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THE CARBOXYLMETHYLATION OF CEREBRAL MEMBRANE-BOUND PROTEINS INCREASES WITH AGE

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SUMMARY

Recently, we have characterized a membrane-bound (mb) component of brain protein carboxylmethyltransferase II (PCMT) which effectively carboxylmethylates endogenous mb methyl-accepting proteins (MAPs). (*Neurochem. Int., 10* (1987) 155). We have also shown that exposing mb-MAPs to mild alkali leads to a marked increase in their recognition by PCMT. Since one of the likely consequences of the alkaline treatment appears to be the deamidation of selected protein-bound asparagines or aspartates, followed by the formation, in their place, of D-or L-isoaspartates, it is reasonable to assume that mb-MAPs constitute unique targets for the mb-PCMT because they contain such unnatural aspartate residues. Testing the relevance of this notion to the aging of cerebral mb-MAPs we focus in this report on age-related changes involving mb-MAPs. When two-or six-times washed (in 50 mM $NaPO_4$ buffer, pH 6.5) 17 500 *g*, 30-min membranes or Percoll-gradient purified synaptic membranes were prepared from young (3—4 months) and old (11—12 months) rat brains and were incubated with 20 μM [^{3}H]methyl *S*-adenosyl-L-methionine at pH 6.0, mb-MAP carboxyl[^{3}H]methylation was significantly more intense in the old than in the young membranes, no additional increase being noted at 28—35 months. Mb-MAP carboxylmethylation increases were confirmed over a wide range of membrane protein concentrations and incubation times and are taken to reflect age-related modifications of the primary structure of susceptible mb-MAPs. To investigate these, we incubated young and old membranes, as well as their Lubrol-P_x(1%) extracts (30 min, 0°C), with 0.05 M NH_4OH for 90 min at 37°C, a treatment which left PCMT activity largely unaffected. Our findings reveal

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Abbreviation: SAM, *S*-adenosyl-L-methinonine.

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that the effect of the NH_4OH treatment on the generation of carboxylmethylatable sites was markedly smaller in "old" than in "young" proteins, suggesting that "new" carboxylmethylatable sites are generated in susceptible mb-MAPs *in situ,* by a process accompanying, or otherwise marking, the natural aging of neural membrane proteins.

Key words: Brain; Protein carboxylmethylation; Membranes

INTRODUCTION

Although the process of cellular aging is admittedly highly complex, [1] evidence is accumulating to suggest that it has a molecular basis and, hence, that it may be profitably studied through investigations of the aging of intracellular proteins. Several types of modification may be incurred by proteins residing in aging cellular systems [2,3] and part of the relevant research has recently been summarized [4—6]. One type of an enzyme-catalyzed age-related protein modification is its carboxylmethylation. The cognate enzyme, protein carboxylmethyltransferase II (EC. 2.1.1.24) (PCMT) [7,8], appears to recognize only those proteins in which, *via* biological mechanisms still incompletely understood [9,10], selected L-aspartate residues have been replaced by D-aspartate [11] and/or L- or D-isoaspartate [12—15]. Although several reports associating the presence of protein-bound D-aspartate with aging have appeared, [16—19] there is no unambiguous evidence for the presence of unnatural isoaspartates in proteins of either young or aged cells.

Recently we have characterized in some detail a membrane-bound (mb) component of brain PCMT [20,21] which effectively carboxylmethylates mb methyl-accepting proteins (MAPs). We have also shown that exposing mb-MAPs or their Lubrol-P_x extracts to mild alkali leads to a marked increase in their recognition by PCMT [22]. Since, *in vitro,* one of the likely consequences of the mild alkaline treatment appears to be the deamidation of selected protein-bound asparagines [23], and/or aspartates [24], followed by the formation, in their place, of unnatural isoaspartate(s) [25,26], it is reasonable to assume that mb-MAPs constitute unique targets for the mb-PCMT, because they contain unnatural aspartate residues. In the present report, we test the relevance of this notion to the aging of cerebral mb-MAPs and find that, in the rat, their treatment with mild alkali results in the generation of significantly fewer protein-bound sites susceptible to carboxylmethylation by the mb-PCMT in middle age and very old brains, than in young brains. Based on this and other findings, also part of this report, we propose that, as selectively vulnerable asparagine and/or aspartate residues of specific brain mb proteins progressively convert to unnatural aspartate forms *in situ,* the resulting increase in their carboxylmethylatability *in vitro* may represent a useful marker with which to trace the time course of their aging.

MATERIALS AND METHODS

S-adenosyl-L-methionine (di-*p*-toluenesulfonate salt) was a gift from Dr. G. Stramentinoli, BioResearch, Liscate, Italy. Crystalline ovalbumin, bovine serum albumin, Lubrol-P_x, Coomassie Blue and Percoll were from Sigma. The MW standards for electrophoresis were from Pharmacia. Acrylamide and *N,-N'*-bis-methylene acrylamide were from Serva. En^3-Hance was from New England Nuclear. [^{3}H]methyl *S*-adenosyl-L-methionine (1 mCi/ml) with a nominal specific radioactivity of 10—15 Ci/mmol was from ICN. All other reagents were of the best analytical grade available.

Animals

Rats were Sprague—Dawley, Charles River males purchased from the local vendor (Portage, Michigan). They were 2—4 (young), 11—12 (middle age) and 28—35 (very old) months old.

Preparation of fractions

Rat cerebra were homogenized in about 10 vols. of ice-cold 50 mM $NaPO_4$ buffer (pH 6.5) and the homogenate centrifuged at 17 500 *g* for 30 min. The large pellet was re-homogenized mechanically (8—10 up-and-down strokes) using a Teflon-tipped pestle rotating in a glass homogenizing tube at approximately 1200 rev./min. Pellet No. 2 was obtained by centrifuging as above. This pellet, one washed six times, or a pellet consisting of highly purified synaptic membranes (see below) were the source of the mb-PCMT-MAP system used throughout the study. Synaptosomes were prepared according to Nagy and Delgado-Escueta [27]. The collected gradient bands (10%—16% Percoll interface) from several tubes were sedimented at 105 000 *g* for 45 min and the pellet containing the synaptosomes was resuspended by manual homogenization in 3 ml of 50 mM $NaPO_4$ buffer (pH 6.5) for 30 min at 0°C. Synaptic membranes were collected by centrifugation at 105 000 *g* for 20 min. They were washed once, before resuspension and aliquoting into 1-ml portions followed by freezing or immediate use.

Analytical

Protein was determined according to Peterson [28], with crystalline bovine serum albumin as standard.

Acidic slab gel electrophoresis

The procedure described by Aswad and Deight [29] was used. A vertical slab gel system (SE-400, Hoefer Scientific), powered by 180 V for 3.5 h at room temperature provided the desired separations. After electrophoresis, portions of the slabs containing the radioactive proteins were treated with En^{3}Hance according to the vendor's instructions and were immediately dried in a slab gel drier (Pharmacia,

GSD-4) at 32v, 1 amp for 2.5 h before being placed in contact with XAR-5 X-ray film for exposure at −70°C. The non-radioactive proteins, run in parallel lanes, were stained with 0.25% (w/v) Coomassie Blue for 30 min and were then destained in 7% (v/v) acetic acid.

The assay of PCMT activity

(a) Acting on endogenous mb-MAPs. The procedure of Diliberto and Axelrod [30] was used. Each tube (255 μl) contained 5—10 μcuries of [^{3}H]methyl SAM and the following, at the final concentration: phosphate-citrate buffer (pH 6.0), 50 mM; SAM, 20 μM; EDTA, 0.4 mM; dithiothreitol, 1.2 mM and tissue, up to 200 μl [22]. 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 rev./min for 15 min. The pellets were suspended 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. After the addition of 1.2 ml of a 3:2 (v/v) mixture of toluene: isoamyl alcohol, the tubes were thoroughly vortexed and centrifuged at 4000 rev./min for 5 min. Two 0.5-ml aliquots of the upper phase, containing the released [^{3}H]CH_3OH were placed into 2 scintillation vials for determination of the total and the carboxyl-[^{3}H]methyl radioactivity.

(b) *Acting on ovalbumin.* The above assay was carried out in the presence of 100 μg of ovalbumin.

(c) *mb-PCMT activity.* PCMT activity is expressed as pmol of [^{3}H]methyl transferred from [^{3}H]methyl SAM to the endogenous mb-MAPs or, when ovalbumin was present, to the sum of the mb-endogenous MAPs + ovalbumin. PCMT specific activity (spec. act.) is defined as activity/mg of protein.

(c) *The mild alkaline treatment.* Six-times washed membrane pellets were resuspended in 50 mM $NaPO_4$ buffer (pH 6.5), containing the mb-PCMT-MAP system [22] and were then treated with 0.05 M NH_4OH (see below) or, first, with 1% Lubrol-P_x at 0°C for 30 min. In the latter case, following centrifugation at 17 500 *g* for 30 min, the resulting supernatant (S_L) and pellet (P_L), (resuspended in 50 mM $NaPO_4$ buffer (pH 6.5)), were incubated in the presence of 0.05 M NH_4OH for 90 min at 37°C, alongside controls containing no ammonia. The pH of all of the samples was re-adjusted to 6.5 with 1 M acetic acid (requiring 35—45 and 3—4 μl for the alkali-exposed and the control samples, respectively) and the amount of NH_4OH-generated mb-MAPs was then determined in the standard assay. Separate experiments established that the above NH_4OH treatment caused no loss of PCMT activity, thus obviating the necessity to use the purified cytosolic PCMT for the quantitation of the NH_4OH-generated mb-MAPs.

RESULTS

Initially, the time course (10—120 min) of carboxyl-[^{3}H]methylation of mb-MAPs by the mb-PCMT was compared in six-times washed membranes of young

and very old brains. The results (data not shown) of these experiments clearly demonstrated higher levels of carboxylmethylation in the very old animals' brains. Thus, after 60 and 120 min of incubation, respectively, we consistently found 10—15 and 20—25 pmol of [^{3}H]methyl transferred to mb-MAPs in the very old, *vs.* 6—7 and 9—12 pmol in the young brains. Interestingly, however, a similar comparison of the mb-PCMT-MAP system (15—180 min of incubation) between synaptic membranes [27] of young and middle age brains revealed no significant differences in carboxylmethylation, whether this was tested in fresh or once liquid nitrogen frozen-thawed membrane preparations (O.Z. Sellinger and S. Fielek, unpublished observations).

The significantly higher levels of protein carboxylmethylation in the six-times washed membranes of the very old brains are further illustrated in Fig. 1.

[SAM]-dependent differences in carboxyl-[^{3}H]methylation between mb-MAPs of young and very old six-times washed membranes are illustrated in Fig. 2. Since more [^{3}H]methyl appeared to be transferred to mb-MAPs in the older membranes, a higher apparent V_{max} value for SAM obtained; however, given our inability to date to identify the nature, number and molecular characteristics of the individual proteins

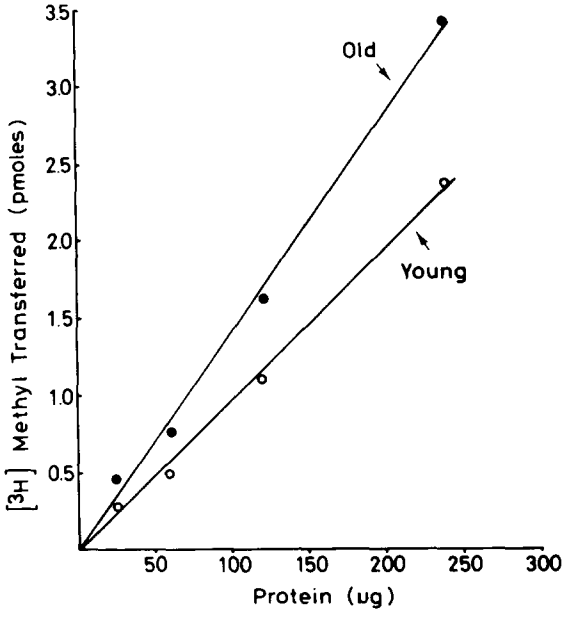

Fig. 1. The effect of membrane protein concentration on the carboxyl-[^{3}H]-methylation of brain MAPs. Six-times washed membranes were prepared (see Methods) from brains of a young (65 days) and an old (341 days) rat. Suspensions containing the amounts of protein indicated were incubated for 60 min in the standard assay (see Methods). The values on the ordinate refer to the number of pmol transferred from [^{3}H]SAM to acceptor proteins during the incubation. In three consecutive experiments the difference between old and young was within 15—20% of the values shown, favoring the old membranes.

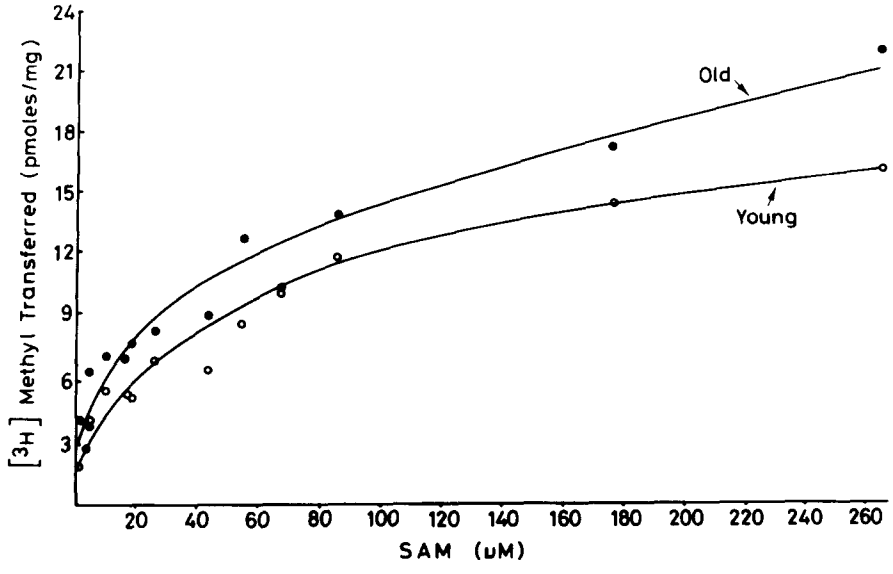

Fig. 2. The effect of varying the concentration of SAM on the carboxylmethylation of rat brain mb-MAPs. The graphs shown are a composite of three separate experiments at each age, in each of which the effect of at least five different concentrations of SAM was compared in the presence of 456 μg of young (65 days) and 467 μg of old (341 days) membrane protein. Incubations were for 30 min in the standard assay (see Methods).

being carboxylmethylated i.e. of the mbMAPs, we refrain from expressing the relevant V_{max} values for [SAM] numerically.

A comparison of the PCMT activity in two- *vs.* six-times washed membranes of young, middle age and very old rat brains is presented in Table I. Although the specific activity of the endogenous mb-[^{3}H]MAPs (experiment 1) diminished at all ages as the concentration of protein in the incubates increased, its absolute value was significantly higher at 11—12 than at 2—4 months at each concentration of protein tested. Conversely, there was little additional change in the specific activity of the mb-[^{3}H]MAPs between 11—12 and 28—35 months of age. Further evidence for the middle age onset of the increase in mb-MAP carboxyl-[^{3}H]methylation is provided by the marked increase (78%) of the specific activity of mb-[^{3}H]MAPs at 11—12 months relative to that noted at 2—4 months (experiment 2), with no further increase at 28—35 months. The findings of experiments 3 and 4 (Table I) also reveal the specific activity of the mb-[^{3}H]MAPs to be clearly higher at 28—35 months than at 2—4 months, both before (experiment 3) and after solubilization (experiment 4) of the relevant MAPs. When assayed in the presence of ovalbumin (Table I), BSA or calmodulin (data not shown), levels of mb-PCMT activity showed no consistent age-related fluctuations.

The effect of treating proteins of young and very old rat brain membranes with

TABLE I

AGE AND MEMBRANE-BOUND PROTEIN CARBOXYLMETHYLATION IN RAT BRAIN: ENDOGENOUS PROTEINS *vs.* OVALBUMIN AS SUBSTRATES

Expt. No.	*Fraction*	*Protein (mg)*	*Endogenous MAPs**					*+ Ovalbumin*		
			Age: months					*Age: months*		
			(2—4)	*(11—12)*	$\Delta_{(age)}$	*(28—35)*	$\Delta_{(age)}$	*(2—4)*	*(11—12)*	*(28—35)*
			(spec. act.)	*(spec. act.)*	*(%)*	*(spec. act.)*	*(%)*	*(spec. act.)*	*(spec. act.)*	*(spec. act.)*
1	M,2	0.72	17.8	24.3	+36.5	19.8	+11.2	45.0	49.4	44.2
	M,2	1.35	5.4	6.8	+25.9	8.5	+57.4	13.0	15.6	16.7
	M,2	2.60	2.9	4.4	+51.7	4.0	+37.0	—	—	—
2	M,6	0.50	13.6	24.2	+77.9	24.4	+79.4	20.6	29.6	36.0
3	M,6	1.13	14.8	—	—	30.0	+103	29.0	—	24.6
4	S_L	0.80	64.0	—	—	89.6	+40.0	—	—	—
								—	—	—

*, Methyl accepting proteins.

spec. act.: pmol [^{3}H]methyl transferred/mg protein per h. The $\Delta_{(age)}$ values are to be compared to the 2—4 month-old group. M, membranes; 2,6: number of buffer washes used to prepare fraction M; S_L, supernatant obtained after centrifugation of the Lubrol-P_x treated M,6 fraction (see Methods).

TABLE II

THE ACTION OF 0.05 M NH_4OH ON METHYL ACCEPTING PROTEINS IN YOUNG AND VERY OLD RAT BRAINS

Fraction	*Young*			*Very old*			*Difference**
	Control spec. act.	*+ NH_4OH spec. act.*	*Increase (%)*	*Control spec. act.*	*+ NH_4OH spec. act.*	*Increase (%)*	*Δ,(NH_4OH, age)*
Mb-MAPS**	13.5	26.0	+ 92.6	16.3	26.9	+ 65.4	− 27.2
S_L-MAPs	(a) 31.9	135	+ 415	44.8	89.4	+ 99.8	− 315
	(b) 29.8	58.6	+ 96.7	46.7	81.1	+ 58.7	− 38
	(c) 11.7	37.3	+ 318	26.5	57.7	+ 226	− 92
P_L-MAPs	(a) 5.28	15.0	+ 284	5.64	12.0	+ 113	− 171
	(b) 5.66	12.7	+ 124	6.0	10.9	+ 93	− 31

*, Δ, (%), (young—very old) (column 3—column 6).

**, MAP: Methyl-accepting proteins.

spec. act., pmol of [^{3}H]-methyl transferred/mg protein per 30 min.

Membranes, 6-times washed (see Methods), exposed to pH 6.5 (control) or to 0.05 M NH_4OH for 90 min at 37 °C.

S_L, P_L, Lubrol-P_x supernatant and pellet (see Methods).

S_L, a,b,c, three consecutive experiments; P_L, a,b: two consecutive experiments.

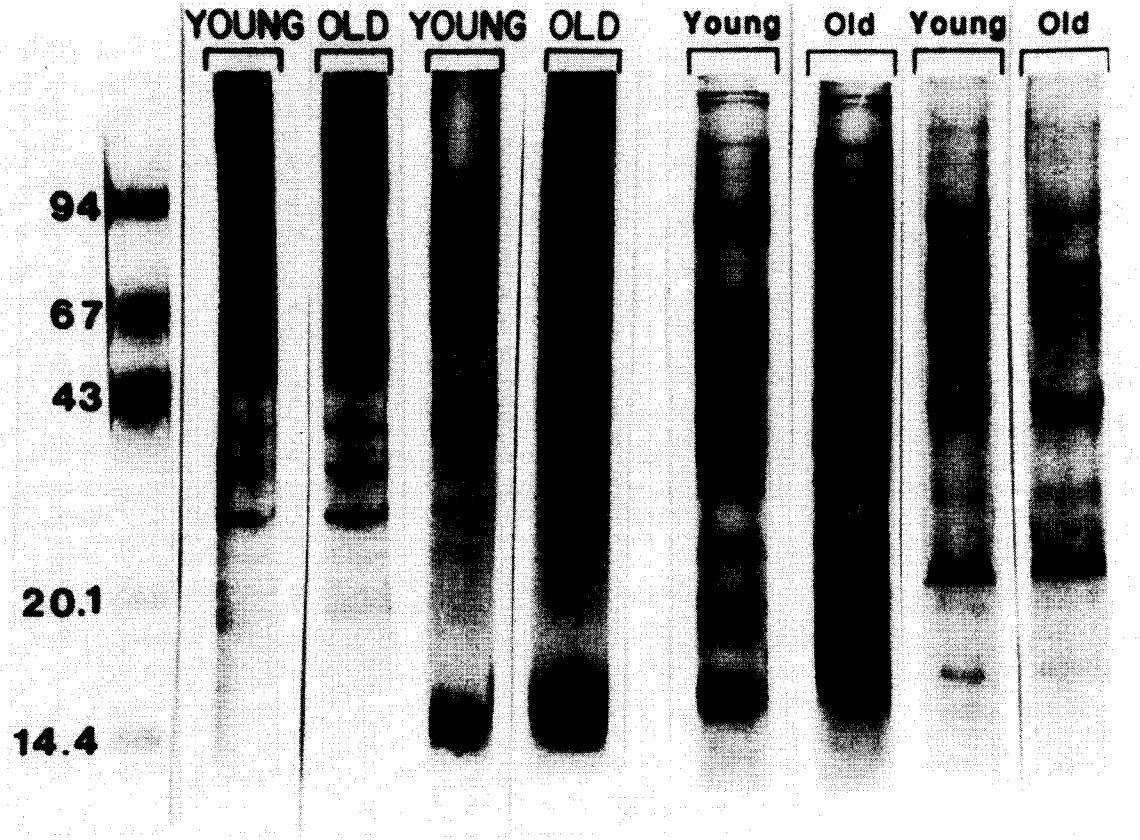

Fig. 3. Acidic slab gel electrophoresis of the carboxyl-[^{3}H]-methylated membrane proteins. *Lanes 2—4:* Young: 60 days old; old: 35 months-old. Six-times washed membranes were prepared and suspensions containing 1.32 mg of protein were incubated in the standard assay (see Methods) for 1 h in the presence of 20 μcuries of [^{3}H]-methyl SAM (65.6 Ci/mmol). Pellets were obtained by centrifugation (6000 rev./min, 30 min) and, after one wash, were resuspended in 300 μl of acidic sample buffer [29]. Sixty-eight μl, containing 300 μg of protien/lane was applied. *Lane 1:* molecular weight standards, from the top down: phosphorylase b (94 kDa); bovine serum albumin (67 kDa); ovalbumin (43 kDa); soybean trypsin inhibitor (20.1 kDa); lactalbumin (14.4 kDa). *Lanes 2 and 3:* Coomassie blue staining pattern of young and old membrane proteins. *Lanes 4 and 5:* [^{3}H]-fluorogram of the carboxyl-[^{3}H]-methylated young and old mb-[^{3}H]MAPs. *Lanes 6—9:* Young: 90 days old; old: 368 day old. Synaptic membranes were purified according to Ref. 27 and 1.3 mg of protein was incubated in the presence of 20 μcuries of [^{3}H]-methyl SAM (12 Ci/mmol) for 60 min (see Methods). After centrifugation and resuspension 69 μl, containing 300 μg of protein/lane was applied. *Lanes 6 and 7:* Coomassie blue staining pattern of young and old synaptic proteins. *Lanes 8 and 9:* [^{3}H]-fluorogram of the carboxyl-[^{3}H]-methylated young and old synaptic mb-[^{3}H]MAPs.

0.05 M NH_4OH for 90 min at 37 °C was tested next. Table II shows that (a) mb-[^{3}H]MAPs were generated in the unfractionated membranes and in the Lubrol-P_x-derived subfractions, S_L and P_L; (b) a greater number of MAPs appeared after the NH_4OH treatment in the young than in the very old brains and, consequently, (c) there was a significant reduction in the magnitude of the NH_4OH effect in the very old, relative to the young brains. The uniformly negative $\Delta_{(NH_4OH,\ age)}$ values shown in the last column of Table II are a quantitative expression of these findings. Figure 3 illustrates the young *vs.* old banding pattern of the proteins in the six-times washed membranes and in the synaptic membranes after Coomassie staining and following 60-min incubation with [^{3}H]SAM. The fluorograms reveal fewer than a dozen mb-[^{3}H]MAPs. Yet, while relative to its young counterpart, the fluorogram of the six-times washed old mb proteins shows higher intensities of labelling in the regions of the slowest and fastest migrating species, the synaptic fluorograms fail to reveal discernible differences in labelling intensity between any of the young and old mb-[^{3}H]MAPs.

DISCUSSION

The present findings document an increase in the carboxylmethylation of methyl-accepting proteins contained in thoroughly washed membranes prepared from rat brain at 11—12 months of age, relative to its levels determined at a single earlier (2—4 months) and a single later (28—35 months) point in life. The observed increase in the intensity of this carboxylmethylation appears to be due, in large measure, although not exclusively, (see Table I, + ovalbumin) to an increased availability of methylatable sites in the relevant mb-MAPs (Fig. 1), rather than in improvements of the ability of the mb-PCMT to recognize preexisting mb-MAPs. We cannot rule out, however, that both components of this newly characterized [22] mb-PCMT-mb-MAP system of brain undergo modifications in membrane structuration and/or in chemistry simultaneously, given our inability to date to cleanly dissociate mb-PCMT from its mb-MAP substrates. At present, it would appear that synaptic mb-MAPs are not involved.

As recently noted by Man *et al.* [19], structural changes in selected proteins of the human brain, such as the racemization of some of their L-aspartate residues to the unnatural D-form, appears to be on ongoing process, with no chronologically precise time-frame; indeed, protein-bound D-aspartate could be detected in the human brain at 10 days post-natally and was noted to peak in or close to midlife (40—49 years of age). Our present findings appear to be consistent with this notion, i.e. that, in rat brain as well, highest intensities of mb-protein carboxylmethylation, presumed to occur exclusively on unnatural aspartate residues of selected proteins, are reached between 1/3 and 1/2 of this (laboratory-reared) rodent's life span.

The results of preliminary HPLC analyses [31] of the acid hydrolysed mb-proteins contained in six-times washed membranes of both young (2—4 months) and

middle-age (11—12 months) rat brains are in further agreement with such possibilities, as they confirm the natural presence of D-aspartate in both sets of samples (O.Z. Sellinger and C.M. Kramer, unpublished observations).

A chronological relationship between increased protein carboxylmethylation and aging has been demonstrated for proteins of the human erythrocyte [5,11,16—18]. Most recently, a detailed study has revealed that intrinsic proteins of the erythrocyte membrane, but not the erythrocyte cytosolic proteins, undergo a most intensive racemization of L- to D-aspartate and, also, that they accumulate other amino acid residues which form D-aspartate upon acid hydrolysis [16]. The latter have been presumed to include D-asparagine and D-isoaspartate. It is also well established, on the other hand, that L-isoaspartate residues, shown to be effective substrates for PCMT [13—15,24,25] when present in synthetic peptides [24] or when generated *in vitro* [32], may form from protein-bound L-aspartate or L-asparagine residues [33], *via* a chemical sequence, the natural occurrence of which would necessitate as a minimum a microenvironment in which foci of local alkalinity would remain sustained over time. It has been shown in this context, and our findings (Table II) prove it for rat brain mb-MAPs, that treatment of MAPs with ammonia [22,25] generates new carboxylmethylatable sites by converting susceptible natural aspartate/asparagines to "atypical" forms of amino acids [5,9]. This test tube reaction may be viewed, therefore, as the single macro event which telescopes, in a very short time and under man-made laboratory conditions, the presumed recurrent micro events which result *in situ* in net increases of mb proteins containing unnatural aspartate residues. The function of these increments in mb-MAP content per se, in the context of biological aging of membranes or in terms of the indirect effects of such age-induced mb-protein modifications on the behavior of cytoskeletal structures into which mb-MAPs are naturally embedded, is unclear at this time. Our findings (Table II) of a significant reduction in the effectiveness of the ammonia treatment to generate new carboxylmethylatable sites in cerebral mb-MAPs of old, relative to young, six-times washed membranes strongly infers the possibility that modifications similar to those ammonia is able to produce in the test tube, do continue to be generated as young membranes mature, reach middle-age and continue on to become old and very old.

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