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SUBSYNAPTOSOMAL DISTRIBUTION OF ENZYMES INVOLVED IN THE METABOLISM OF LIPIDS

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SUMMARY

Subsynaptosomal fractions, a microsomal fraction and a mitochondrial fraction enriched in nonsynaptosomal mitochondria were prepared from rat brain and were analyzed for phosphatidic acid phosphatase (EC 3.1.3.4), inositol-CMP-phosphatidyltransferase (EC 2.7.8), phosphatidic acid cytidyltransferase (EC 2.7.7), β-galactosidase (EC 3 2.1.23), and carnitine acetylase (EC 2.3.1.7) activities. Carnitine acetylase and phosphatidic acid cytidyltransferase were mitochondrial. The other enzymes were contained in membrane rich fractions. Inositol-CMP-phosphatidyltransferase was microsomal, while phosphatidic acid phosphatase was most concentrated in a plasma membrane fraction derived from synaptosomes. The distinctive distributions of phosphatidic acid phosphatase, inositol-CMP-phosphatidyltransferase and phosphatidic acid cytidyltransferase in different subcellular compartments suggests that the capacity for the biosynthesis of all of the major phospholipids is not restricted to one type of membrane nor does it reside in all membranes in brain.

INTRODUCTION

In this paper we survey the synaptic plasma membrane fraction (SPM fraction) and other subsynaptosomal fractions for the presence and organization of various enzymes involved in lipid metabolism. The objective is 2-fold: first, to determine the subcellular locus of the chemical reactions catalyzed by the enzymes studied and second, to find satisfactory marker enzymes for subcellular particles from brain so as to facilitate further improvements in the purification of membrane fractions. We have analyzed the subcellular distribution of the enzymes phosphatidic acid phosphatase, inositol-CMP-phosphatidyltransferase, phosphatidic acid cytidyltransferase, carnitine acetylase and β-galactosidase.

Lipids are a major component of nerve cell membranes; they play a major role in structuring the membrane and probably in the control and specificity of ion permeability[1-3]. Membrane fractions, rich in plasma membranes, derived from brain tissues are particularly rich in phosphatidyl ethanolamine and phosphatidyl choline[1,4]. Phosphatidyl serine and phosphatidyl inositol are also present but in lower quantities.

Abbreviation: SPM fraction, synaptic plasma membrane fraction.

Phosphatidyl inositol has been of particular interest in nervous tissue since its turnover is increased in post synaptic neurons by synaptic transmission[5,6] or acetylcholine[7-10].

The pathways for the biosynthesis of various phospholipids has been studied in some detail and reviewed[11]. Phosphatidic acid is dephosphorylated to form 1,2-diglyceride by the enzyme phosphatidic acid phosphatase. Diglycerides serve as precursors for the biosynthesis of phosphatidyl choline and phosphatidyl ethanolamine. Alternately, phosphatidic acid can react with CTP to form CDP-diglyceride in the presence of phosphatidic acid cytidyltransferase. CDP-diglyceride is an intermediate used in the biosynthesis of phosphatidyl inositol, a reaction catalyzed by inositol-CMP-phosphatidyltransferase. Another aspect of lipid metabolism is the synthesis of fatty acids. Synaptic plasma membrane fractions have unusually high quantities of long-chain unsaturated fatty acids[1,12]. The synthesis of fatty acids requires acetyl-CoA. A portion of the cytoplasmic acetyl-CoA is derived from intramitochondrial stores through carnitine mediated transport across the mitochondrial membrane, although in certain tissues such as rat liver the main acetyl carrier is citrate not carnitine.[13] The mitochondrial membrane is not permeable to acetyl-CoA, but it is permeable to acetylcarnitine, which is formed from acetyl-CoA and carnitine by the enzyme carnitine acetyltransferase. Hence acetyl groups are transferred across the mitochondrial membrane in the form of acetylcarnitine, which is then reconverted to the corresponding CoASH derivative.

Various enzymes are involved in the degradation of lipids. One such enzyme is β-galactosidase. Galactosidases can hydrolyze the galactosidic bond of cerebrosides[14] to produce ceramide and galactose or they can degrade certain gangliosides[15]. The subcellular distribution of all these enzymes in brain has been partially studied. Essentially all the phosphatidic acid phosphatase activity is particulate. McCAMAN *et al.*[16] found that 37 % of the activity of the total homogenate was in the crude mitochondrial fraction (12000 $\times$ *g* for 20 min) and 48 % was microsomal (100000 $\times$ *g* for 30 min). By differential centrifugation phosphatidic acid cytidyltransferase is found primarily in the crude mitochondrial fraction[17,18] suggesting a localization in mitochondria, lysosomes or synaptosomes. Interestingly, in guinea pig liver this enzyme is primarily microsomal[19]. Inositol CMP phosphatidyltransferase in homogenates prepared from guinea pig brain appears to be microsomal[20]. Carnitine acetylase is probably mitochondrial in brain based on studies of fractions prepared by differential centrifugation[21]. β-Galactosidase has been found to be at least in part in lysosomes[22]. The isopycnic banding pattern, however, is disperse and it is not clear whether or not all β-galactosidase is lysosomal.

MATERIALS AND METHODS

Materials

All radioactive substrates were purchased from New England Nuclear, Boston, Mass. except for [^{3}H]CTP which was purchased from Schwartz Bio Research, Orangeburg, N. Y. All other chemicals used were of reagent grade.

Preparation of fractions

Synaptosomes and synaptosomal subfractions were prepared as in the accompanying paper[23] from rat brain. Synaptosomes were isolated from a crude mito-

chondrial fraction on a Ficoll–sucrose gradient. The synaptosomal fraction was osmotically shocked at alkaline pH and resolved in a discontinuous sucrose gradient into separate subsynaptosomal fractions which were rich in both membranes and mitochondria.

Microsomes (P_3) were prepared by removing a crude mitochondrial fraction at 17000 × *g* for 30 min and sedimenting the resulting supernatant at 100000 × *g* for 1 h.

A special mitochondrial fraction enriched in non-synaptosomal mitochondria was isolated in the following way. The mitochondria were obtained from the 13 % Ficoll–sucrose pellet formed in the Ficoll–sucrose gradient used to prepare synaptosomes[23]. The pellet was osmotically shocked and centrifuged on a gradient identical to that used for the subfractionation of synaptosomes. This procedure assured that both mitochondrial fractions received similar treatment and also removed contaminating synaptosomal membranes so that non-synaptosomal and synaptosomal mitochondria could be compared. The pellet sedimenting through 38 % sucrose (w/w) and forming at the bottom of the tube was taken for comparison to synaptosomal mitochondria.

Finally a total particulate fraction (HP) was prepared from a crude homogenate to permit comparison of the enzyme content of the various particulate fractions to the total particulate protein of the homogenate. This fraction was obtained by pelleting the total homogenate at 100000 × *g* for 1 h.

Enzyme assays

The activities of β-galactosidase[23] (EC 3.2.1.23) and phosphatidic acid phosphatase[16] (EC 3.1.3.4) were measured as previously described. Protein determinations were carried out using the microprocedure of LOWRY *et al.*[25].

Acetylcholinesterase (EC 3.1.1.7). Acetylcholinesterase activity was measured as previously described[26], but the separation of the enzymatic product ([1-^{14}C]acetate) from the substrate ([1-^{14}C]acetylcholine) involved a micro modification of a procedure described by FONNUM[27]. One μl of sample was added to 20 μl of ice-cold buffer–substrate consisting of 2 mM [1-^{14}C]acetyl choline (0.7 mC/mmole) in 0.1 M sodium phosphate (pH 7.1). After 30 min of incubation at 38°, the tubes were returned to the ice bath. The reaction was stopped by the addition of 100 μl of 3-heptanone containing sodium tetraphenyl boron (50 mg/ml) at room temperature. The tubes were vigorously mixed, then centrifuged at room temperature to separate the phases. The organic phase, containing the residual substrate, was aspirated and discarded. A measured aliquot (10 μl) of the aqueous phase was removed and placed in a scintillation counting vial for assay of the enzymatically liberated [1-^{14}C]acetate[26].

Carnitine acetylase (EC 2.3.1.7). Carnitine acetylase activity was measured as previously described[21] but the separation of the enzymatic product [1-^{14}C]acetylcarnitine) from the substrate ([^{14}C]acetyl-CoA) involved a micro modification of a procedure described by FONNUM[27]. The product ([1-^{14}C]acetylcarnitine) was extracted into 100 μl of 3-heptanone containing sodium tetraphenyl boron (50 mg/ml). 75 μl of this organic layer were removed and transferred to a clean tube containing 50 μl of 20 mM sodium phosphate (pH 7.3). This "washing" step was necessary in order to remove the last traces of the radioactive substrate. After thorough mixing and centrifugation, 50 μl of the organic phase were removed and placed in a scintilla-

tion vial for assay of the enzymatically produced [1-^{14}C]acetylcarnitine. This modified procedure results in significantly lower blanks and a slightly higher percent recovery (98–102 %) of the radioactive acetylcarnitine than the procedure previously described[21], for the measurement of carnitine acetylase activity.

Inositol CMP phosphatidyltransferase (EC 2.7.8). Inositol CMP phosphatidyltransferase activity was determined by using a procedure to be published[44]. The procedure consisted of adding 1 μl of sample to 10 μl of ice-cold buffer–substrate which contained the following constituents: 100 mM 2-amino-2-methyl-1,3-propandiol–HCl buffer (pH 8.6), 1 mM mercaptoethanol,0.5 % Triton X-100, 0.5 mM $MnCl_2$, 1 mM CDP-diglyceride and 5 mM [^{3}H]inositol spec. act. 30 mC/mmole). After 30 min of incubation at 38° the reaction was stopped by the addition of 2 μl of 3 M HCl followed by 40 μl of methanol with mixing after each addition at room temperature. Next, 40 μl chloroform were added and mixed, followed by the addition of 60 μl of 2 M KCl. After thorough mixing and centrifugation to separate the phases, the overlying aqueous phase was aspirated and discarded. After a final "wash" with 100 μl of 2 M KCl, a 15-μl portion of the organic (chloroform) phase was removed and placed in a scintillation counting vial for assay of the enzymatic product, phosphatidyl [^{3}H]inositol.

Phosphatidic acid cytidyltransferase (EC 2.7.7). Phosphatidic acid cytidyltransferase activity was measured using a modification, suitable for the mammalian enzyme (B. GOLDBERG AND R. E. MCCAMAN, unpublished results), of the procedure employed by MCCAMAN AND FINNERTY[28] to assay this activity in bacterial preparations. The procedure consisted of adding 3 μl of the sample to 7 μl of ice-cold buffer–substrate which contained 300 mM Tris–acetate (pH 7.6), 33 mM $MgCl_2$, 15 mM [^{3}H]CTP (spec. act. 10 mC/mmole), 0.6 % Triton X-100 and 4 mM purified "soy" phosphatidic acid[16]. After 60 min of incubation at 38° the reaction was stopped and the radioactive product, [^{3}H]CDP-diglyceride, was extracted as described above for the phosphatidyl inositol.

RESULTS

Synaptosomes are the predominant component in the basic synaptosomal preparation which has been characterized by electron microscopy[23]. This basic preparation, which is isolated on a Ficoll–sucrose gradient, contains approx. 13 % of the total particulate protein of the homogenate. This synaptosomal preparation contained approx. 12 % of the phosphatidic acid phosphatase, carnitine acetylase and phosphatidic acid cytidyltransferase of the total particulate protein of the homogenate. It also contained approx. 9 % of the acetylcholine esterase, 8 % of the β-galactosidase and 7 % of the inositol-CMP-phosphatidyltransferase.

Subsynaptosomal fractions were isolated from this basic synaptosomal preparation and analyzed for their enzymatic content. The enzyme distributions were grouped into two general patterns. The first pattern was characterized by a high recovery in upper portions of the gradient in fractions rich in membranes. These upper fractions contained 60 % or more of phosphatidic acid phosphatase, inositol-CMP-phosphatidyltransferase and β-galactosidase. In contrast, 80 % or more of the phosphatidic acid cytidyltransferase and carnitine acetylase was found in the lower gradient fractions enriched in mitochondria (Table I). We also analyzed the sedimentation profile of acetylcholinesterase in order to provide continuity between the en-

zyme distribution patterns in this study and those of the preceeding one[23]. The agreement between the data was within a few percent even though different assay methods were used for acetylcholinesterase.

Phosphatidic acid phosphatase, inositol-CMP-phosphatidyltransferase and β-galactosidase are resolved from mitochondrial fractions. As indicated in Table I, Fractions 1 and 2 contain 58% of the phosphatidic acid phosphatase, 72% of the inositol CMP phosphatidyltransferase, 62% of the β-galactosidase and less than 10% of the carnitine acetylase, a mitochondrial enzyme. The largest percent (39–44%) of total enzyme activity was found in Fraction 2 for the first 3 of the above enzymes.

Phosphatidic acid phosphatase, inositol-CMP-phosphatidyltransferase, and β-galactosidase are partially, but not completely resolved from each other. Inositol-CMP-phosphatidyltransferase shows its highest relative specific activity at the top of the gradient in Fraction 1, while phosphatidic acid phosphatase has its highest relative specific activity in Fraction 2, the fraction richest in synaptic plasma membranes (Table II). Inositol-CMP-phosphatidyltransferase sediments like acetylcholinesterase. Most of the inositol-CMP-phosphatidyltransferase and acetylcholinesterase activity is found in classes of particles which either sediment slowly because they are small or which isopycnically band at relatively light densities. Phosphatidic acid phosphatase sediments to a greater extent into more dense fractions than either inositol-CMP-phosphatidyltransferase of acetylcholinesterase. Less of the phosphatidic acid phosphatase activity is found in Fraction 1 and more in Fraction 3 than is the case for inositol-CMP-phosphatidyltransferase or acetylcholinesterase. Fraction 3 contains 19% of the total phosphatidic acid phosphatase activity of the gradient and approx. 11% of both inositol-CMP-phosphatidyltransferase and acetylcholinesterase (Table I). The distribution pattern for β-galactosidase is intermediate. Although β-galactosidase s considered a lysosomal enzyme it does not sediment in this study with mitochondria

TABLE I

DISTRIBUTION OF VARIOUS ENZYMES IN SUBSYNAPTOSOMAL FRACTIONS AS PERCENTAGE OF TOTAL RECOVERED ACTIVITY

Values are mean and average deviation. Fraction 1 25% band, Fraction 2 (25–32.5%), Fraction 3 (32.5–35%), Fraction 4 (35–38%), and Fraction 5 (38% pellet). The percentage recovery of the various enzymes from the gradient input (synaptosomal particulate applied to gradient) was: phosphatidic acid phosphatase 85% (5 determinations), β-galactosidase 75% (2 determinations), acetylcholinesterase 66%, inositol CMP phosphatidyltransferase 63% (2 determinations), carnitine acetylase 97% (3 determinations), and protein 79% (4 determinations). Enzyme distributions were determined from at least two separate experiments. Duplicate gradients were prepared and used for confirmatory analysis when it appeared to be warranted.

Enzyme	% *Total activity*				
	1	*2*	*3*	*4*	*5*
Phosphatidic acid phosphatase	14.8 ± 6.2	44.1 ± 4.7	19.4 ± 4.1	16.0 ± 2.6	5.8 ± 1.1
β-Galactosidase	23.4 ± 5.1	39.4 ± 0.4	15.9 ± 5.8	13.0 ± 1.3	8.3 ± 1.6
Acetylcholinesterase	40.8 ± 5.8	41.3 ± 0.1	11.2 ± 4.2	5.6 ± 1.3	1.2 ± 0.1
Inositol-CMP-phosphatidyltransferase	28.4 ± 1.5	44.7 ± 0.1	10.9 ± 0.2	11.3 ± 1.2	4.7 ± 0.5
Phosphatidic acid cytidyltransferase	2.8 ± 1.8	13.8 ± 0.0	20.5 ± 4.3	36.1 ± 1.7	26.8 ± 1.8
Carnitine acetylase	1.5 ± 0.1	7.2 ± 1.6	23.4 ± 2.5	40.6 ± 2.0	27.2 ± 2.8
Protein	16.8 ± 1.4	29.1 ± 1.8	16.4 ± 1.5	22.2 ± 1.0	15.5 ± 3.5

TABLE II

COMPARISON OF RELATIVE SPECIFIC ACTIVITIES OF SYNAPTOSOMAL SUBFRACTIONS

Relative specific activity is defined as percent total activity in fraction divided by percent of total protein in the fraction. Fraction notations are given in Table I.

Enzyme	*Relative specific activity*				
	1	*2*	*3*	*4*	*5*
Phosphatidic acid phosphatase	0.82 ± 0.33	1.50 ± 0.46	1.18 ± 0.19	0.78 ± 0.18	0.52 ± 0.14
β-Galactosidase	1.28 ± 0.23	1.26 ± 0.13	0.89 ± 0.25	0.61 ± 0.01	0.72 ± 0.32
Acetylcholinesterase	2.35 ± 0.56	1.22 ± 0.01	0.71 ± 0.19	0.27 ± 0.09	0.11 ± 0.02
Inositol-CMP-phosphatidyltransferase	1.63 ± 0.24	1.32 ± 0.01	0.71 ± 0.09	0.54 ± 0.11	0.40 ± 0.08
Phosphatidic acid cytidyltransferase	0.17 ± 0.11	0.41 ± 0.03	1.31 ± 0.14	1.70 ± 0.10	2.29 ± 0.10
Carnitine acetylase	0.09 ± 0.01	0.23 ± 0.03	1.21 ± 0.02	2.00 ± 0.02	2.61 ± 0.35

as is often the case[29]. The relative specific activity for β-galactosidase is highest in Fraction 1, but is followed very closely by Fraction 2. (Table II). Thus while the profiles for phosphatidic acid phosphatase, inositol-CMP-phosphatidyltransferase and β-galactosidase are distinguishable, there is considerable overlap.

In Table III we compare the specific activity of total particulate protein of the homogenate to that of the synaptosome subfractions (Fractions 1–5), microsomes (P_3) and a mitochondrial fraction enriched in non-synaptosomal mitochondria (M). The synaptic plasma membrane fraction (Fraction 2) has the highest specific activity for phosphatidic acid phosphatase, indicating a purification of this enzyme with the purification of synaptic plasma membranes. The specific activity in Fraction 2 is 1.7 times greater than the homogenate (total particulate protein of the homogenate) and 1.5 times greater than microsomes (P_3). Inositol-CMP-phosphatidyltransferase activity is highest in microsomes in contrast to the SPM fraction which has only one third of this activity. The highest specific activity of acetylcholinesterase is in Fraction 1, whereas Fraction 2 contains one half this activity. Both the purified mitochondrial fraction and Fraction 5 have a 4–5-fold higher specific activity for carnitine acetylase than that of the total particulate protein of the homogenate. Similarly phosphatidic acid cytidyltransferase shows a 3-fold enrichment in the purified mitochondrial fraction and Fraction 5.

DISCUSSION

The general conclusions that one can derive from the examination of not only the subsynaptosomal fractions but the microsomal and nonsynaptosomal mitochondrial fractions are as follows: (1) inositol-CMP-phosphatidyltransferase is predominately microsomal with some contamination in Fraction 1, which is not unexpected because Fraction 1 contains small membrane fragments. (2) The distribution patterns of carnitine acetylase and phosphatidic acid cytidyltransferase argue clearly for their mitochondrial localization.

In these studies we report a number of new observations regarding the distribution of certain enzymes involved in lipid metabolism. Phosphatidic acid phos-

TABLE III

COMPARISON OF SPECIFIC ACTIVITY (MEAN ± S.D.) OF VARIOUS FRACTIONS TO THAT OF THE TOTAL PARTICULATE FROM A HOMOGENATE (SPECIFIC ACTIVITY IN FRACTION/SPECIFIC ACTIVITY IN HOMOGENATE PELLET)

Microsomes (P_3), M (purified mitochondrial fraction), NEP.t (synaptosomes prior to osmotic shock), NEP.p (synaptosomal particulate applied to gradient) and gradient fractions: 1 (25% sucrose band), 2 (25–32.5% band), 3 (32.5–35% band), 4 (35–38% band), and 5 (38% pellet). The absolute values of enzymes in μmoles/h per g protein in the particulate derived for a homogenate were: phosphatidic acid phosphatase, 888 ± 72 μmoles phosphate liberated; inositol-CMP-phosphatidyltransferase, 293 ± 64 μmoles CMP formed; acetylcholinesterase, 7355 ± 2453 μmoles [^{14}C]acetate formed; β-galactosidase, 120 ± 34 μmoles 4-methylumbelliferone liberated; carnitine acetylase, 1380 ± 255 μmoles [^{14}C]acetylcarnitine formed; phosphatidic acid cytidyltransferase, 6.6 ± 1.0 μmoles [^{3}H]CDP diglyceride formed.

Enzyme	*Fraction*				*Gradient subfractions*				
	P_3	*M*	*NEP.t*	*NEP.p*	*1*	*2*	*3*	*4*	*5*
Phosphatidic acid phosphatase	1.13 ± 0.08	0.42 ± 0.07	0.96 ± 0.19	1.27 ± 0.22	1.13 ± 0.56	1.75 ± 0.11	1.56 ± 0.30	1.04 ± 0.29	0.70 ± 0.28
Inositol-CMP-phosphatidyltransferase	1.80	0.14	0.56 ± 0.05	0.59 ± 0.06	0.75 ± 0.04	0.61 ± 0.12	0.34 ± 0.03	0.26 ± 0.10	0.19 ± 0.07
Acetylcholinesterase	1.58	0.09	0.75 ± 0.12	1.23 ± 0.05	2.49 ± 1.06	1.26 ± 0.26	0.68 ± 0.10	0.27 ± 0.03	0.11 ± 0.01
β-Galactosidase	0.65 ± 0.08	0.62 ± 0.01	0.62 ± 0.03	0.35 ± 0.04	0.41 ± 0.10	0.38 ± 0.03	0.27 ± 0.03	0.20 ± 0.02	0.26 ± 0.04
Carnitine acetylase	0.14 ± 0.03	5.52 ± 0.38	0.91 ± 0.03	1.34 ± 0.22	0.15 ± 0.04	0.38 ± 0.03	2.02 ± 0.43	3.35 ± 0.81	4.28 ± 0.48
Phosphatidic acid cytidyltransferase	0.12	3.0	0.88	1.98	0.20	0.52	1.70	2.20	2.96

phatase is localized at least in part in synaptic plasma membranes. Morphological analysis shows that Fractions 2 and 3 contain the most synaptic thickenings. It is these fractions which are richest in phosphatidic acid phosphatase. The specific activity of phosphatidic acid phosphatase in the SMP fraction (Fraction 2) is higher than in microsomes, an observation similar to that previously observed for (Na^+–K^+)-ATPase[30,31]. Also both of these enzymes are found to a considerable extent in the more dense regions of this gradient. Since phosphatidic acid phosphatase, which synthesizes the 1,2-diglyceride, is present in the synaptic plasma membrane fraction (SPM fraction), it would be of particular interest to determine if the enzymes which utilize the diglyceride in the synthesis of phosphatidyl choline and phosphatidyl ethanolamine are also present. Recently it has been shown that phosphatidyl choline can be biosynthesized by isolated synaptosomes and synaptic vesicles[32], suggesting that these particles contain the required enzymes.

Since phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl inositol are ubiquitous membrane lipids in brain[5], it is of interest to determine whether the capacity for the synthesis of these essential phospholipids is present in all membranes. However, in brain as in most other tissues, separate compartments appear to exist for many of these enzymes[16–20]. From the results obtained in the present study phosphatidic acid phosphatase and inositol-CMP-phosphatidyltransferase may be contained to differing degrees in plasma membranes and microsomes and possibly even in mitochondria, but phosphatidic acid cytidyltransferase appears to be exclusively mitochondrial. Microsomes contain less than 1 % of the total activity of phosphatidic acid cytidyltransferase and carnitine acetylase found in the particulate protein of the homogenate. Thus the synthesis of phosphatidyl inositol from CDP-diglyceride and myoinositol by the enzyme inositol CMP phosphatidyltransferase would require the export of CDP-diglyceride from mitochondria to a microsomal particle where it would react with myoinositol. The exchange of phospholipids which have been biosynthesized in one fraction and then transported to another has in fact been observed[33,34] and may also occur in brain. Furthermore, if inositol-CMP-phosphatidyltransferase is exclusively microsomal, phosphatidyl inositol for presynaptic terminal membranes would need to be provided by axonal flow.

With the exception of β-galactosidase and acetylcholinesterase, the enzymes studied remain insoluble after osmotic shock at alkaline pH. Only 60 % of the total enzyme activity of β-galactosidase remained insoluble and 80 % of the acetylcholinesterase. Carnitine acetylase and phosphatidic acid cytidyltransferase were significantly activated by osmotic shock in both total activity and specific activity (Table III). Any increase in specific activity (basic synaptosomal preparation *vs.* synaptosomal particulate applied to gradient) larger than 20 % would be due to enzyme activation or removal of an inhibitor, since osmotic shock releases approx. 20% of the soluble protein.

In brain β-galactosidase is extremely important because of its involvement in lipid degradation. Deficiencies in such hexosaminidases have been proposed as a possible etiological factor in Tay–Sachs disease[36–39]. The β-galactosidase contained within synaptosome fractions is partially released by osmotic shock. Like other "lysosomal" enzymes studied in the previous paper (acid phosphatase and β-*N*-acetylglucoaminidase), β-galactosidase was most concentrated and had the highest relative specific activity in membrane fraction. Recently β-galactosidase has been

found in liver plasma membranes[35]. This enzyme is optimally active at pH 7.0 in the presence of Mg^{2+}, whereas the lysosomal β-galactosidase is active at pH 5.0 and does not require Mg^{2+}. Brain membranes do not appear to have such an enzyme. We found that the β-galactosidase in these fractions is optimally active at pH 4.0 in agreement with data obtained by others on homogenates[24] and this pH optimum is the same in the presence or absence of Mg^{2+} irrespective of the buffer used (Table IV). It is appropriate to point out that galactosidases are a class of enzymes with different substrate specificities[15] and specific galactosidases might not be detected. We conclude that to define the subcellular particle containing "lysosomal" enzymes in these fractions, a histochemical analysis is required.

TABLE IV

THE ACTIVITY OF β-GALACTOSIDASE IN DIFFERENT BUFFERS AT pH 4.0 AND pH 7.0 IN THE PRESENCE OF Mg^{2+}

Enzyme activity was determined using *p*-nitrophenyl-β-D-galactopyranoside as substrate. Both *para* and *ortho* derivatives give similar results. HEPES = *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

Buffer	*Specific activity**	
	pH 4.0	*pH 7.0*
0.1 M phosphate–citrate, 2 mM $MgCl_2$	4.7	0.9
0.1 M HEPES, 2 mM $MgCl_2$	3.4	1.2
0.1 M cacodylate, 2 mM $MgCl_2$	4.1	0.2

* μmoles *p*-nitrophenol formed per h per mg protein.

As discussed previously large differences in specific activity of enzymes rarely arise in brain and never have been achieved with a good recovery of total enzyme activity. Part of the explanation for this is that fractions are diluted with assorted membrane fragments masking other differences. Yet the presence of inositol-CMP-phosphatidyltransferase in synaptosomal fractions is puzzling if this enzyme is localized in an intracellular membrane such as endoplasmic reticulum. Either the enzyme belongs to contaminating microsomal particles or synaptosomes, and possibly even synaptic plasma membranes, have their own activity as has been suggested by HARWOOD AND HAWTHORNE[39] even though the enzyme is less active in synaptosomes and SPM fractions than in microsomes. Microsomes should be removed by virtue of their small sedimentation coefficients during differential centrifugation and by rate separation on Ficoll–sucrose gradients. Various investigators have in fact shown by reconstitution experiments with labelled microsomes that microsomal particles are in fact effectively removed[40,41]. Nonetheless microsomal enzymes are found in the crude mitochondrial fraction though, as expected, at a lower specific activity. A possible explanation for this may be that during homogenization plasma membranes enclose sections of cell cytoplasm and intracellular membranes in the manner that synaptosomes are formed. The presence of extensive processes in brain cells favors entrapping of intracellular components during shear. These vesicular structures are relatively large particles and sediment like synaptosomes. After osmotic shock of the synaptosomal fraction, microsomes are released from these structures and contaminate subsequent fractions. At present we cannot be certain whether inositol-

CMP-phosphatidyltransferase exists in synaptosomes and synaptic plasma membranes at low levels of activity or arises from small quantities of microsomal contamination in these fractions.

In this paper and the previous one[23] we survey subfractions from synaptosomes for certain enzymatic activities. Based on the relative content of various marker enzymes, we want to determine how homogeneous these subfractions are, particularly the synaptic plasma membrane fraction. The specific activity of microsomal enzymes in the SPM fraction is one third that of microsomes. Two examples of this are NADH cytochrome *c* reductase (in presence of antimycin A) and inositol-CMP-phosphatidyltransferase. One possible interpretation of this is that microsomal membranes comprise 33 % of the SPM fraction. By similar reasoning, mitochondria may comprise 10 % of the SPM fraction based on cytochrome oxidase or carnitine acetylase content. While the mitochondrial estimate may be reasonable, the microsomal contamination is probably an over estimate because the microsomal intracellular membranes are diluted by various plasma membrane fragments which sediment with the microsomes. There are in addition more subtle contaminates such as those plasma membranes derived from other portions of neurons and other cell types which cannot as yet be distinguished by morphological or enzyme analysis. Glial membranes most likely do not constitute a major contaminate since these membranes do not sediment with synaptosomes in Ficoll–sucrose gradients[42]. This argument is indirect, however, and at present there is no way to distinguish the contribution of glial, dendritic, soma and axonal plasma membranes.

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