_L and P_C time courses. The action of CHAPS, relative to that of Lubrol-P_x, appeared to result in a doubling of the amount of mb-MAPs which could be carboxylmethylated in the initial 30 min; yet, at the end of the 120 min incubation period, the carboxylmethylation of the Lubrol-P_x treated MAPs matched that of the buffer-treated controls, while that of the CHAPS-treated MAPs still proved inferior. These apparently different effects of Lubrol-Px and CHAPS on the ability of mb-MAPs to act as endogenous substrates of the mb-PCMT were further assessed in another experiment in which Lubrol-P_x and CHAPS pellets were resuspended not in buffer, as above, but in 1% Lubrol-P_x and 30 mM CHAPS and were then placed to incubate in the PCMT assay medium at 37°C (Fig. 3A). After 60 min, 200 μl aliquots of the cold-preincubated, detergent- or buffer-containing suspensions of MAPs were added and incubation continued for an additional 60 min at 37°C. Figure 3A shows that the addition of the buffer-suspended MAPs to the detergent-treated membrane suspensions led to markedly higher PCMT activities than were obtained by adding to them detergent-suspended MAPs. Conversely, the time course of the detergent-solubilized PCMT acting on the equally solubilized endogenous MAPs (Fig. 3B) reflects comparable effects of the two detergents on this form of the enzyme.

(b) *Effect of pH, buffer species and detergent.* We also compared the specific activity of PCMT acting on endogenous MAPs in 20 mM Tris-citrate and MOPS buffers at pH 6.5 and in 20 mM MES buffer at pH 5.5 in the absence and presence of 0.1% Nonidet P-40. Increases in enzyme activity of 3.5-, 2.2- and 1.4-fold over controls were noted in the detergent's presence (data not shown).

Table 6. The carboxyl-[^{3}H]methylation of purified proteins by the mb-protein carboxylmethyltransferase (PCMT)

Experiment No.	Addition	[^{3}H]methyl transferred (pmol)
1.	None*	3.29
	+ ovalbumin, 10 μg	7.79
	+ synapsin, 5 μg	5.11
	+ synapsin, 10 μg	9.44
2.	None†	3.41
	+ ovalbumin, 10 μg	5.15
	+ calmodulin, 5 μg	5.05
3.	None‡	12.5
	+ BSA, 100 μg	21.3
	+ ovalbumin, 10 μg	33.0

*mb-PCMT from neuronal microsomes (Sellinger and Santiago, 1972), 3.51 μg of protein.
† Pellet No. 6 membranes (see Table 3, A1), 0.50 mg of protein.
‡ Pellet No. 3 membranes (see Table 3, A1), 0.92 mg of protein.
The PCMT assay volume was 255 μl (see Methods).

(c) *Purified proteins as substrates for the mb-PCMT.* Ovalbumin, BSA, calmodulin and synapsin were compared. The results of experiments (1) and (2) (Table 6) demonstrate that the mb-PCMT of rat brain recognizes 5–10 μg of synapsin and 5 μg of calmodulin somewhat more effectively than 10 μg of ovalbumin, while experiment No. 3 reveals that BSA is a much poorer substrate for the mb-PCMT than ovalbumin.

(d) *The effect of NH_4OH.* Recently, treatment of the purified protein calmodulin with 0.1 M NH_4OH was shown to result in a substantial increase in its carboxylmethylation by the cytosolic type I isozyme of bovine brain PCMT (Aswad and Deight, 1983b). As shown in Table 7, we tested the effectiveness of this mild alkaline treatment on the generation of new carboxylmethylatable sites in brain MAPs by treating

Table 7. Mild alkaline treatment of detergent-solubilized brain MAPs greatly increases their carboxyl-[^{3}H]methylation by the equally detergent-solubilized PCMT.

Treatment	Protein (μg)	[^{3}H]-MAPs (pmol/mg)	Δ, NH_4OH %
Lubrol-P_x			
Control	613	9.32	—
+0.05 M NH_4OH	582	37.8	+405
Nonidet P-40			
Control	616	10.9	—
+0.05 M NH_4OH	558	39.5	+362

Brain membranes contained in pellet No. 6 (see, Table 3, A1) were suspended in 5 ml of 1% Lubrol-P_x or Nonidet P-40. The resuspensions were kept on ice for 30 min and were centrifuged for 30 min at 17,500 *g*. Duplicate 400 μl aliquots of each supernatant were mixed with either 40 μl of 0.55 M Na-phosphate buffer, pH 6.6 (control) or 40 μl of 0.55 M NH_4OH and were preincubated at 37°C for 90 min. The pH of all samples was adjusted to 6.5 and 220 μl aliquots, containing the amounts of protein indicated, were then transferred to tubes containing 45 μl of the ovalbumin-free PCMT assay medium. Incubation was for 30 min at 37°C.

the Nonidet P-40 and the Lubrol-P_x supernatants derived from pellet No. 6 (see Table 3, A1) with NH_4OH for 90 min at 37°C. However, because we found 0.1 M NH_4OH to severely inactivate the solubilized PCMT, the concentration of NH_4OH was lowered to 0.05 M. The alkaline treatment led to 3.6–4.0-fold elevations in carboxylmethylation of the detergent solubilized MAPs by an apparently alkali-resistant and largely unscathed PCMT (Kim, 1984).

(e) *The combined BTI + NH_4OH pretreatment.* The protein modifying reagent BTI (see Materials) which converts the carboxyamide group of protein asparagine to the corresponding primary amine (Soby and Johnson, 1981; Johnson *et al.*, 1985), was incubated with a Lubrol-P_x solubilized extract of pellet No. 6 (see Table 3A, 1) (1.36 mg of protein) in a volume of

Table 8. Effect of BTI pretreatment on the NH_4OH-induced methyl accepting capacity of Lubrol-P_x- solubilized rat brain MAPs.

First preincubation (60°C), in:	Second preincubation (37°C), in:	[^{3}H]methyl transferred to MAPs (pmol)	
		Uncorrected	*Corrected*
1. Buffer*	Buffer	58.7	—
2. DMF + TFA†	Buffer	28.7	—
3. BTI†† in DMF + TFA	Buffer	43.2	14.5
4. as in 2.	NH_4OH, 0.05 M	90.3	61.6
5. as in 3.	NH_4OH, 0.05 M	74.7	31.5

*Buffer: 50 mM Na-phosphate, pH 6.5; †DMF: dimethylformamide; TFA: trifluoroacetic acid; ††BTI: bis(I,I-trifluoroacetoxy)-iodobenzene.
The basal conditions describing this experiment were taken from Johnson *et al.* (1985). Further details are listed in Methods and text. The source of MAPs was the 17,500 *g* (30 min) supernatant derived from a 30 min (0°C) treatment of pellet No. 6 (see Table 3, A1) with 1% Lubrol-P_x. Following the first preincubation (BTI: 36 mg; 60°C, 4 h in the dark), dialysis, the second preincubation (37°C, 90 min) and pH adjustment to 6.5, an identical amount of protein (0.271 mg) was incubated in the presence of 20 μl of the purified cytosolic rat brain PCMT and MAP capacity was determined. The corrected picomole values were obtained by subtracting (2) from (3), (2) from (4) and (3) from (5).

1 ml in the dark for 4 h at 60°C, with appropriate controls incubated alongside (lines 2 and 3). All samples were then dialysed overnight against distilled water and were preincubated for 90 min at 37°C either in the presence of 50 mM Na phosphate buffer, pH 6.5 or of 0.05 M NH_4OH. Samples were then adjusted, as required, to pH 6.5 with 1 M acetic acid.

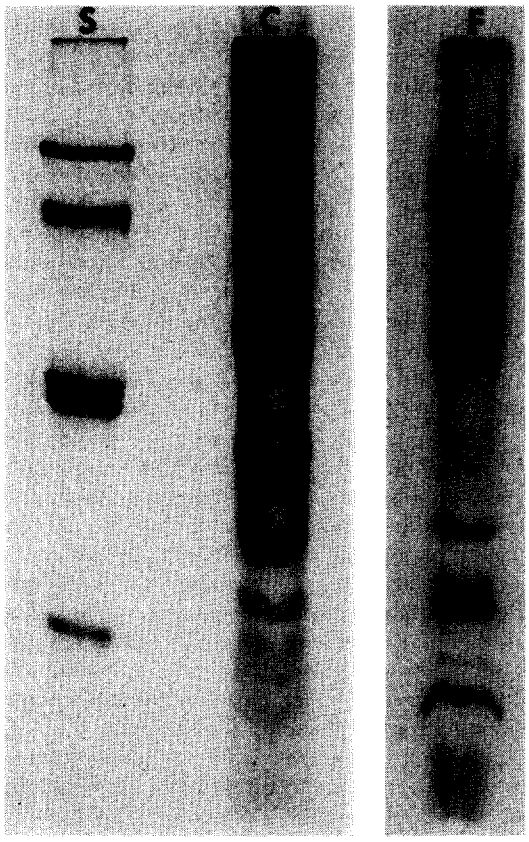

Fig. 4. Acidic slab gel electrophoresis of the endogenous carboxyl-[^{3}H]-methylated rat brain membrane-bound proteins. Pellet No. 6 (see legend to Table 3A) (1.3 mg of protein) was subjected to carboxyl-[^{3}H]-methylation, as described in Methods, for 2 h at 37°C, in the presence of 20 μcuries of [^{3}H]-methyl SAM (19 Ci/mmol). Pellets were obtained by centrifugation (6,000 rpm, 30 min) and after one wash were resuspended in 300 μl of acidic sample buffer (Aswad and Deight, 1983a). Sixty-nine μl, containing 300 μg of protein/lane, was applied. Lane S: molecular weight standards, from the top down: phosphorylase b (94 KDa); bovine serum albumin (67 KDa); ovalbumin (43 KDa); carbonic anhydrase (30 KDa but, in confirmation of Aswad and Deight (1983a), running in this gel system as a 40 KDa protein) and soybean trypsin inhibitor (20.1 KDa), Lane C: Coomassie blue staining pattern of pellet No. 6 proteins; Lane F: [^{3}H]-fluorogram of the carboxyl-[^{3}H]-methylated pellet No. 6 proteins.

For the subsequent determination of MAPs, the carboxyl-[^{3}H]-methylation assay was fortified by the addition of 20μl of the purified, cytosolic rat brain PCMT (see Materials). All tubes contained an identical amount of protein (0.271 mg). The results show an apparent effect of BTI *per se*, relative to the control containing the acidic solvent alone (compare lines 3 and 2), on the generation of carboxyl-[^{3}H]-methylatable sites; we have no cogent explanation for this observation, which we have made repeatedly. However, the principal feature of the findings shown in Table 8 is the marked reduction of the ability of NH_4OH to generate new MAP sites for PCMT action, following BTI treatment (compare corrected values in lines 5 and 4). This finding, replicated in 3 consecutive experiments, is consistent with the notion that BTI converts a portion of MAP amide residues, such as those present in glycine-linked asparagines (Johnson *et al.*, 1985), to the corresponding primary amines, thus rendering them unavailable for the subsequent NH_4OH-induced conversion to novel carboxyl-[^{3}H]-methylatable sites.

(f) *The nature of the mb-MAPs.* The results of acidic slab gel electrophoresis of the carboxyl-[^{3}H]-methylated mb-MAPs are illustrated in Fig. 4, together with a Coomassie Blue staining profile of the proteins present in the membranes contained in pellet No. 6. The figure illustrates the fact that relatively few membrane proteins appear to act as MAPs for the mb-PCMT, and that fewer than a dozen, ranging in molecular weights from under 20 to about 90 KD, share the greater portion of the radioactivity associating with the mb-carboxyl-[^{3}H]-methylated proteins.

DISCUSSION

The present findings aim to characterize the mb-protein carboxyl-methylation system of brain tissue and constitute evidence for its association with membranes of all 3 principal cell types of rat brain, namely the neurons, the glial astrocytes and the oligodendroglial cells. In addition, the findings emphasize the self-sufficiency of the mb-protein carboxylmethylation system under study, in that they show a selective capacity of some, but not all of the mb-proteins, to act as MAPs for mb-PCMT (Fig. 4). The findings thus greatly expand the observations of Diliberto and Axelrod (1976), who first noted the existence of "endogenous" protein carboxylmethylation in brain tissue and those of Iqbal and Steenson (1976), who first documented the fact that cerebellar membranes contain PCMT activity able to recognize exogenously

added MAPs. It is indeed important to point out that since brain mb-PCMT activity has so far been assayed exclusively in the presence of added MAPs, no information on its intrinsic ability to use endogenous mb-MAPs as its effective and specific substrates has been presented to date.

Recently, Brown (1984) reported that the PCMT activity associated with whole rat brain synaptosomes, isolated on sucrose-density gradients, became non-sedimentable after exposure to 0.2% Triton X-100 at 37°C for 30 min and high-speed centrifugation. Brown (1984) also reported activity ratios between the PCMT acting on added vs endogenous MAPs of about 3 for total synaptosomes. We report values approximating 7 (Table 2) in synaptosomes isolated on Percoll-gradients. Our data also reveals (Table 2) a doubling of the activity of synaptosomal mb-PCMT acting on endogenous MAPs when assayed in the presence of 1% Nonidet P-40, a finding suggesting a topographic location for the mb-PCMT-MAP system deep within the matrix of the synaptic membrane. The presence of such an mb-PCMT component in synaptic membranes could not be inferred from the published literature to date (Diliberto and Axelrod, 1976; Brown, 1984).

The presence of PCMT activity in homogenates, membranes and cytosolic fractions derived from glial cells is also reported in Table 2. These findings document an extraneuronal localization for brain protein carboxylmethylation and point to a quantitatively significant activity in the oligodendroglial cells. Since bovine and rat myelin and bovine basic myelin protein have recently been shown to be excellent substrates for cytosolic PCMT (Johnson and Aswad, 1985a), the present findings are not overly surprising. They also cast doubt on the notion of a special role of protein carboxylmethylation in brain areas abounding in specialized neuronal elements (Billingsley and Lovenberg, 1985; Billingsley *et al.*, 1985a; Billingsley and Balaban, 1985).

As previously noted with cerebral mb-catechol-O-methyltransferase (Jeffery and Roth, 1984), the PCMT activity of pellet No. 1 (Table 3, A1) accounted for no more than 30% of the total PCMT; moreover, it resisted solubilization more effectively with successive buffer washes until, after wash No. 5, only 18.1% if its residual mb-activity became non-sedimentable. Treatment of the membranes in pellet No. 6 with detergents resulted in effective solubilization of PCMT as well as in a major "activation" of the solubilized enzyme (Table 3, A2) as evidenced by its excessive recoveries after 1% Na-deoxycholate, 1% Nonidet P-40 and 1% Lubrol-P_x.

The more detailed study of the differential sensitivity of the mb-PCMT activity toward detergents (Table 4) revealed that its exposure to Na-deoxycholate and, to a lesser extent, CHAPS, led to significant stimulation of mb-PCMT acting on added MAPs, while a similar exposure to Triton X-100 and Nonidet P-40 had no such effect. Different effects of detergents on mb-brain enzymes have been reported (Percy *et al.*, 1982; Singh *et al.*, 1985), and it is of particular interest that Na-deoxycholate, which markedly stimulates mb-PCMT (Table 4), was shown to totally inhibit the phospholipid methyltransferase activities in brain membranes (Percy *et al.*, 1982).

In an attempt to determine whether the stimulation of the mb-PCMT activity by CHAPS at 0°C (Table 4) could be modulated by raising the temperature, the experiment described in Table 5 was performed. Comparison of the effect of temperature on the s.a. of the mb-PCMT acting on endogenous MAPs reveals its decrease in the absence, and its slight increase in the presence of CHAPS (II vs I). Table 5 also shows that the s.a. of the CHAPS-resistant mb-PCMT component decreased markedly as a function of the temperature of preincubation, while, conversely, the s.a. of the CHAPS-solubilized PCMT underwent no such temperature-dependent decrease. The overall findings of Table 5 reflect a complex response of the mb-PCMT component to the dual challenge of temperature and 30 mM CHAPS. Whether this response is in turn a manifestation of the existence of the mb-PCMT as a glycosylated (Sweetnam and Tallman, 1986), or otherwise post-translationally modified, membrane-integral protein cannot be answered at this time.

The nature of the mb-MAPs was investigated in several ways, by examining effects of detergents on their quality as mb-PCMT substrates (Figs 2 and 3) and by chemically modifying them (Tables 7 and 8). This was done in an attempt to learn more about the structural requirements necessary for a protein to act as an effective substrate for endogenous mb-PCMT. We also confirmed that purified proteins which are excellent MAPs for the cytosolic PCMT (Gagnon *et al.*, 1981; Aswad and Deight, 1983b) are also excellent MAPs for mb-PCMT (Table 6).

Treating MAP-containing brain membranes with Lubrol-P_x and CHAPS (Fig. 2) revealed kinetically different time courses of MAP "inactivation" and a 30% loss in the ability of the CHAPS-treated MAPs to be recognized by the mb-PCMT. The results depicted in Fig. 3 suggest a more complex scenario, however, inasmuch as they demonstrate that buffer-pretreated mb-MAPs added to either Lubrol-P_x or

CHAPS-pretreated mb-MAPs provided for higher levels of mb-PCMT activity than did the homologous additions of P_L to P_L and P_C to P_C (see legend, Fig. 3). These findings suggest that the action of the 2 detergents damaged the mb-MAPs as mb-PCMT substrates whereas their prolonged exposure to buffer at pH 6.5 had no such negative effect.

As recently suggested by Clarke and associates (Clarke, 1985; Murray and Clarke, 1986), Aswad and associates (Aswad, 1984; Johnson and Aswad, 1985a, b; Johnson *et al.*, 1985) and jointly by both groups (O'Connor *et al.*, 1984), the carboxylmethylation of proteins (peptides) by the cytosolic PCMT of brain and other tissues (O'Connor *et al.*, 1984) may occur at the α-carboxyl group of atypical β-linked aspartyl (L-isoaspartyl) residues. These atypical L-isoaspartyl residues may be readily generated *in vitro* in proteins (peptides) containing asparagine-glycine bonds through a mild base-catalyzed deamidation, the ensuing formation of a cyclic succinimide intermediate and its spontaneous ring opening to yield the normal (aspartyl) and the atypical (isoaspartyl) peptides in variable ratios (Murray and Clarke, 1986). Since Johnson *et al.* (1985) have shown that exposure of pure bovine brain calmodulin to 0.1 M MH_4OH dramatically increased its carboxylmethylation by cytosolic PCMT, we subjected a Lubrol-P_x extract of the membrane proteins contained in pellet No. 6 (Table 3, A1) to 0.05 M NH_4OH and noted a 4-fold increase in the carboxyl-[^{3}H]-methylation of its MAPs by the co-present, solubilized PCMT, which appeared to survive the NH_4OH treatment largely unscathed (Kim, 1984). Furthermore, we also noted that pretreatment of the Lubrol-P_x and Nonidet P-40 extracts of the membrane proteins of pellet No. 6 with BTI greatly diminished the subsequent effectiveness of 0.05 M NH_4OH to generate novel carboxyl-[^{3}H]-methylatable residues in the solubilized brain MAPs. This finding is taken as strong evidence in support of the notion that the increase in MAP carboxyl-[^{3}H]-methylation noted after NH_4OH (Table 7) reflects the generation in the affected MAPs of atypical L-isoaspartate residues which are known to be the preferred target of PCMT action. Conclusive evidence in favor of this notion is being gathered presently.

Acknowledgements—The research was supported in part by a pilot grant from the National Institute on Aging (NIH), AG-05022.

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