Characterization of phospholipid methylation in rat brain myelin

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Highly purified rat brain myelin was solubilized in Triton X-100 and myelin phospholipid N-methyltransferase was characterized. The enzyme activities were separated by isoelectric focusing and ion-exchange chromatography. The phospholipid methyltransferase has shown at least four peaks of activity with pI_{app} values of 4.5, 5.2, 6.2 and 8.4. After affinity purification each of these activities revealed a close set of bands of approx. 65 kDa on SDS/PAGE. These data together with those from preparative

SDS/PAGE separations suggested that rat brain myelin contains three acidic and at least one basic phospholipid-methylating isoenzymes and that the major isoenzyme in each case is approx. 65 kDa in size. While the predominant product of the reaction catalysed by all detected isoforms was monomethylated phosphatidylethanolamine, the least acidic isoform (pI_{app} 6.2) also formed about 20% phosphatidylcholine, suggesting that these isoenzymes may play different roles *in vivo*.

INTRODUCTION

Phospholipid N-methyltransferase (PLMTase; EC 2.1.1.17) catalyses phosphatidylcholine (PC) synthesis by three successive methylations of phosphatidylethanolamine (PE) through the intermediate phospholipids, phosphatidyl-N-monomethylethanolamine (PME) and phosphatidyl-N,N-dimethylethanolamine (PDE), with S-adenosylmethionine (SAM) as a methyl donor [1]. Methylation of PE has been associated with a number of cellular events, including signal transduction and synthesis of arachidonate-rich PC [2]. On the other hand, conversion of PE into PC is the only known pathway for the biosynthesis of choline in the body, other than from the diet, which may be of special importance for brain tissue [3].

Different forms of PLMTase were reported to exist in rat brain synaptosomes [4], rat [5] and mouse liver [6], erythrocyte membranes [7] and adrenal medulla [8]. In yeast, two genes encoding different methyltransferases were cloned, one catalysing PE monomethylation and another all three methylation steps [9]. On the other hand, a number of reports maintained the view that one enzyme is responsible for all three methylation steps and that conclusions based upon kinetic data and bivalent cation requirements may be misleading [10]. The purification of a single polypeptide of basic nature from rat liver microsomes, which catalysed all three steps of PE N-methylation [11], appeared to confirm that view. However, the same authors noticed later that extended electrophoresis resolved two (or possibly three) isoforms of purified methyltransferase [12]. The most recent report also clearly indicated the presence of at least two different forms of PLMTase in rat liver [13].

Myelin is a specialized extension of the plasma membrane of oligodendrocytes in the central nervous system (CNS) which wraps around axons and provides insulation for, and facilitation of, axonal saltatory conduction. It consists of 70–75% of lipids and the earlier concept of myelin as an inert membrane sheath has been revised and it is known to contain numerous enzyme activities, more than half of which are involved in lipid metabolism [14]. The importance of myelin studies comes from the involvement of myelin in a number of severe demyelinating

diseases to which the CNS is susceptible. Hattori with co-authors [15] documented the presence of phospholipid methylation activities in both axolemma- and myelin-enriched fractions from rat brain and methylation of PE was found to be very active in axon [16]. The presence of PLMTase in highly purified rat brain myelin has recently been reported [17] and it was claimed that two forms of the enzyme existed which differed in their affinity for SAM, solubilization characteristics and mode of inactivation by trypsin.

Here we report evidence for the presence of differently charged forms of PLMTase in detergent-solubilized rat brain myelin. We also present data on the molecular mass of these forms. Some preliminary results have been reported [18].

MATERIALS AND METHODS

Materials

Phospholipids (PE, PME, PDE and PC), S-adenosylhomocysteine (SAH) and S-adenosyl-L-methionine (SAM) were purchased from Sigma and [3H -methyl]SAM (specific radioactivity 70–75 Ci/mmol) was from New England Nuclear–Du Pont. Bio-Lyte 3/10 ampholyte for isoelectric focusing (IEF) was obtained from Bio-Rad. Triazine-activated agarose 4XL was purchased from Affinity Chromatography Ltd. Silica gel plates (Alugram Sil G/UV₂₅₄) were supplied by Camlab. Ultrafiltration membranes YM-10 (cut-off 10000 Da) were from Amicon. GELCODE colour silver stain kit was from Pierce. Water was of MilliQ quality. Other reagents were obtained from commercial sources and were of reagent grade.

Isolation and solubilization of myelin

Myelin was isolated from Wistar rat brains according to the method of Norton and Poduslo [19] with the modification described in [20] for greater purity. All manipulations were performed at 4 °C unless otherwise specified. The brains from two rats (10 weeks old) were usually used for each preparation. For solubilization the highly purified myelin was suspended in

Abbreviations used: CNS, central nervous system; IEF, isoelectric focusing; pI_{app} , apparent isoelectric point; PE, phosphatidylethanolamine; PDE, phosphatidyl-N,N-dimethylethanolamine; PME, phosphatidyl-N-monomethylethanolamine; PC, phosphatidylcholine; PLMTase, phospholipid N-methyltransferase; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine.

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6–8 ml of 20 mM phosphate buffer containing 0.5 % Triton X-100 (w/v), pH 8.0, and incubated at 4 °C for 40–60 min. Unsolubilized material was pelleted at 100 000 *g* for 60 min and the supernatant used as a source of solubilized enzyme. Triton X-100 in the solubilization buffer may be substituted with CHAPS (0.3 %, w/v).

Assay for PLMTase and TLC of products

PLMTase was assayed by measuring the radioactive methyl group incorporation into phospholipids. The assay medium contained the enzyme source (0.2 mg of protein/ml or less) in 20 mM K_2HPO_4 (pH 8.0), 0.5 % Triton X-100 (w/v) and 10 μ l of PE/PME mixture (concentrations 8 mg/ml and 1 mg/ml respectively) in a final volume of 440 μ l and the assay was performed in duplicate.

The reaction was started by the addition of [*methyl*- H^3]SAM (12.5 mCi/mmol) to a concentration of 200 μ M [21] and carried out at 40 °C for 30 min in a shaking water-bath. To stop the reaction and extract phospholipids 2 ml of chloroform/methanol (2:1, v/v) mixture was added and vials were vortexed for 30 s. The time control of extraction was essential for the reproducibility of the assay and the accuracy of the duplicates. The lipid-rich chloroform extract was washed twice with 2 ml of 0.5 M KCl in 50 % methanol. Aliquots (500 μ l) were transferred into clean vials, dried under a stream of N_2 and total chloroform-soluble radioactivity was measured by scintillation counting (Beckman LS 5000 CE).

Individual phospholipid products of the reaction were analysed by applying aliquots of organic phase to a silica gel TLC plate which was then developed either in a solvent system of chloroform/ethanol/triethylamine/water (4:5:4:1, by vol.) (system A) or *n*-propanol/propionic acid/chloroform/water (3:2:2:1, by vol.) (system B). Phospholipids were visualized by 0.12 % Rodamine 6G solution and distribution of radioactivity on a plate was determined by radiometric scanning on the Berthold Tracemaster 20 radioactivity scanner. Spots corresponding to products of PE *N*-methylation were scraped off a TLC plate and radioactivity associated with them was determined by scintillation counting. HPLC analysis of the extracted phospholipids, performed as described [22], was used to further confirm the identity of radiolabelled phospholipids.

Isoelectric focusing of myelin PLMTase

Solubilized myelin was brought to 50 ml with water, Bio-Lyte 3/10 ampholytes and Triton X-100 were added to a final concentration of 2 % and 0.5 % respectively, and the proteins were focused in a Rotofor IEF Preparative Cell (Bio-Rad) for 4–5 h (until the voltage stabilized) at 4 °C. Harvested fractions were assayed for PLMTase activity after adjusting their pH to 8 without removal of the ampholytes. Active fractions were pooled, diluted with water (according to the manufacturer's recommendation) and refocused over a narrower pH gradient. Fractions obtained thus, containing PLMTase activity, were concentrated by ultrafiltration on YM-10 membrane and subjected to affinity chromatography.

Affinity chromatography on SAH-agarose

Triazine-activated agarose 4XL (0.2 g) was wetted in water for 30–40 min, filtered and dispersed in 4 ml of 100 mM $NaHCO_3$ buffer (pH 9) containing 5 mg of SAH. The suspension was slowly spun for 14–16 h at room temperature and excess SAH was removed by washing the gel with 20 vol. of 0.5 M KCl. Unreacted groups on agarose were blocked by a 2 h incubation

with 2 M ethanolamine. The gel (1 ml) was finally washed with 0.5 M KCl (20 vol.) and then equilibrated with 5 mM K_2HPO_4 (pH 6.2) buffer containing 5 mM EDTA and 10 mM 2-mercaptoethanol in a small column.

The sample (1 ml) was applied to the column and recirculated 8–10 times and the column then washed with the equilibration buffer (up to 10 ml) to elute non-specifically bound proteins. PLMTase was eluted with 10 mM K_2HPO_4 (pH 8.0) buffer/1 M NaCl/10 mM 2-mercaptoethanol/0.5 % Triton X-100.

Preparative SDS/PAGE of myelin

Myelin was mixed with 5 ml of standard SDS/PAGE sample buffer (0.0625 M Tris/HCl, pH 6.8, 2 % SDS, 5 % 2-mercaptoethanol, 10 % glycerol, 0.002 % Bromophenol Blue) and incubated for 30 min at 40 °C for solubilization. The sample was centrifuged at 25 700 *g* for 5 min in a microcentrifuge and filtered prior to electrophoresis. The 10 % T gel column was polymerized in a 491 Prep Cell (Bio-Rad) and SDS/PAGE was performed according to Laemmli [23]. The fractions were collected (4 ml) at a flow rate of 1 ml/min and protein elution was monitored at 280 nm. All fractions which were eluted after the dye front were pooled, two at a time, concentrated by ultrafiltration through a YM-10 membrane, washed with 20 % ethylene glycol to remove SDS, and finally reconstituted in the standard PLMTase assay buffer. Concentrated fractions were assayed for PLMTase activity in the presence of 10 μ l of PE/PME mixture.

Ion-exchange chromatography on Q-Sepharose Fast Flow

Solubilized myelin (2 ml) was loaded on the Q-Sepharose Fast Flow packed column (1.6 cm $\times$ 11 cm) equilibrated with 20 mM phosphate buffer (pH 8.0) containing 0.5 % Triton X-100 (buffer A). The column was washed with 60 ml of buffer A at a flow rate of 2 ml/min and then with 60 ml of a linear gradient, 0–100 %, of buffer A containing 1 M NaCl (buffer B) and finally with 40 ml of buffer B. Protein elution was monitored by absorbance at 280 nm. Fractions (4 ml) were collected and assayed for PLMTase activity.

SDS/PAGE and protein concentration determination

Rapid protein gel electrophoresis analysis of the samples was performed on Mini-Protean II Cell mini-gels according to Laemmli [23]. The gels were silver stained with a GELCODE colour kit. The intensity and colour of protein bands on the gel were used for protein quantity estimation after re-focusing and affinity chromatography steps when protein concentration was extremely low. Otherwise proteins were quantified by the method of Bradford [24] with BSA as a standard (Triton X-100 was included in the buffer).

RESULTS

Separation of differently charged PLMTase isoforms

The procedure employed for myelin isolation allows us to obtain highly purified myelin where the sum of all contaminants is well below 5 % [25]. The presence of PLMTase in rat brain myelin, in agreement with the reported data [17], was confirmed by the increasing specific activity of the enzyme during the 4-step myelin isolation and its constant level (28 pmol/min per mg of protein) during repetitive washing of myelin. Phospholipid methylation was linear up to 60 min at the protein concentration of 10–12 mg/ml and SAM concentration of 200 μ M. The assay

Figure 1 Isoelectric focusing of myelin PLMTase

(a) Triton X-100-solubilized myelin proteins were subjected to IEF in the Preparative IEF Cell (Bio-Rad) as described in the Materials and methods section and harvested fractions were assayed for PLMTase activity (○). pH gradient formed during the IEF is depicted by ■. The fractions NN 5–12 ('4–5 PLMTase') and 13–19 ('8–10 PLMTase') were pooled separately and refractionated. The PLMTase activity profile of 4–5 PLMTase is shown in (b) after refraction over the pH range 3–10. Similar refractionation of 8–10 PLMT methylase yielded the activity profile shown in (c). The results are typical of two individual experiments performed in duplicate.

conditions were chosen to be identical throughout the purification and, although neither PE nor PME nor PDE affected PLMTase activity of the highly purified myelin and the product formation (not shown), the addition of PE/PME mixture was made to provide an exogenous substrate for the enzyme at the later stages

Figure 2 Incorporation of [^3H]methyl groups in the phospholipids catalysed by the myelin PLMTase isoforms

Phospholipids were separated on a TLC plate in system A as described in the Materials and methods section, following the PLMTase assay of the activities recovered at $pI_{app.}$ 4.5 (a), 5.2 (b) and 6.2 (c) after two individual experiments. The radioactivity distribution in phospholipids was determined by radioscanning the tracks for 20 min each. The positions of the authentic phospholipids were visualized with Rodamine 6G staining. Abbreviations: Or, origin at 2 cm; Fr, solvent front at 20 cm.

of purification where the loss of endogenous lipids was anticipated [11].

The IEF of Triton X-100-solubilized myelin over the pH range 3–10 separated at least two forms of PLMTase (Figure 1a) which are referred to as '4–5 methyltransferase' [apparent pI ($pI_{app.}$) around 4–5] and '8–10 methyltransferase' ($pI_{app.}$ around 8–10). Some phospholipid methylation activity was also detected at highly acidic $pI_{app.}$ below 4. When the '4–5 methyltransferase' peak of activity (fractions 4–12) was refractionated, over the effective pH gradient 4–7, three distinct peaks of phospholipid methylation activities were detected (Figure 1b), corresponding to $pI_{app.}$ 4.5, 5.2 and 6.2. TLC analysis of the products of PLMTase showed (Figure 2) predominant formation of PME (more than 90% of total phospholipid-incorporated radioactivity) by all acidic isoforms with the exception of the $pI_{app.}$ 6.2 isoform, which incorporated about 20% of radioactive methyl group in PC along with about 80% in PME under identical assay conditions (Figure 2c). The R_f value of the formed radiolabelled PME was just slightly higher than that of standard diacyl-PME, possibly suggesting the labelling of plasmalogen monomethyl ethanolamines. Since the efficiency of ^3H scanning is low, the pattern of the incorporation of [^3H]methyl groups into the

Table 1 Purification of phospholipid N-methyltransferase (PLMTase) from rat brain myelin

Stage		Volume (ml)	Protein (mg/ml)	Activity ¹	Total activity ²	Yield ³	Purification
Triton X-100 solubilization		7	10	0.84	58	38	1
Centrifuge supernatant		6	7.5	1.1	49	31	1.3
IEF (pH 3–10)	pI _{app.} 4–5	24	0.10	24	57	100	21.8
	pI _{app.} 8–10	21	0.18	26	98	100	23.6
Re-IEF of 'acidic PLMTase' (pH 3–7)	pI _{app.} 4.5	2.8	0.01	448	12.5	21	533
	pI _{app.} 5.2	3.6	0.01	341	12.2	20	405
	pI _{app.} 6.2	4.8	0.01	437	21	35	520
Re-IEF of 'basic PLMTase' (pH 3–10)	pI _{app.} 4.5–5.2	4.3	0.01	486	20.8	21	578
	pI _{app.} 8.5	5.2	0.005	1986	51.6	52	2364
Affinity chromatography	pI _{app.} 4.5	2	0.001	2024	4.1	7	2414
	pI _{app.} 5.2	2	0.002	1979	7.9	14	2355
	pI _{app.} 6.2	2	0.001	2004	4	7	2385
	pI _{app.} 8.5	2	0.002	2052	8.2	8	2442

¹ PLMTase activity is expressed in nmol of [³H]methyl transferred per mg of protein per 30 min assay.

² Total activity is in nmol of [³H]methyl transferred per 30 min assay.

³ Activity of both acidic and basic forms of the enzyme after IEF is taken as 100% due to an increase of activity after this step of purification.

Figure 3 Preparative column SDS/PAGE of myelin PLMTase

Myelin protein was subjected to preparative reducing SDS/electrophoresis on a 10% acrylamide gel column and collected fractions were assayed for the PLMTase activity as in the Materials and methods section. The elution positions of the prestained molecular-mass standard proteins were determined by running them on an identical gel column. The dye front was eluted mainly in fraction 7. The estimated molecular mass of the eluted PLMTase forms is indicated (in kDa).

products of PE methylation was confirmed by using another TLC system and HPLC (results not shown). Less than 5% of radioactivity, as determined by liquid scintillation of the corresponding scraped TLC spots, was incorporated in PDE by all detected isoforms of PLMTase. The refocusing of the '8–10 methyltransferase' over the pH gradient 7–10 did not yield a tighter focusing, and subsequent IEF over the full pH gradient (3–10) yielded instead a broad peak of PLMTase activity with maximum at pI_{app.} 8.4 (Figure 1c). TLC analysis of the products of the reactions catalysed by this activity showed that, similarly to the pI_{app.} 4.5 and 5.2 PLMTase activities, PME was the major radioactive product (results not shown). The results of purification are given in Table 1. The ion-exchange chromatography of solubilized myelin on Q-Sepharose revealed several peaks of PLMTase activity: (a) a sharp peak in non-bound protein fractions (basic proteins) and (b) a much broader peak of activity eluted with 0.2–0.6 M NaCl (acidic proteins) along with (c) the

Figure 4 Affinity purification of rat brain myelin PLMTase forms

The PLMTase activities recovered at different pI_{app.} values (lanes A, B and C are pI_{app.} 5.2, 4.5 and 6.2 respectively; 0.5 μg of protein per lane) were subjected to SAH-affinity purification and analysed on SDS/12% PAGE (lanes 1–4 are pI_{app.} 4.5, 5.2, 6.2 and 8.4 activities respectively; 1 μg of protein per lane). The gel was silver stained. Lane 5 shows the effect of 2-mercaptoethanol omission from the sample buffer on the set of bands at 65 ± 2 kDa for pI 6.2 activity. The positions of molecular-mass standards in kDa are indicated.

remainder which was eluted with 1 M salt (highly acidic proteins) (not shown).

Molecular mass of PLMTase isoforms

In order to estimate the molecular mass of myelin PLMTase column SDS/PAGE was used. The results showed that the major forms of the myelin PLMTase activity were eluted from the column with molecular masses of 60–70 kDa and approx. 100 kDa (Figure 3). A little PLMTase activity was detected at approx. 20 kDa. Total recovered PLMTase activity, however, was poor, most likely due to the incomplete renaturation of the enzyme after SDS treatment.

The high affinity of PLMTase for SAH has been previously used for partial purification of the enzyme by affinity chromatography [6,26]. Using conditions similar to those used by Kim et al. [27] for SAH coupling to the affinity medium, we were able to characterize further the active fractions from the IEF experiments. Thus, the active fractions containing PLMTase recovered at different pI_{app.} values were purified on the SAH-agarose column (Figure 4). Although under these conditions not all PLMTase bound to the support (possibly due to the presence of

detergent), this problem was alleviated by recirculating the sample through the column. The bound PLMTase activity could not be eluted by merely including 0.2 mM SAM in the elution buffer in the absence of detergent. The best elution was achieved by the combination of changed pH, ionic strength and inclusion of detergent in the elution buffer. SDS/PAGE of the specifically eluted fractions revealed that all four activities (pI_{app} 4.5, 5.2, 6.2 and 8.4) contained the set of closely situated bands at 65 ± 2 kDa, which appeared to converge to a single band at the same position on the gel under non-reducing conditions.

DISCUSSION

The most probable but not the only explanation of the data from IEF and chromatography experiments is the presence of differently charged isoforms of PLMTase in myelin or different enzymes, responsible either for separate methylation steps (e.g. formation of PME only or going through PDE to form PC, see Figure 2) or, perhaps, for catalysing the transfer of the methyl group to different ethanolamine phospholipids, plasmalogens for example [28]. It seems unlikely, although not impossible, that this sort of distribution of the enzyme activity can be caused by heterogeneity of the micelles formed during solubilization or by the different extent of protein aggregation. The reason for that was the reproducible separation profile which was independent of the type of detergent (Triton X-100 or CHAPS) and so presumably reflected the real ratio of differently charged isoforms. Besides, a number of other chromatography experiments supported the notion of charge heterogeneity of myelin PLMTase (results not shown).

It has been demonstrated that two forms of liver PLMTase (purified and cloned) may have close molecular masses and catalyse the same reactions but differ at least in part of their C-terminal sequences [13]. Assuming the same is true for the myelin PLMTase, one can imagine how these differences in the sequence may result in the different pI_{app} values. The possible acidic nature of liver PLMTase (pI 4–5) was noticed once [29] but subsequently the enzyme was characterized as a basic protein [11].

It is possible, however, that strongly bound phospholipids may change the charge of the PLMTase. By analogy, the isoelectric point of two different isoforms of phosphatidyl-inositol/phosphatidylcholine transfer protein (PI-TP) depends on which of its phospholipid substrates is non-covalently bound to the protein, which suggests that the two forms are otherwise identical [30,31]. It is also possible that a different modification pattern (phosphorylation or glycosylation) may have resulted in the different pI values for methyltransferases, since it is known that liver PLMTase at least can be phosphorylated [12].

Quite unexpectedly, it was noticed during the experiments that the total recovered PLMTase activity increased after the first separation step (either IEF or Q-Sepharose chromatography), although this was also noted with liver PLMTase purification [11]. Possible explanations of this phenomenon may include removal of the endogenous inhibitor(s) for PLMTase or the more favourable (for PLMTase activity) detergent-lipid-protein ratio which resulted from separation forces. These propitious conditions may also explain, at least partially, the dramatic increase in the enzyme activity after refocusing, although the increase could be provided by the superior resolving power of IEF alone.

SDS/PAGE analysis of the active fractions obtained after refocusing of '4–5 PLMTase activity' revealed heterogeneity in their protein composition. However, the approx. 65 kDa band

was the major band detected on SDS/PAGE of the active IEF fractions after final purification on SAH-agarose (Figure 4).

The column SDS/PAGE results (Figure 3) further reinforced the hypothesis that the ~ 65 kDa band (or close set of bands) on slab SDS/PAGE corresponded to rat brain myelin PLMTase. The nature of other detected molecular mass peaks of the myelin PLMTase activity is unclear at present. It is possible, however, that the small amount of activity recovered at around 20 kDa corresponds to a similar form of PLMTase in liver.

Maintenance of the PE/PC ratio (which changes in chronic multiple sclerosis tissue plaques [32]) may be a role of phospholipid methylation in myelin. This may be important as it was shown that the charged polar groups of the phospholipids can interact with myelin basic protein *in vivo* and modifications of these groups may affect the structure and function of the membrane [33]. Therefore, activity of the PLMTase may have a part in maintaining myelin structure. The observation that a decreased level of SAM is associated with demyelination [34,35] also supports this view.

In summary, the results of this study indicate the heterogeneity of PLMTase in rat myelin. The existence of three acidic and at least one basic phospholipid-methylating isoenzymes, with the major isoenzyme in each case being approx. 65 kDa in size, provides one explanation for this heterogeneity. The phospholipids synthesized by these differently charged forms of PLMTase were predominantly monomethylated ethanolamine lipids, possibly plasmalogens, although one isoform (pI_{app} 6.2) also formed appreciable amounts of PC. The availability of the pure enzyme should allow further understanding of the roles and physiological significance these forms of PLMTase may have.

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REFERENCES

- Vance, D. E. and Schneider, W. J. (1981) *Methods Enzymol.* **71**, 581–588
- Hirata, F. and Axelrod, J. (1980) *Science* **209**, 1082–1090
- Blusztajn, J. K. and Wurtman, R. J. (1983) *Science* **221**, 614–620
- Crews, F., Hirata, F. and Axelrod, J. (1980) *J. Neurochem.* **34**, 1491–1498
- Rama Sastry, B. V., Statham, C. N., Axelrod, J. and Hirata, F. (1981) *Arch. Biochem. Biophys.* **211**, 762–773
- Tanaka, Y., Amano, F., Maeda, M., Nishijima, M. and Akamatsu, Y. (1990) *Jpn. J. Med. Sci. Biol.* **43**, 59–73
- Hirata, F. and Axelrod, J. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 2348–2352
- Hirata, F., Viveros, O., Diliberto, E. J. and Axelrod, J. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 1718–1721
- Kodaki, T. and Yamashita, S. (1987) *J. Biol. Chem.* **262**, 15428–15435
- Audubert, F. and Vance, D. E. (1983) *J. Biol. Chem.* **258**, 10695–10701
- Ridgway, N. D. and Vance, D. E. (1987) *J. Biol. Chem.* **262**, 17231–17239
- Ridgway, N. D. and Vance, D. E. (1989) *Biochim. Biophys. Acta* **1004**, 261–270
- Cui, Z., Vance, J. E., Chen, M. H., Voelker, D. R. and Vance, D. E. (1993) *J. Biol. Chem.* **268**, 16655–16663
- Ledeer, R. W. (1992) in *Myelin: Biology and Chemistry* (Martenson, R. E., ed.), pp. 531–570, CRC Press Inc., Boca Raton
- Hattori, H., Bansal, V. S., Orihel, D. and Kanfer, J. N. (1984) *J. Neurochem.* **43**, 1018–1024
- Vance, J. E., Pan, D., Vance, D. E. and Campenot, R. B. (1991) *J. Cell Biol.* **115**, 1061–1068
- Chung, K., Yu, B. S. and Choi, M.-U. (1990) *Korean Biochem. J.* **23**, 103–109
- Tsvetnitsky, V., Yeboah, F. A. and Gibbons, W. A. (1993) *Biochem. Soc. Trans.* **21**, 489S
- Norton, W. and Poduslo, S. (1973) *J. Neurochem.* **21**, 749–757
- Haley, J. E., Samuels, F. G. and Ledeen, R. W. (1981) *Cell. Mol. Neurobiol.* **1**, 175–187
- Ridgway, N. D. and Vance, D. E. (1992) *Methods Enzymol.* **209**, 366–374

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- 22 Samet, J. M., Friedman, M. and Henke, D. C. (1989) *Anal. Biochem.* **182**, 32–36
- 23 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- 24 Bradford, M. (1976) *Anal. Biochem.* **72**, 248–254
- 25 Norton, W. T. and Cammer, W. (1984) in *Myelin* (Morell, P., ed.), pp. 147–195, Plenum Press, New York
- 26 Makishima, F., Toyoshima, S. and Osawa, T. (1985) *Arch. Biochem. Biophys.* **238**, 315–324
- 27 Kim, S., Nochumson, S., Chin, W. and Paik, W. K. (1978) *Anal. Biochem.* **84**, 415–422
- 28 Percy, A. K., Moore, J. F. and Waechter, C. J. (1982) *J. Neurochem.* **38**, 1404–1412
- 29 Ridgway, N. D. and Vance, D. E. (1986) *Fed. Proc. Am. Soc. Exp. Biol.* **45**, 1670
- 30 Helmkamp, G. M. (1985) *Chem. Phys. Lipids* **38**, 3–16
- 31 van Paridon, P. A., Visser, A. J. W. G. and Wirtz, K. W. A. (1987) *Biochim. Biophys. Acta* **898**, 172–180
- 32 Wilson, R. and Tocher, D. R. (1991) *Lipids* **26**, 9–15
- 33 Salvati, S., Attorri, L., Confaloni, A. and Di Biase, A. (1990) *Neurochem. Res.* **15**, 1051–1053
- 34 Hyland, K., Smith, I., Bottiglieri, T., Perry, J., Wendel, U., Clayton, P. T. and Leonard, J. V. (1988) *Neurology* **38**, 459–462
- 35 Reynolds, E. H. (1992) *J. Neuroimmunol.* **40**, 225–230
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