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REDISTRIBUTION OF RAT BRAIN ESTERASES DURING SUBCELLULAR FRACTIONATION

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SUMMARY

Carboxylic ester hydrolases (nonspecific esterases) and acetylcholinesterase were determined in particulate fractions of rat cerebrum after differential and density-gradient centrifugation. The crude mitochondrial fraction was osmotically shocked by distilled water. When related to the total activity of the original homogenate, 12% of nonspecific esterases and 18.5% of acetylcholinesterase were recovered in the crude mitochondrial fraction. Assays of mitochondrial subfractions showed that 68 and 63.8% of these enzyme activities respectively were recovered in the myelin. The high activity of the myelin subfraction appeared to be due to artifactual redistribution of enzyme. The amount of enzyme released and subsequently bound to subcellular membranes during preparative manipulations might depend on protein–lipid interactions. The synaptic membrane subfraction retained by 0.9 M sucrose differed from the other subfractions in the degree of retention of membrane-bound enzymes during suspension in media of varying pH, osmolality and ionic concentration. These membranes may have a special and physiologically significant molecular organization which differs from that of other membrane fractions.

INTRODUCTION

The carboxylic ester hydrolases (nonspecific esterases) are widely distributed in neural and other tissues and are present early in evolutionary development[1]. However, their naturally occurring substrates are unknown. In contrast, a function for acetylcholinesterase in neural transmission seems reasonably well established. Presumably its physiological substrate is acetylcholine, a neurohumoral transmitter. Acetylcholinesterase is concentrated at synapses although by no means limited to this site. It is demonstrable also in the cell body of neurons where it occurs in the endoplasmic reticulum[2].

Few investigations have dealt with the distribution of nonspecific esterases in subcellular fractions of brain[3,4]. By use of differential centrifugation, SELLINGER AND DE BALBIAN VERSTER[4] localized the enzyme mainly to the microsomal fraction of rat cerebral cortex. BERNSOHN *et al.*[3], employing the density-gradient centrifugation technique of DE ROBERTIS *et al.*[5], recovered nonspecific-esterase activity not only

in the microsomal fraction but also in other subcellular particulates of rat cerebrum, including synaptic membranes.

Detailed studies have been performed on the subcellular localization of acetylcholinesterase and acetylcholine[5–8]. A comparison of the acetylcholinesterase distribution data of DE ROBERTIS *et al.*[5] and of RODRIGUEZ DE LORES ARNAIZ *et al.*[6] reveals that, in the latter report, the myelin fraction was much richer in enzyme content and that it had a higher relative specific enzyme activity than in the earlier study from the same laboratory. The density gradient used in the two investigations differed only slightly. However, the experiments of RODRIGUEZ DE LORES ARNAIZ *et al.*[6] included exposure of the crude mitochondrial fraction to distilled water. It is possible that this hypoosmotic treatment led to a redistribution of enzyme protein. A similar phenomenon has been described by BEINERT[9].

The interpretation of results obtained by the assay of subcellular fractions must be restrained by the knowledge that a number of physicochemical factors, which are operative during the disruption of the tissue in media of varying ionic strength and osmolality, may lead to redistribution of enzyme protein. Charged lipids are known to bind proteins[10–12]. In view of this fact, it is surprising that only little effort has been made to explain the relatively high activity of various enzymes[6,13–15] in the myelin fraction, especially since myelin is believed by some to be enzymatically inert[16]. Possibly the presence of enzymes in myelin fractions could be due to the interaction of the charged phospholipids of myelin with enzyme protein released from other sites.

The purposes of this study were as follows: (a) to examine the localization of nonspecific esterases, acetylcholinesterase and protein in subcellular fractions of rat cerebrum; (b) to assess, by the use of media of varying ionic composition and osmolality, the extent and nature of artifactual enzyme redistribution; (c) to attempt a correlation between the phospholipid content of subcellular membranes and the degree and stability of enzyme binding.

MATERIALS AND METHODS

Sources of chemicals

α-Naphthol (recrystallized): Fisher Scientific Co., Fairlawn, N.J.; α-naphthyl acetate: Mann Research Lab., New York, N.Y.; 4-aminoantipyrine: Eastman Organic Chemicals, Rochester, N.Y.; acetylthiocholine iodide and 5,5′-dithio-bis-(2-nitrobenzoic acid): Sigma Chemical Co., St. Louis, Mo.

Subcellular fractionation of rat cerebrum

The cerebral hemispheres of four adult, white, male rats were removed quickly after spinal fracture, washed in ice-cold 0.32 M sucrose, stripped of meninges and blotted to remove blood and excess moisture. The tissue was homogenized in 5 vol. 0.32 M sucrose (w/v) in a glass homogenizer fitted with a tight teflon pestle. Twelve vertical strokes at approx. 1000 rev./min were used to disrupt and disperse the tissue. An aliquot of the homogenate was frozen for later assay of enzyme activity and protein content.

Further processing of the homogenate followed the procedure of RODRIGUEZ DE LORES ARNAIZ *et al.*[6]. Differential and density-gradient centrifugation were carried

out in a B-60 International centrifuge. The homogenate was centrifuged in the A-211 rotor at 900 × *g* for 10 min. The supernatant was aspirated. The loosely packed pellet was washed once with 0.32 M sucrose and recentrifuged, the supernatants were combined, and the remaining pellet was designated as the debris fraction. The combined supernatant was centrifuged in the same rotor at 11500 × *g* for 20 min. The pellet was washed once in 0.32 M sucrose, recentrifuged and the combined supernatants were designated as Supernatant 1. Enzyme and protein were assayed on this fraction without further attempts to separate the contained cellular components (endoplasmic reticulum, ribosomes, cytoplasm).

The pellet of the preceding step is the crude mitochondrial fraction of previous reports[5,6]. It was subjected to hypoosmotic treatment by suspending it in 10 vol. (v/v) of ice-cold distilled water. Exposure was allowed to take place for 30 min at 0–4°. Following this "osmotic shock", the suspension was centrifuged at 20000 × *g* for 30 min. The pellet was designated M_1. The slightly opalescent supernatant (Supernatant 2) was aspirated and was used for assay of protein and enzyme without further fractionation.

The M_1 fraction was dispersed by homogenization in a measured volume of 0.32 M sucrose, and an aliquot was taken for enzyme assay, protein determination and lipid extraction. The resuspended M_1 fraction was layered over a discontinuous sucrose gradient (0.8, 0.9, 1.0, 1.2 M). Centrifugation of the gradient at 50000 × *g* for 2 h in the SB-110 rotor developed four distinct bands and a pellet, the latter deposited at the bottom of the tube. These isodensity bands were aspirated by curved pasteur pipettes and were designated $M_1$0.8, $M_1$0.9, $M_1$1.0, $M_1$1.2. The pellet was termed M_1p. It was resuspended, made to known volume, and redispersed.

Electron microscopy

For electron-microscopic examination, the mitochondrial subfractions were diluted with water to lower the density of the sucrose solution and were centrifuged at 100000 × *g* for 1 h to form firmly packed pellets. The supernatants were discarded. A 1% OsO_4 solution was poured directly into the centrifuge tubes that contained these pellets. After 90 min of fixation, portions of each pellet were selected and suspended for 20 min in 2% uranyl acetate, were dehydrated in increasing concentrations of ethanol and were embedded in Epoxy resin[17]. Ultrathin sections were cut with a Porter–Blum ultramicrotome MT-2, "stained" by lead hydroxide[18] and examined with an RCA electron microscope EMU 3F.

Lipid extraction and phospholipid-phosphorus assay

For the determination of phospholipid phosphorus, aliquots of M_1, the isodensity bands and the M_1p fraction were diluted with water and centrifuged at 100000 × *g* for 30 min to eliminate sucrose. The pellets were suspended in 1.0 ml water and extracted according to FOLCH *et al.*[19] at room temperature for 24 h. The lipid extracts, washed with water, were quantitatively transferred into kjeldahl flasks and evaporated to approx. 2.0 ml at 70°. Digestion and phosphorus assay were performed according to BARTLETT[20].

Enzyme assays and protein determinations

Nonspecific esterases were assayed according to the method of ALDRIDGE[21] with modifications. α-Naphthyl acetate in a final concentration of 1.0 μmole/ml was

used as the substrate instead of phenyl esters. 0.1 M Tris–HCl buffer at pH 7.4 and 37° replaced the phosphate buffer of the original description. Acetone and propylene glycol were used to solubilize the substrate. The final concentration of these solvents in the assay tubes were 4 and 8% respectively. α-Napththol released by enzyme hydrolysis was reacted with added 4-aminoantipyrine, and a red color was developed with $K_3Fe(CN)_6$. The color was rapidly extracted into 10.0 ml ethyl acetate by vigorous shaking. Centrifugation cleared the organic phase, and absorption was determined at 510 mμ in a Beckman DB spectrophotometer. Recrystallized α-naphthol served as a standard. α-Naphthyl acetate hydrolysis was determined in the presence and absence of 5 μM eserine sulfate. The release of α-naphthol in the presence of this inhibitor was considered to be due to nonspecific esterases. Acetylcholinesterase was assayed according to ELLMAN *et al.*[22] using acetylthiocholine as substrate at a concentration of 3 mM. Specific inhibitors were not used since ELLMAN *et al.*[22] have shown that butyrylcholinesterase of whole rat brain accounts for only 3% of the hydrolysis of acetylthiocholine. Relative specific enzyme activities (rel. spec. activities) of individual fractions were determined as percent of enzyme recovery/percent of protein recovery.

For proteins the procedure of LOWRY *et al.*[23] was employed. Solutions that were opalescent due to contained lipid were cleared by extraction with chloroform following color development[24]. Where Triton X-100 was used to liberate enzyme from particles, the method of LOWRY *et al.* was complicated by the formation of a precipitate. Color development, however, took place and the solutions could be cleared by centrifugation. A standard curve was plotted with standards containing identical concentrations of Triton X-100.

Washing experiments

In an attempt to study the interaction of enzyme protein with the particles obtained by subcellular fractionation, the freshly prepared isodensity bands $M_1$0.8 - $M_1$1.2 and the M_1p fraction were diluted by the addition of the various media listed below. After mixing, an aliquot of the suspension was assayed for nonspecific esterases, acetylcholinesterase and protein and the values obtained were considered to represent 100% of the enzyme activity or of the protein content of that fraction. Subsequent to centrifugation at 100000 × *g* for 30 min, the pellets were redispersed in media containing identical buffer and electrolyte concentrations, and the process was repeated twice. The percentage of retention of nonspecific esterases, acetylcholinesterase and protein was determined after each washing, but only the data obtained on the reconstituted final pellet are presented except for one representative example (Fig. 6). To eliminate the possibility that the loss of enzyme activity from washed particles was due to denaturation, assays were performed on the supernatants in several experiments. It was found that the amount of activity recovered in solution accounted for the loss from the particulates. The following media were used: distilled water; 0.32 M sucrose; 0.32 M sucrose + 0.1 M NaCl; 0.1 M Tris–HCl (pH 8.5) at 10°; 0.1 M Tris–HCl (pH 8.5) at 10° + 0.02 M $CaCl_2$; 0.1 M Tris–HCl (pH 8.5) at 10° + 0.001 M EDTA; 1% Triton X-100; 0.05 M phosphate (Na_2HPO_4 + NaH_2PO_4) buffer (pH 5.7 and 7.8).

RESULTS

Electron microscopy of subcellular fractions

Fractions $M_1$0.8 (myelin) (Fig. 1) and M_1p (mitochondria) (Fig. 5) were the most homogeneous. The fraction retained by 0.9 M sucrose contained numerous membrane fragments with clubbed ends (Fig. 2) which are believed to arise from synaptic structures. The electron-microscopic appearance of $M_1$0.9 and $M_1$1.0 was

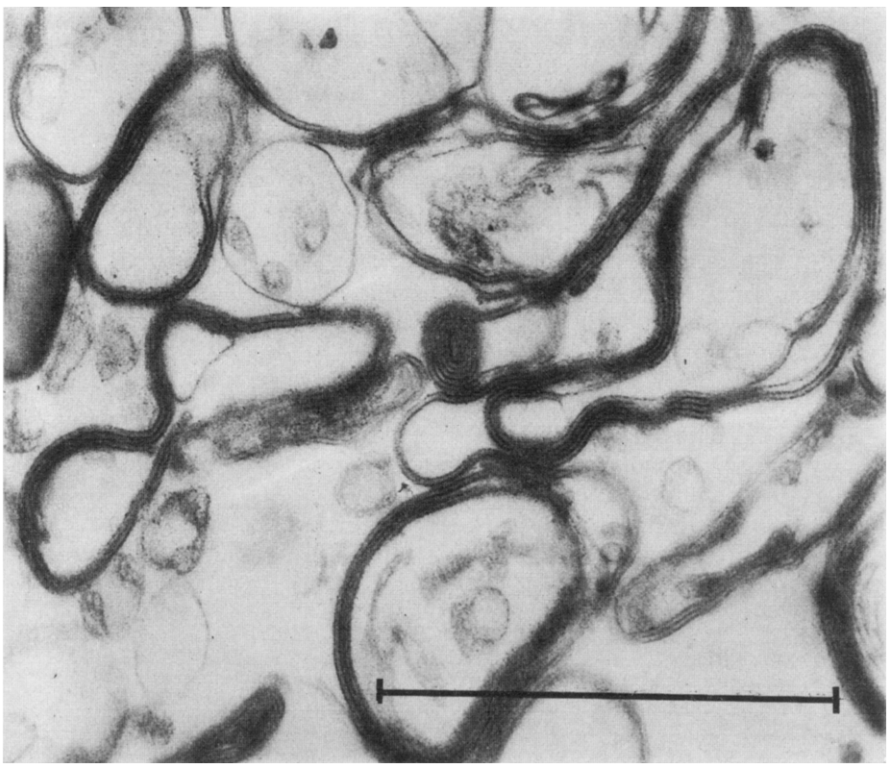

Fig. 1. Fraction $M_1$0.8 containing myelin with little contamination by other constituents. Marker = 1 μ. 60000 ×.

different. Membrane adherence and the formation of circular profiles was more prominent in $M_1$1.0 (Fig. 3). The $M_1$1.2 fraction also contained numerous membrane fragments (Fig. 4), but mitochondria and dense bodies were present.

Enzyme activities and protein content of the fractions obtained by differential centrifugation

Determinations of nonspecific esterase and acetylcholinesterase activities and protein are summarized in Table I. Supernatant 1 which contained the bulk of the microsomes had the highest proportion of acetylcholinesterase and nonspecific esterase activities.

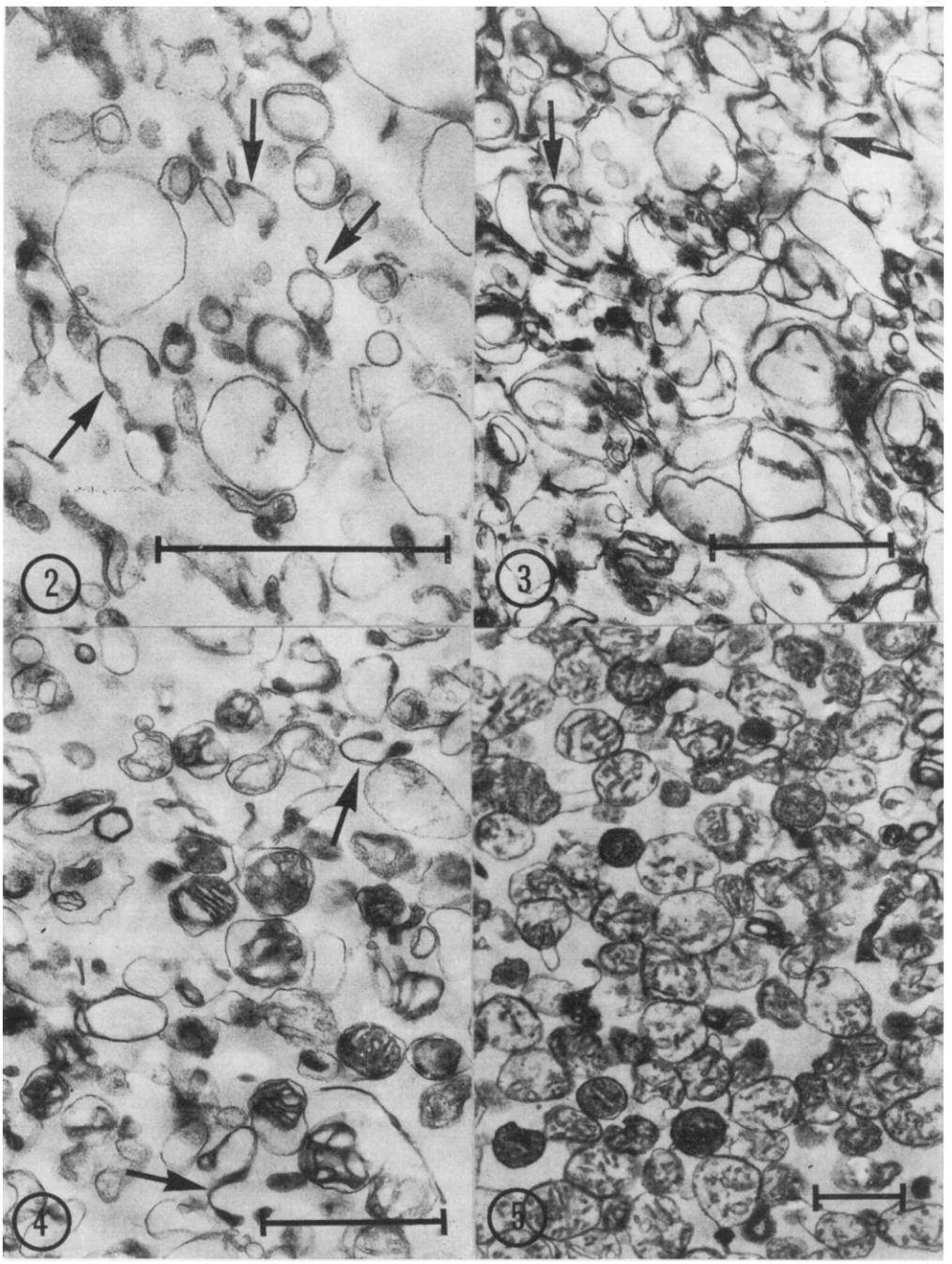

Fig. 2. Fraction $M_1$0.9. Some membrane profiles believed to be of synaptic origin are marked by arrows. Marker = 1 μ. 40 600 ×.
Fig. 3. Fraction $M_1$1.0. Membrane profiles. Arrows indicate synaptic membranes. Marker = 1 μ. 24 400 ×.
Fig. 4. Fraction $M_1$1.2. Membranes and mitochondria. Marker = 1 μ. 24 400 ×.
Fig. 5. Fraction M_1p. Mitochondria. Marker = 1 μ. 12 000 ×.

TABLE I

PER CENT DISTRIBUTION OF NONSPECIFIC ESTERASES, ACETYLCHOLINESTERASE AND PROTEIN IN DEBRIS, M_1, SUPERNATANTS 1 AND 2

Nonspecific esterases were determined in the presence of 5 μM eserine sulfate. Number of experiments indicated in parentheses. Percentages are related to the total homogenate as 100%. For the total homogenate the nonspecific esterase activity was 813.0 ± 44.2 μmoles α-naphthol/g wet tissue/h, the acetylcholinesterase activity 9.5 ± 0.4 μmoles thiocholine/g wet tissue/min, the protein content 156.6 ± 7.8 mg/g wet tissue.

Fraction	*Nonspecific esterases (8)*		*Acetylcholinesterase (6)*		*Protein (8)* (%)
	Distribution (%)	*Rel. spec. activity**	*Distribution* (%)	*Rel. spec. activity**	
Debris	30.1 ± 4.4**	0.84	34.0 ± 8.2**	0.95	35.8 ± 5.5**
M_1	12.2 ± 2.0	0.85	18.5 ± 1.8	1.29	14.3 ± 1.1
Supernatant 1	32.5 ± 2.6	0.99	42.8 ± 7.2	1.30	32.9 ± 2.4
Supernatant 2	5.4 ± 1.0	0.57	3.9 ± 1.4	0.41	9.5 ± 2.2
Recovery (%)	80.2		99.2		92.5

* % enzyme recovery/% protein recovery.
** Standard error of the mean.

TABLE II

PER CENT DISTRIBUTION OF NONSPECIFIC ESTERASES, ACETYLCHOLINESTERASE, PROTEIN AND PHOSPHOLIPID PHOSPHORUS IN THE FRACTIONS $M_1$0.8, $M_1$0.9, $M_1$1.0, $M_1$1.2 AND M_1p

Nonspecific esterases determined in the presence of 5 μM eserine sulfate. Number of experiments indicated in parentheses. Percentages related to M_1 as 100%.

Fraction	*Nonspecific esterases (8)*		*Acetylcholinesterase (6)*		*Protein (8)*	*Phospholipid phosphorus (4)*
	Distribution (%)	*Rel. spec. activity**	*Distribution* (%)	*Rel. spec. activity**		
$M_1$0.8	68.0 ± 10.1**	1.66	63.8 ± 11.8	1.56	41.0 ± 2.2	40.3 ± 0.9
$M_1$0.9	11.6 ± 1.0	1.93	13.2 ± 2.6	2.20	6.0 ± 0.5	10.3 ± 1.1
$M_1$1.0	7.0 ± 0.8	1.52	5.7 ± 0.8	1.24	4.6 ± 0.5	10.2 ± 1.3
$M_1$1.2	13.0 ± 2.2	1.11	10.1 ± 1.0	0.86	11.7 ± 1.1	16.0 ± 2.5
M_1p	10.7 ± 1.2	0.41	5.7 ± 0.8	0.22	26.1 ± 2.0	16.3 ± 3.1
Recovery (%)	110.3		98.5		89.4	93.1

* % enzyme recovery/% protein recovery.
** Standard error of the mean.

Enzyme activities and rel. spec. activities, protein and phospholipid phosphorus in isodensity bands derived from M_1

The distribution of nonspecific esterases, acetylcholinesterase, protein and phospholipid phosphorus in the isodensity bands is presented in Table II. The myelin fraction $M_1$0.8 contained over 60% of the activity of both enzymes and over 40% of the phospholipid phosphorus and protein. The rel. spec. activity for both enzymes was highest in the synaptic membrane fraction ($M_1$0.9); the next highest rel. spec. activities were found in the myelin fraction.

With the exception of M_1p nonspecific esterases and acetylcholinesterase have an almost parallel distribution. The proportionality in the distribution of the two

enzymes is reflected in the values for inhibition of α-naphthyl acetate hydrolysis by eserine. In Fractions $M_1$0.8–$M_1$1.2, the inhibitor reduced hydrolysis by nearly 20%. In M_1p the inhibition was 14%.

Washing experiments

When the percentage of retention of enzyme activity was measured after each of three individual washes, it proved to decline in an exponential-like fashion (Fig. 6). The curve in Fig. 6 is derived from results of the procedure on the myelin fraction.

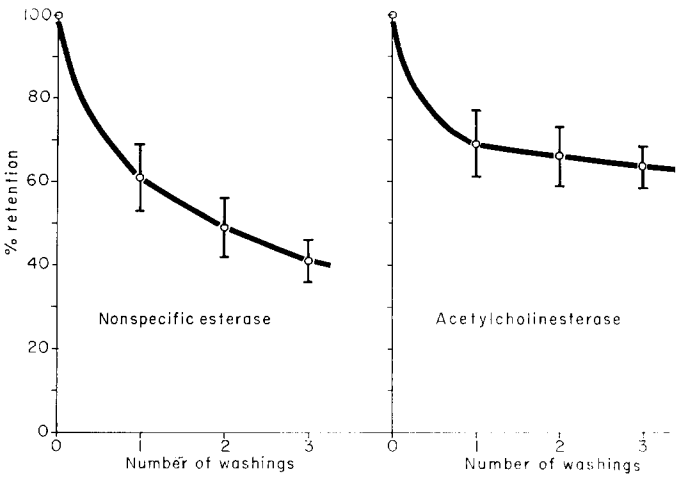

Fig. 6. Retention of enzyme activity in the myelin fraction after successive washings in 0.32 M sucrose. Activity declines in exponential-like fashion. Bars indicate standard errors of the mean. Retention of acetylcholinesterase is greater than retention of nonspecific esterases.

Similar curves could be obtained for Fractions $M_1$0.9 and $M_1$1.0. Since the values obtained after the third wash were subject to the least experimental variation (Fig. 6), results for all media are based on analysis of thrice-washed fractions (Fig. 7). Assays of enzyme activities for Fractions $M_1$1.2 and M_1p are not reported because of the following: (a) comparable aliquots have low activity on a per unit volume basis compared to the other fractions as reflected in the rel. spec. activities (Table II); (b) technical difficulty occurred in obtaining uniform dispersion of the particles for assay.

The protein of the washed fractions was not readily released from the particles. Passage into the supernatant was rarely greater than 10–20% of the initial content with the exception of the 1% Triton X-100 experiment where 50–70% of the protein of the particulates was extracted.

With the exception of Triton X-100 which solubilized all particle-bound enzyme and, hence, is not represented in Fig. 7, distilled water was most effective in accomplishing the release of particle-bound enzyme in all fractions (Fig. 7). Generally, nonspecific esterases appeared to separate more easily from the particles than acetylcholinesterase. Retention of activity was greater with 0.32 M sucrose than with distilled water. The addition of 0.1 M NaCl to the 0.32 M sucrose solution resulted in more retention of nonspecific esterases and acetylcholinesterase in Fractions $M_1$0.8 and $M_1$1.0; however, the $M_1$0.9 fraction behaved differently in that less nonspecific esterases were retained while acetylcholinesterase retention by this fraction was pro-

moted. The addition of 0.02 M $CaCl_2$ to Tris–HCl appeared to enhance the fixation of both enzymes to the membranes of $M_1$0.8 and $M_1$1.0. In contrast, in the case of acetylcholinesterase, less enzyme was retained in all fractions when EDTA was included in the same buffer. The results obtained with phosphate buffer indicate that in $M_1$0.8 and $M_1$1.0 there was less displacement of nonspecific esterases at pH 5.7 than at 7.8. The opposite was true for $M_1$0.9. Little effect of pH on acetylcholinesterase retention by Fractions $M_1$0.8 and $M_1$0.9 was noted. In Fraction $M_1$1.0 alkalinity enhanced the retention of acetylcholinesterase.

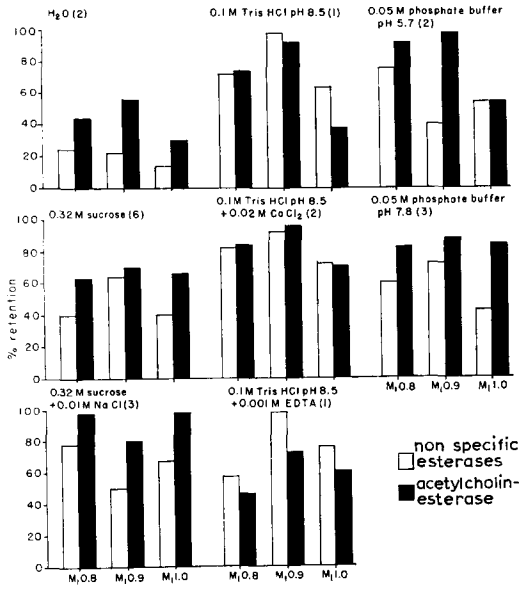

Fig. 7. Retention of nonspecific esterases and acetylcholinesterase in Fractions $M_1$0.8–$M_1$1.0 at the conclusion of three washings in various media. Number of experiments indicated in parentheses.

DISCUSSION

Distribution of enzymes and phospholipid

In the fractions obtained by differential centrifugation, nonspecific esterases and acetylcholinesterase are preferentially localized in the Supernatant 1 fraction which contains microsomes. This is in confirmation of the reports by BERNSOHN *et al.*[3] and SELLINGER AND DE BALBIAN VERSTER[4] for nonspecific esterases and WHITTAKER[25] for acetylcholinesterase. Previously, it has been pointed out, that the per cent distribution of nonspecific esterases and acetylcholinesterase is roughly parallel (Table I). Since both enzymes also have a similar distribution in the mitochondrial subfractions (Table II) and since both hydrolyze acetyl esters preferentially[3], it seems reasonable to suggest that they may have interrelated roles in brain function and metabolism.

In the mitochondrial subfractions, obtained by density-gradient centrifugation, the highest recovery for both enzymes lies in the myelin fraction (Table II). The high activity of the myelin fraction seems unlikely to be due to contamination by other cellular constituents since more than 60% of both nonspecific esterases and acetyl-

cholinesterase are recovered there (Table II). Morphologically this fraction appeared rather homogeneous. Some curvilinear and circular membranous profiles were present but these may have arisen, at least in part, from myelin lamellae disrupted and reaggregated during fractionation. In a previous study by RODRIGUEZ DE LORES ARNAIZ *et al.*[6], 30.5% of acetylcholinesterase was recovered from the $M_1$0.8 fraction. The comparable figure in our study is 63.8%. This discrepancy may well be due to differences in the experimental conditions. RODRIGUEZ DE LORES ARNAIZ *et al.* dissected cerebral cortex from rat brain whereas we used whole cerebrum. Thus, M_1 of our material contains a greater amount of myelin which could provide a large number of binding sites for the attachment of released enzymes. Furthermore, our homogenates contain corpus striatum which is known to be particularly rich in acetylcholinesterase activity[26]. In studying the subcellular distribution of enzymes by methods that involve osmotic shock as used for the release of trapped cytoplasm[27] or disruption of "nerve-ending particles"[25,28], the possibility that marked alterations of the conditions *in situ* occur must be considered. Nevertheless, in agreement with previous studies[3,6], the comparable fraction of this investigation, $M_1$0.9, has a higher specific activity for both acetylcholinesterase and nonspecific esterases than any other membrane fraction.

The myelin fraction contains the greatest amount (40.3%) of the phospholipids of M_1 and has the highest enzyme recovery of any M_1 subfraction (Table II). However, there is no direct correlation between phospholipid and enzyme distribution (Table II). Thus, the fractions retained by 0.9 and 1.0 M sucrose each contain about 10% of the total phospholipid, but the per cent distribution of the enzyme activities in these two subfractions is markedly different (Table II). The phospholipid distributions given for $M_1$0.9 and $M_1$1.0 are in agreement with a recent study by LAPETINA *et al.*[29].

Possible mechanism in phospholipid–enzyme interactions

There is evidence that ionic phospholipids account for fixation of enzyme protein to membranes[10–12]. In aqueous media myelin behaves like an anion[30]. It derives its charge from contained phospholipids. The extent of the bonding between an enzyme and the charged phospholipid of a subcellular membrane involves the titration of the negatively-charged, available phospholipid by the positively-charged site of an enzyme[12]. The failure of mitochondrial lipids to bind proportional amounts of enzyme (Table II) may be due to the location of much of the membrane surface in the interior of the organelle.

The results obtained with distilled water and isotonic sucrose indicate that more enzyme protein is retained in the latter medium (Fig. 7). The ready release of enzyme protein from the particulates into distilled water, as demonstrated in the washing experiments, supports the suggestion that the osmotic shock employed in the preparation of the M_1 subfractions causes artifactual redistribution of enzyme. The resuspension of the osmotically treated M_1 fraction in isotonic sucrose, prior to density-gradient centrifugation, would tend to retain enzyme in its new and artificial locus. The stabilizing effect of isotonic sucrose on the attachment of different enzymes to tissue fractions has been noted previously[9,31,32].

The addition of NaCl to sucrose results in better retention of nonspecific esterases in $M_1$0.8 and $M_1$1.0 but not in $M_1$0.9 (Fig. 7). Acetylcholinesterase is better retained in all fractions after addition of 0.1 M NaCl to isotonic sucrose. BEINERT[9]

found that NaCl prevented the redistribution of cytochrome *c* in rat liver subcellular fractions, whereas FONNUM[31] stated that lactate dehydrogenase was almost entirely released from rat brain synaptosomes in media containing 0.32 M sucrose and 0.03 M NaCl. FONNUM[31] also demonstrated that, in contrast, NaCl inhibited the release of glutamate decarboxylase.

The addition of 0.02 M $CaCl_2$ leads to better retention of nonspecific esterases in $M_1$0.8 and $M_1$1.0. No significant change is noted for acetylcholinesterase. The effect of Ca^{2+} may be due to the reaction of the divalent cation with negatively-charged phospholipids[33–36]. Based on experimental evidence with glutamate decarboxylase, FONNUM[31] postulated that phospholipid–Ca^{2+}–enzyme complexes form. EDTA (Fig. 7) seems to accomplish the preferential release of acetylcholinesterase from subcellular membranes.

Acetylcholinesterase retention is generally less sensitive to pH than nonspecific esterases retention, though significantly more acetylcholinesterase is retained by $M_1$1.0 at pH 7.8. With decreasing H^+ concentration, the synaptic membrane fraction $M_1$0.9 retains more nonspecific esterases activity whereas Fractions $M_1$0.8 and $M_1$1.0 tend to release this enzyme more readily at alkaline pH's. Reasons for the differing results obtained by washing in phosphate buffer at pH 5.7 and 7.8 can only be speculated upon. From the interaction of catalase and trypsin with natural and synthetic lipids at oil–water interfaces, FRASER *et al.*[10] and FRASER AND SCHULMAN[11] concluded that the adsorption of enzyme is related to the charge and nature of the lipid polar groups at the interface and to the charge and nature of the polar groups of the enzyme protein. While increasing acidity tends to repress the negative charges on phospholipids, phosphatidylserine still has available negative polar groups at pH 5.7 (ref. 37). Phosphatidylethanolamine may have net negative charge at pH 7.8 (ref. 35). The charges on nonspecific esterases and acetylcholinesterase at these H^+ concentrations are not known.

The $M_1$0.9 fraction, which consists mainly of synaptic membranes, appears to be unique in that the membrane–enzyme association is firmer than that obtaining in the other fractions including $M_1$1.0 which also contains synaptic structures. Furthermore, $M_1$0.9 has the highest rel. spec. activity for both enzymes of any fraction studied. In view of the physical and chemical differences that exist between $M_1$0.9 and $M_1$1.0, it may be suggested that there are two classes of synaptic membranes which differ in membrane organization, chemical composition and function[6,29].

The high enzyme activities found in the myelin fraction deserve specific comment. In previous work reported from this laboratory, where density-gradient separation of the crude mitochondria of rat brain was accomplished without osmotic shock, the subfraction rich in myelin and associated axoplasm contained a lesser proportion (approx. 17%) of the total nonspecific esterase activity of the crude mitochondrial fraction. In contrast, the myelin subfraction studied in the present investigation contained respectively 68 and 64% of the nonspecific esterase and acetylcholinesterase activities of the crude mitochondria. Reasons for suggesting that osmotic shock may lead to release of enzyme from natural sites have been adduced in the foregoing. It seems likely that most, if not all, myelin-bound enzyme activity is due to artifactual redistribution. However, despite a report that myelin is enzymatically inert[16], the possibility remains that some myelin-bound nonspecific esterases occur *in vivo*[38].

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