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UDP-Galactose–Ceramide Galactosyltransferase in Rat Brain Myelin Subfractions During Development

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The localization and activity of the enzyme UDP-galactose–hydroxy fatty acid-containing ceramide galactosyltransferase is described in rat brain myelin subfractions during development. Other lipid-synthesizing enzymes, such as cerebroside sulphotransferase, UDP-glucose–ceramide glucosyltransferase and CDP-choline–1,2-diacylglycerol cholinephosphotransferase, were also studied for comparison in myelin subfractions and microsomal membranes. The purified myelin was subfractionated by isopycnic sucrose-density-gradient centrifugation. Four myelin subfractions, three floating respectively on 0.55 M- (light-myelin fraction), 0.75 M- (heavy-myelin fraction) and 0.85 M-sucrose (membrane fraction), and a pellet, were isolated and purified. At all ages, 70–75% of the total myelin proteins was found in the heavy-myelin fraction, whereas 2–5% of the protein was recovered in the light-myelin fraction, and about 7–12% in the membrane fraction. Most of the galactosyltransferase was associated with the heavy-myelin and membrane fractions. Other lipid-synthesizing enzymes studied appeared not to associate with purified myelin or myelin subfractions, but were enriched in the microsomal-membrane fraction. During development, the specific activity of the microsomal galactosyltransferase reached a maximum when the animals were about 20 days old and then declined. By contrast the specific activity of the galactosyltransferase in the heavy-myelin and membrane fractions was 3–4 times higher than that of the microsomal membranes in 16-day-old animals. The specific activity of the enzyme in the heavy-myelin fraction sharply declined with age. Chemical and enzymic analyses of the heavy-myelin and membrane myelin subfractions at various ages showed that the membrane fraction contained more proteins in relation to lipids than the heavy-myelin fraction. The membrane fraction was also enriched in phospholipids compared with cholesterol and contained equivalent amounts of 2′:3′-cyclic nucleotide 3′-phosphohydrolase compared with heavy- and light-myelin fractions. The membrane fraction was deficient in myelin basic protein and proteolipid protein and enriched in high-molecular-weight proteins. The specific localization of galactosyltransferase in heavy-myelin and membrane fractions at an early age when myelination is just beginning suggests that it may have some role in the myelination process.

Galactosylceramides (cerebrosides) are one of the major lipid components of myelin membranes. During myelination rapid deposition of cerebrosides occurs in the brain (Hauser, 1968; Norton & Poduslo, 1973). The enzyme UDP-galactose–ceramide galactosyltransferase (UDP-galactose–2-hydroxyacylsphingosine galactosyltransferase, EC 2.4.1.45) catalyses the last step in the biosynthesis of cerebrosides and the enzyme in brain has been extensively studied (Morell & Radin, 1969; Morell *et al.*, 1970; Basu *et al.*, 1971; Brenkert & Radin, 1972). *In vitro*, it preferentially catalyses the transfer of galactose from UDP-galactose to hydroxy fatty acid-containing ceramide rather than to non-hydroxy fatty acid-containing ceramide as the acceptor (Shah, 1971; Brenkert & Radin, 1972). The enzyme has been reported to be primarily localized in the microsomal fraction of brain, similar to most of the lipid-synthesizing enzymes *de novo*. However, Nes-

Abbreviations used: SDS, sodium dodecyl sulphate; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]amino}ethanesulphonic acid.

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kovic *et al.* (1973) and Costantino-Ceccarini & Suzuki (1975) reported its presence in purified myelin fraction.

Since the galactosyltransferase may have some role in the formation and maintenance of myelin, here we describe the localization and activity of this enzyme in various subfractions of purified myelin during the active myelination period of rat brain. The subfractionation of myelin was done by isopycnic density-gradient centrifugations, unlike most previous investigations. The subfractions were characterized by enzymic activity and by morphological and chemical analyses. Other lipid-synthesizing enzymes, such as cerebroside sulphotransferase (EC 2.8.2.11), UDP-glucose–ceramide glucosyltransferase and CDP-choline–1,2-diacylglycerol cholinephosphotransferase (EC 2.7.8.2) were also studied for comparison in myelin subfractions and microsomal membranes. Part of this work was presented previously (Chou & Jungalwala, 1976; Koul & Jungalwala, 1977, 1978, 1979).

Experimental

Materials

UDP - [U - ^{14}C]galactose (sp. radioactivity 200 mCi/mmol), UDP-[U-^{14}C]glucose (sp. radioactivity 200 mCi/mmol), cytidine diphospho[*Me*-^{14}C]choline (sp. radioactivity 50 mCi/mmol), phosphoadenosine 5′-phospho[^{35}S]sulphate (sp. radioactivity 2.5 mCi/mmol) and DL-[4,5-^{3}H]leucine (sp. radioactivity 50 mCi/mmol) were from New England Nuclear, Boston, MA, U.S.A. Hydroxy fatty acid- and non-hydroxy fatty acid-containing ceramides, and cerebrosides, were purchased from Applied Science Laboratories, State College, PA, U.S.A., or Supelco, Bellefonte, PA, U.S.A. ATP, NADPH, adenosine 5′-phosphate, adenosine 2′:3′-cyclic phosphate ammonium salt were from P-L Biochemicals, Milwaukee, WI, U.S.A. Cytochrome *c* (horse heart), alkaline phosphatase, UDP-galactose, UDP-glucose, CDP-choline, *N*-acetylglucosamine, EGTA, Tris and Tes were from the Sigma Chemical Co., St. Louis, MO, U.S.A. 1,2-Dioleoyl-*sn*-glycerol and steroid standards were from either Supelco, or Serdary Research Laboratories, London, Ont., Canada. Silica-gel G t.l.c. plates were from Merck. Unisil silica gel (100–200 mesh) was from Clarkeson Chemical Co., Williamsport, PA, U.S.A. Solvents and other chemicals were from Fisher Scientific Co., Fair Lawn, NJ, U.S.A. Acrylamide and other chemicals for gel electrophoresis were from Bio-Rad Laboratories, Richmond, CA, U.S.A.

Methods

Isolation of myelin and myelin subfractions. Sprague–Dawley albino rats from Charles River Laboratories, Wilmington, MA, U.S.A., or from our own colony were used. Mylein was isolated and purified from rat forebrains essentially by the method of Jungalwala & Dawson (1971) as modified by Ruenwongsa *et al.* (1979). All operations were carried out at 0–4°C. Briefly, a 10% brain homogenate in 0.32 M-sucrose was centrifuged at 12500 *g* for 20 min to obtain a combined nuclear and crude mitochondrial pellet. This was successively centrifuged twice at 75000 *g* for 45 min through a discontinuous gradient of 0.32 M/0.85 M-sucrose. The myelin obtained at the interface of the gradient was washed three times with 0.32 M-sucrose by centrifugation at 11000 *g* for 10 min. This myelin was termed 'unshocked myelin'. The myelin was osmotically shocked three times with water, each time for 20 min and collected by centrifugation. The osmotically-shocked myelin was further purified on a discontinuous gradient of 0.32 M/0.85 M-sucrose centrifuged at 75000 *g* for 1 h (Jungalwala & Dawson, 1971).

The osmotically shocked myelin suspended in 0.32 M-sucrose (15 ml) was subfractionated by a modification of the method of Agrawal *et al.* (1974) by isopycnic density-gradient centrifugation on 0.55 M- (13 ml), 0.75 M- (15 ml) and 0.85 M-sucrose (12 ml) at 76900 *g* for 12 h. The fractions that floated on 0.55 M-, 0.75 M- and 0.85 M-sucrose, except the pellet, were collected by centrifugation after appropriate dilution of sucrose with water. Each of these subfractions, except the pellet, was again centrifuged, on a similar sucrose density gradient, but in a total volume of 16 ml, at 82000 *g* for 2 h.

The fractions floating on 0.55 M-, 0.75 M- and 0.85 M-sucrose were termed light-myelin, heavy-myelin and membrane fractions respectively. These purified myelin subfractions were collected by centrifugation and frozen at −20°C until used.

EGTA-treated myelin. Myelin obtained (from 16-day-old rat brains) after one osmotic shock in water was reshocked for 1 h in the presence of 40 ml of 10 mM-EGTA, pH 7.5 (DeVries *et al.*, 1978). The re-shocked myelin was collected by centrifugation and fractionated by density-gradient centrifugation on 12 ml each of 0.85 M-, 1.0 M- and 1.2 M-sucrose, containing 1 mM-EGTA, pH 7.5, and 1 mM-Tes, pH 7.5, as described by DeVries *et al.* (1978). Most of the myelin membrane material floated on 0.85 M-sucrose. The small amount of material that floated on 1.0 M- and 1.2 M-sucrose was combined.

Preparation of the microsomal fraction. The microsomal fractions was prepared from the post-nuclear and crude mitochondrial supernatant (Ruenwongsa *et al.*, 1979). This supernatant was re-centrifuged at 12000 *g* for 10 min and the resulting supernatant was subjected to high-speed centrifugation at 102000 *g* for 1 h. The pellet obtained was used as the microsomal fraction.

Assay of galactosyltransferase. The method of Brenkert & Radin (1972) was used with some modifications. Appropriate amounts of enzyme proteins were freeze-dried in reaction tubes. Benzene (1 ml) was added and the mixture was sonicated in a Bransonic 200 Sonicator bath for about 1 min. After adding either α-hydroxy fatty acid- or non-hydroxy fatty acid-containing ceramide (0.2 ml/tube, 1 mg/ml in benzene), the mixture was dried under N_2. The final reaction mixture in 0.2 ml volume contained 0.1 M-Tris/HCl buffer, pH 7.4 (at 37°C) 15 mM-$MnCl_2$, 0.17 mM-UDP-[^{14}C]galactose (sp. radioactivity 3000 d.p.m./nmol) together with 200 μg of α-hydroxy fatty acid- or non-hydroxy fatty acid-containing ceramide and enzyme protein. The reaction mixture was briefly sonicated to get a dispersion and incubated at 37°C for 1 h in a shaking water bath. The reaction was stopped by addition of 10 ml of chloroform/methanol (2:1, v/v). Rat brain lipids (2.5 mg) were added to each tube to act as a carrier and the extract was washed with 0.2 vol. of 0.9% NaCl. The upper phase was removed and the lower phase was washed three times with 0.2 vol. of theoretical upper phase consisting of 0.9% NaCl/methanol/chloroform (47:48:3, by vol.), containing 0.01 mM-galactose (Folch *et al.*, 1957). The lower phase was washed three more times with theoretical upper phase without galactose. The final lower phase was dried under N_2 and the radioactivity was determined in a Packard liquid-scintillation counter with a toluene-based solution as scintillant. The results are expressed as nmol of [^{14}C]galactose transferred to ceramide/mg of protein per h.

Product identification. Radioactive reaction products of the galactosyltransferase were identified by benzoylation and high-pressure liquid chromatography of the corresponding perbenzoylated cerebrosides by the method of Jungalwala *et al.* (1977). More than 90% of the radioactivity appeared in α-hydroxy fatty acid-containing cerebrosides when α-hydroxy fatty acid-containing ceramide was the acceptor.

Other enzyme assays. 2′:3′-cyclic nucleotide 3′-phosphohydrolase (EC 3.1.4.16) was assayed by the method of Prohaska *et al.* (1973), except that 0.2 ml of absolute ethanol was added to the collected butan-2-ol/benzene (1:1, v/v) extract to prevent cloudiness at ambient temperatures on cold days. NADPH–cytochrome *c* reductase (EC 1.6.2.4) assay was by the method of Jungalwala & Dawson (1971). Cerebroside sulphotransferase and CDP-choline–1,2-diacylglycerol cholinephosphotransferase were determined by the procedure of Jungalwala (1974*a*,*b*). However, 1.32 mM-dithiothreitol was added to the reaction mixture for the assay of cholinephosphotransferase. 5′-Nucleotidase (EC 3.1.3.5) was determined by the method of Mitchell & Hawthorne (1965). UDP-Glucose–ceramide glucosyltransferase was measured by the method of Brenkert & Radin (1972). ($Na^+ + K^+$)-activated ouabain-sensitive ATPase (EC 3.6.1.3) was assayed by the method of Barnett (1970), except that after the assay, the P_i was determined by the method of Prohaska *et al.* (1973)

Protein. This was determined by the method of Lowry *et al.* (1951), as modified by Lees & Paxman (1972), with bovine serum albumin as standard. Delipidated proteins were prepared for SDS/polyacrylamide gels by the method of Agrawal *et al.* (1974).

Electron microscopy. The subcellular fractions were fixed in 10% glutaraldehyde in cacodylate buffer at 4°C overnight. The material was post-fixed in OsO_4 and then embedded. The sections were cut on an LKB Ultratome with a glass knife, stained with uranyl acetate and lead citrate and viewed under a Philips EM300 electron microscope.

Determination of myelin purity with labelled microsomal fraction. Five 27-day-old rats were injected with [^{3}H]leucine (50 μCi each; sp. radioactivity 5 Ci/mmol) intracranially. After 3 h, brains were removed and the microsomal fraction was prepared as described previously. A portion of the material was taken for counting radioactivity and protein determination. The rest of the microsomal fraction was mixed with unlabelled rat brain homogenate. This mixture was processed for the isolation of myelin. A portion of the material was taken for counting radioactivity and cross contamination was determined by the extent of the microsomal radioactivity in myelin membrane fraction.

Lipid analysis. Lipids from known amounts of heavy-myelin and membrane fractions were extracted essentially by the method of Folch *et al.* (1957). The lipid extracts were subfractionated into non-polar lipids, galactolipids and phospholipids by silicic acid-column chromatography by the method of Vance & Sweeley (1967). The phospholipid content was estimated after phosphate determinations by the method of Bartlett (1959). Total galactolipids were estimated by the orcinol method (Balazs *et al.*, 1971). Cholesterol and cholesteryl ester were separated by t.l.c. and determined by g.l.c. by the method of Kishimoto & Hoshi (1972). A portion of the total galactolipid fraction was benzoylated. The benzoylated cerebrosides were resolved into non-hydroxy fatty acid and α-hydroxy fatty acid types and determined by high-pressure liquid chromatography as previously described (Jungalwala *et al.*, 1977).

Results

Purity of isolated myelin

The assessment of the purity of the isolated

myelin is of utmost importance, since contamination by other membrane fractions could lead to error in the interpretation of the results. For this reason myelin and myelin subfractions were extensively purified and monitored for microsomal and other membrane contamination routinely. In some of the early experiments (Tables 1 and 2) myelin was prepared from the 'nuclear pellet', instead of the usual nuclear and crude mitochondrial fraction, to minimize the microsomal contamination (Jungalwala & Dawson, 1971). Three separate methods were used to monitor the microsomal contamination in myelin preparations. NADPH–cytochrome *c* reductase has been conventionally used as the microsomal marker enzyme. However, technical difficulties are encountered in the spectrophotometric assay of this enzyme (Ruenwongsa *et al.*, 1979). The specific activity of the enzyme in brain microsomal fraction is much lower than that in the liver. This necessitates the use of larger amounts of insoluble myelin proteins for the determination of measurable changes in the absorbance. CDP-choline–1,2-diacylglycerol cholinephosphotransferase is localized in the microsomal membranes and has little or no activity in myelin (Jungalwala, 1974*a*). We have used this enzyme as a microsomal marker (Ruenwongsa *et al.*, 1979). The radioactive assay of this enzyme is simple and sensitive and has been recently used as a microsomal membrane marker also by other workers (Possmayer *et al.*, 1979). The third way to determine the microsomal contamination was by adding [^{3}H]leucine-labelled microsomal fraction to brain homogenates and measuring the amount of ^{3}H radioactivity present in the re-isolated myelin. As can be seen from Tables 1 and 2, the estimated percentage of microsomal contamination of myelin by the three methods was different. Usually, the percentage of microsomal contamination as measured by the NADPH–cytochrome *c* reductase assay gave much higher values than that obtained with either cholinephosphotransferase assay or by the [^{3}H]leucine method. Similar observations were also made by Possmayer *et al.* (1979) and Ruenwongsa *et al.* (1979). Owing to technical difficulties mentioned previously for the NADPH–cytochrome *c* reductase assay, and because the results obtained with the other two methods were consistent, the latter was considered more satisfactory and reliable. There was no detectable mitochondrial marker enzyme cytochrome *c* oxidase activity in our myelin preparations.

Galactosyltransferase in myelin

The specific activities of the enzyme galactosyltransferase in myelin and microsomal fractions obtained from the whole brain of rats at various ages are given in Table 1. The specific activity in extensively purified myelin isolated from nuclear

Table 1. *Galactosyltransferase of rat brain myelin and microsomal fractions*

Percentage microsomal protein contamination in purified myelin was determined by three different methods as described in the text. The activity of galactosyltransferase was measured with α-hydroxy fatty acid-containing ceramide as an acceptor. The values represent means ± S.E.M. for three to four determinations with various age groups.

Age (days)	Galactosyltransferase activity (nmol/mg per h)		Method ...	Microsomal protein contamination in myelin (%)			Residual galactosyltransferase activity in myelin after correction (nmol/mg per h)		
	Myelin	Microsomal fraction		NADPH–cytochrome *c* reductase	Cholinephosphotransferase	[^{3}H]Leucine	NADPH–cytochrome *c* reductase	Cholinephosphotransferase	[^{3}H]Leucine
24	1.1 ± 0.3	4.4 ± 0.4		10 ± 0.9	1.4 ± 0.1	0.5 ± 0.01	0.66	1.04	1.08
30	1.9 ± 0.7	3.7 ± 0.5		4.8 ± 1.5	1.1 ± 0.1	0.6 ± 0.01	1.7	1.86	1.88
88	0.95 ± 0.5	2.3 ± 0.2		6.0 ± 0.8	1.4 ± 0.2	0.3 ± 0.02	0.81	0.92	0.94

Table 2. *Distribution of galactosyltransferase and other enzymes in subfractions of myelin and microsomal fractions from 23-day-old rat brain*

Purified myelin (approx. 20mg of protein), prepared from cerebral hemispheres of a group of ten 23-day-old rats, was subfractionated as described in the text. The microsomal fraction was also subfractionated in a similar way. Microsomal subfractions are represented by the molarity of sucrose on which the respective fraction floated. The activity of galactosyltransferase was measured with α-hydroxy fatty acid containing ceramide as an acceptor. The values are means ± S.E.M. for two to three determinations.

Fraction	Yield (mg of protein/ 10 brains	Galactosyl-transferase (nmol/mg per h)	NADPH–cytochrome *c* reductase (unit/mg per min)	Cholinephospho-transferase (nmol/mg per h)	Cerebroside sulphotransferase (pmol/mg per h)	Cyclic nucleotide 3′-phosphohydrolase (μmol/mg per min)	5′-Nucleotidase (nmol/mg per min)
Light-myelin	0.6 ± 0.2	1.6 ± 0.05	13.6 ± 0.01	0.59 ± 0.02	0.35 ± 0.1	28.6 ± 1.2	28.7 ± 0.9
Heavy-myelin	11.6 ± 0.5	5.1 ± 0.4	38.0 ± 2.4	0.92 ± 0.09	0.89 ± 0.05	21.8 ± 1.5	17.0 ± 0.6
Membrane	2.0 ± 0.3	6.3 ± 0.3	37.3 ± 0.02	0.82 ± 0.06	0.25 ± 0.05	23.2 ± 2.0	22.6 ± 0.8
Pellet	1.2 ± 0.3	1.1 ± 0.08	20.7 ± 1.1	0.36 ± 0.05	0.16 ± 0.04	15.8 ± 1.8	10.5 ± 2.3
Microsomal	110 ± 5	3.5 ± 0.3	368 ± 59	58.0 ± 0.84	12.53 ± 0.6	2.2 ± 0.08	21.4 ± 0.9
Microsomal subfractions on sucrose:							
0.55M	34.3 ± 2.0	3.2 ± 0.2	348 ± 30	—	—	3.9 ± 0.06	38.7 ± 1.3
0.75M	35.1 ± 2.5	2.6 ± 0.3	287 ± 27	—	—	1.4 ± 0.11	21.8 ± 0.9
0.85M	17.6 ± 3.1	0.8 ± 0.05	144 ± 15	—	—	0.4 ± 0.02	12.7 ± 1.1
Pellet	1.8 ± 0.5	2.3 ± 0.1	21.0 ± 5	—	—	0.7 ± 0.21	11.3 ± 1.0

fraction was about 25–50% of that observed in the microsomal membranes. Even after accounting for the contamination by microsomal membranes, the remaining activity in myelin was approx. 15–46% of that in the microsomal membranes isolated from 24–88-day-old animals.

Galactosyltransferase in myelin subfractions

Myelin obtained from the cerebral hemisphere of rat brain at various ages was subfractionated by isopycnic sucrose-density-gradient centrifugations into four main subfractions. Three of these subfractions were further purified by sucrose density-gradient centrifugation. The distribution of the galactosyltransferase activity in myelin subfractions obtained from 23-day-old rat brain is given in Table 2 as a representative example. The activity of the microsomal marker enzyme, as well as other membrane enzymes, such as 5′-nucleotidase, cerebroside sulphotransferase and 2′:3′-cyclic nucleotide 3′-phosphohydrolase, is also given in Table 2. Of the total myelin protein 70–75% was found in the heavy-myelin fraction at all ages, whereas 2–5% was recovered in the light-myelin fraction and about 7–12% in the membrane fraction. Most of the galactosyltransferase activity was associated with the heavy-myelin and membrane fractions. The galactosyltransferase activity in the myelin subfractions could not be accounted for as being caused by contamination by the microsomal membranes (as estimated by the microsomal marker enzyme activity). For 23-day-old rat brain myelin, the specific activity of the galactosyltransferase in heavy-myelin and membrane fractions was about 1.5–2-fold higher than that in the microsomal membranes, whereas in the light-myelin and pellet fractions it was about one-half to one-third of that in the microsomal membranes.

The cerebroside sulphotransferase activity in myelin subfractions varied from about 1 to 7% of that found in the microsomal membranes. 5′-Nucleotidase, which is considered to be a plasma-membrane marker, was distributed about equally in all the myelin subfractions and microsomal fractions, except in the pellet, whereas 2′:3′-cyclic nucleotide 3′-phosphohydrolase was found mostly in the light-myelin, heavy-myelin and membrane fractions, with low activity in pellet and microsomal fractions.

In view of the much higher specific activity of galactosyltransferase observed in heavy-myelin and membrane fractions compared with microsomal activity, it was necessary to determine if a particular microsomal subfraction with high galactosyltransferase activity was contaminating the myelin subfractions. Microsomal membranes were subfractionated exactly as described for myelin on sucrose density gradients. It was observed that most of the

galactosyltransferase activity was present on top of the 0.55 M- and 0.75 M-sucrose layers and there was no significant enrichment of the galactosyltransferase specific activity in the microsomal subfractions (Table 2).

Galactosyltransferase in myelin subfractions during development

The distribution of galactosyltransferase in myelin subfractions and in the microsomal fraction during development is summarized in Fig. 1. The specific activity of galactosyltransferase in the microsomal fraction in brain from 8-day-old animals was very low, reached a maximum when the animals were about 20 days old and then declined. By contrast, the specific activity of the galactosyltransferase in heavy-myelin and membrane fractions was 3–4 times higher than that of the microsomal membranes, in 16-day-old animals. The activity in the heavy-myelin fraction then declined with age. The activity in the membrane fraction was still high in 20-day-old animals compared with microsomal membrane activity and then declined with age. Light-myelin fraction had generally lower enzyme activity compared with microsomal enzyme activity at all ages studied. The enzyme in the pellet fraction was also low compared with the microsomal fraction.

Other glycosyltransferases in myelin subfractions

The specificity of various glycosyltransferases in myelin and myelin subfractions was studied for 16-day-old animals. The activity of galactosyl- and glucosyl-transferase with non-hydroxy fatty acid- and hydroxy fatty acid-containing ceramides as substrates is given in Table 3. The galactosyltransferase in the microsomal fraction, as well as that in myelin and myelin subfractions, preferentially utilized hydroxy fatty acid-containing ceramide as opposed to non-hydroxy fatty acid-containing ceramide as the acceptor and the specific activity in heavy-myelin and membrane fractions was either equal to or higher than that found in microsomal membranes. The glucosyltransferase in microsomal membranes had no preference for the type of ceramide acceptor, and the activity for this enzyme in myelin or myelin subfractions was less than 5% of that found in the microsomal membranes. This would indicate that glucosyltransferase was not localized in purified myelin. Similar conclusions have been reached by Costantino-Ceccarini & Suzuki (1975).

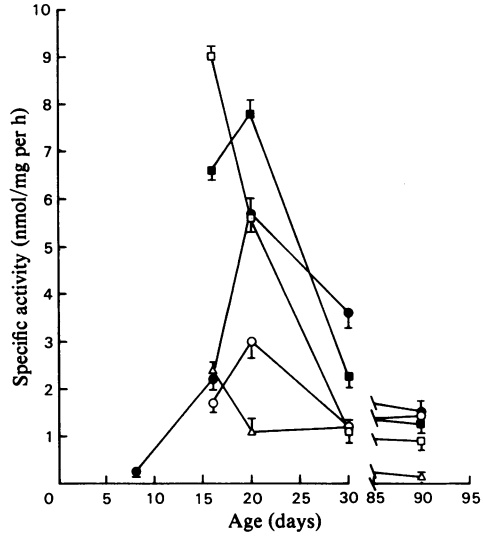

Fig. 1. *Specific activity of galactosyltransferase in rat brain myelin subfractions and microsomal membranes during development*

The average specific activity of UDP-galactose–hydroxy fatty acid-containing ceramide galactosyltransferase of microsomal membranes (●), light-myelin fraction (○), heavy-myelin fraction (□), membrane fraction (■) and pellet (△), with age are plotted. Values for myelin subfractions at 8 days of age are not available. The assay of galactosyltransferase is described in the text. The points represent an average ± S.E.M. for two to three separate determinations.

Table 3. *Activity of glycosyltransferase in microsomal fraction, myelin and myelin subfractions from 16-day-old rat brain*

The myelin subfractions were prepared as described in the text. The glycosyltransferase activity was measured with either non-hydroxy fatty acid (NFA)- or α-hydroxy fatty acid (HFA)-containing ceramide as the acceptor. The values are means ± S.E.M. for three to four determinations.

	Galactosyltransferase (nmol/mg per h)		Glucosyltransferase (nmol/mg per h)	
Fraction	NFA-ceramide	HFA-ceramide	NFA-ceramide	HFA-ceramide
Microsomal	0.5 ± 0.11	2.2 ± 0.3	5.9 ± 0.4	6.0 ± 1.3
Myelin	0.7 ± 0.1	8.0 ± 0.6	—	0.3 ± 0.05
Light-myelin	0.3 ± 0.05	1.6 ± 0.2	—	—
Heavy-myelin	0.7 ± 0.01	8.7 ± 0.1	0.1 ± 0.02	0.1 ± 0.07
Membrane	1.1 ± 0.1	6.3 ± 0.3	—	0.2 ± 0.05

Chemical analyses of myelin subfractions

The chemical analysis of heavy-myelin and membrane fractions isolated at various ages is given in Table 4. The data on light-myelin and pellet fractions were not reliable due to a paucity of the material and are not given in the present paper. The chemical composition of heavy-myelin and membrane fractions was slightly different and it also varied with age. The heavy-myelin fractions contained relatively less protein (18–30%) and more lipids (70–82%) compared with the membrane fraction (Table 4). The membrane fraction had about 40–45% protein by weight. The ratio of protein/lipid appeared to increase with age both in the case of the heavy-myelin and membrane fractions.

Generally, the membrane fraction had slightly less cholesterol and more phospholipid than the heavy-myelin fraction. The mole percentage ratio of cholesterol/galactolipids/phospholipids varied with age in both the myelin subfractions. In the case of the heavy-myelin fraction, the mole percentage ratio of galactolipids slightly increased, whereas that of phospholipids decreased with the increase in the age of animals. In the membrane fraction, the mole percentage ratio of cholesterol increased and that of phospholipids decreased with age. In both heavy-myelin and membrane fractions, the ratio of non-hydroxy fatty acid- to α-hydroxy fatty acid-containing galactolipids decreased with age. The mole percentage ratio of cholesterol/galactolipids/phospholipids for the heavy-myelin fraction from 15-day-old animals as reported by Agrawal *et al.* (1974) was similar to that of the heavy-myelin fraction from 16-day-old animals.

Morphology and protein profile of myelin subfractions

The myelin subfractions (heavy myelin, light myelin and membrane) from 20-day-old rat brain appeared to be similar morphologically when viewed under an electron microscope at several magnifications. Typical myelin multilamellar structures were seen. No significant difference was observed in the architecture of these membrane subfractions. The pellet fraction, on the other hand, was completely different. This fraction contained mostly unilamellar membranes with vesicular structures.

The protein profile of the light-myelin, heavy-myelin and membrane fractions of myelin from 16-day-old rats on SDS/polyacrylamide gels is shown in Fig. 2. Generally, the protein profile of the light-myelin and heavy-myelin fractions appeared to be similar, with typical myelin protein bands. However, the membrane fraction appeared to be different in that it contained considerably larger amounts of high-molecular-weight protein relative to the proteolipid protein and the two myelin basic proteins. The light-myelin fraction was more enriched in basic proteins compared with proteolipid proteins. The relative proportions of the latter proteins were determined by a gel-scanner of the stained gels. The ratio of proteolipid protein to the basic proteins was 0.48, 1.38 and 1.73 for the light-myelin, heavy-myelin and membrane fractions respectively. The ratio of small to large basic proteins was 1.5, 1.4 and 1 for the light-myelin, heavy-myelin and membrane fractions respectively. Similar results have been published by Fujimoto *et al.* (1976) for light- and heavy-myelin subfractions.

Galactosyltransferase in myelin treated wth EGTA

Considerable amounts of galactosyltransferase activity in axons and 'axolemma-enriched fractions' have been reported (Costantino-Ceccarini & De Vries, 1976; Costantino-Ceccarini *et al.*, 1977). Since these fractions could possibly contaminate purified

Table 4. *Chemical analysis of heavy-myelin and membrane fractions at various ages*

Galactolipids include cerebrosides and sulphatides. The ratio of non-hydroxy fatty acid-containing galactolipids (NFA)/hydroxy fatty acid-containing galactolipids (HFA) was determined by high-pressure liquid chromatography of the benzoylated glycolipids. The values are means ± S.E.M. for two to three determinations.

Age (days)	Protein/lipids (w/w)	Mole percentage ratio			NFA/HFA
		Cholesterol	Galactolipids	Phospholipids	
			Heavy-myelin fraction		
16	0.23 ± 0.02	37.9 ± 0.9	16.6 ± 1.3	45.9 ± 0.01	—
20	0.31 ± 0.05	39.8 ± 0.5	16.3 ± 0.8	43.9 ± 0.04	0.74 ± 0.005
30	0.39 ± 0.02	38.4 ± 0.4	19.0 ± 1.3	43.0 ± 0.03	0.60 ± 0.01
90	0.44 ± 0.01	36.0 ± 1.0	21.8 ± 0.5	42.1 ± 0.02	0.55 ± 0.01
			Membrane fraction		
20	0.63 ± 0.02	26.7 ± 0.4	19.8 ± 1.3	53.9 ± 0.02	0.73 ± 0.09
30	0.84 ± 0.03	31.1 ± 0.03	18.8 ± 0.6	50.4 ± 0.01	0.58 ± 0.01
90	0.85 ± 0.01	33.2 ± 2.5	19.3 ± 0.01	47.3 ± 0.03	0.48 ± 0.03

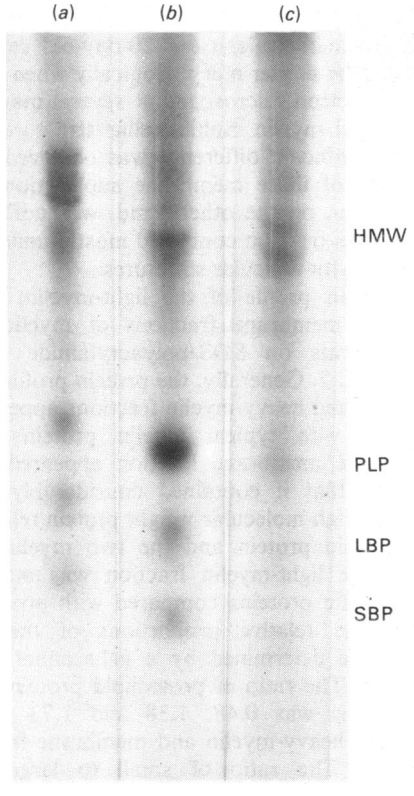

Fig. 2. *Gel disc electrophoresis pattern of myelin subfractions*

Delipidated myelin subfractions (35 μg of protein each) were electrophoresed on 10% polyacrylamide gels containing 0.2% SDS for 18 h with a current of 1.0 mA per tube and stained with Coomassie Blue. (*a*), Light-myelin fraction; (*b*), heavy-myelin fraction; (*c*), membrane fraction. Abbreviations: HMW, high-molecular-weight proteins; PLP, proteolipid proteins; LBP, large basic protein; SBP, small basic protein.

myelin, it was necessary to check this possibility. We prepared purified myelin by our procedure and also essentially by the procedure of DeVries *et al.* (1978) from 16-day-old rat brains.

For the latter procedure, most of the membrane material floated on 0.85 M-sucrose. The small amounts of material floating on 1.0 M- and 1.2 M-sucrose might correspond to the 'axolemma-enriched fractions' described by DeVries *et al.* (1978). The specific activity of galactosyltransferase in myelin prepared normally and in that treated with EGTA is given in Table 5. It is evident that after treatment of myelin with EGTA and density-gradient fractionation the specific activity of the enzyme was not decreased, but in fact slightly increased, possibly due to removal of some extraneous proteins. The material that floated on 1.0 M- and 1.2 M-sucrose had very low galactosyltransferase activity and contained only about 25% of the $(Na^+ + K^+)$-activated ATPase activity of the 'axolemma-enriched fraction' reported by DeVries *et al.* (1978). This would indicate that the myelin fraction was not contaminated by the 'axolemma-enriched fractions'.

Discussion

Studies *in vitro* have shown that the enzymes responsible for the synthesis *de novo* of phospholipids and glycolipids are mainly localized in the endoplasmic reticulum (Dawson, 1973; McMurray & Magee, 1972; Van Den Bosch, 1974) and Golgi complex (Van Golde *et al.*, 1971, 1974). Other subcellular structures such as mitochondria (McMurray & Magee, 1972), plasma membranes (Victoria *et al.*, 1971), synaptosomal membranes (Miller & Dawson, 1972) and myelin (Miller & Dawson, 1972; Jungalwala, 1974*a*,*b*) appeared to

Table 5. *Galactosyltransferase activity in myelin and other fractions after EGTA treatment*

Myelin and microsomal fractions were prepared from 16-day-old rat brains as described in the text. The precipitate fraction was the residue obtained when osmotically-shocked myelin was centrifuged on a discontinuous gradient of 0.32–0.85 M-sucrose. EGTA-treated myelin was prepared as described in the text. The material floating on 1.0 M- and 1.2 M-sucrose was collected after centrifuging EGTA-treated myelin on a 0.85 M-, 1.0 M- and 1.2 M-sucrose step gradient. The activity of the galactosyltransferase was measured with hydroxy fatty acid-containing ceramide as the acceptor. The values are means ± S.E.M. for three to four determinations.

Fraction	Galactosyltransferase (nmol/mg per h)	$(Na^+ + K^+)$-activated ATPase (nmol/mg per h)
Microsomal	3.4 ± 0.68	20.5 ± 0.01
Myelin	7.8 ± 0.24	3.9 ± 0.01
Precipitate	1.75 ± 0.05	14.4 ± 0.02
EGTA-treated myelin	9.4 ± 1.1	5.8 ± 0.03
Membranes on 1.0 M and 1.2 M-sucrose	1.62 ± 0.8	10.4 ± 1.0

have limited capability to synthesize complex lipids. Several kinetic studies *in vivo* have suggested that phospholipids, cerebrosides and sulphatides are synthesized in the endoplasmic reticulum and are transported to other subcellular structures, such as myelin (Jungalwala & Dawson, 1971; Jungalwala, 1974*a*,*b*; Hayes & Jungalwala, 1976; Herschkowitz *et al.*, 1968; Pasquini *et al.*, 1975; Sun & Horrocks, 1973). We have also demonstrated a protein-catalysed exchange of phosphatidylinositol and phosphatidylcholine between microsomal and myelin membranes (Ruenwongsa *et al.*, 1979).

However, UDP-galactose–ceramide galactosyltransferase, which is involved in the synthesis of cerebrosides, has been reported to be present in purified myelin (Neskovic *et al.*, 1973; Costantino-Ceccarini & Suzuki, 1975). Our experimental results also demonstrate that although purified myelin *in vitro* was not capable of catalysing the synthesis of phosphatidylcholine, glucocerebrosides or sulphatides, it contained considerable amounts of the galactosyltransferase activity. This activity in myelin could not be accounted for as due to microsomal or mitochondrial contamination, as measured by several marker enzymes discussed previously. Contamination by other membranes having considerably higher activity than in myelin or microsomal membranes is possible. However, results obtained with subfractionation of microsomal membranes and with myelin prepared in the presence of EGTA to eliminate axolemma did not support such a possibility.

Of the four operationally defined subfractions of myelin, i.e. light-myelin, heavy-myelin, membrane and pellet fractions, most of the myelin galactosyltransferase was localized in the heavy-myelin and membrane fractions. During development, significant changes were observed in the specific activity of the enzyme in the myelin subfractions and in the microsomal membranes. As it has been shown previously by several investigators (Shah, 1971; Brenkert & Radin, 1972), during the myelination period the activity of the microsomal galactosyltransferase increased and reached a maximum around 21 days followed by a decline in the activity However, the developmental profile of the enzyme in the heavy-myelin membrane fractions was not the same. The earliest myelin and myelin subfractions one could isolate from rat brain is at about 15–16 days after birth. The specific activity of the galactosyltransferase in heavy-myelin and membrane fractions at 16 days was 3–4-fold higher than that in the microsomal fraction. The rate of decline in the specific activity of the enzyme in these subfractions with age was also different from that in the microsomal membranes. The higher specific activity of the galactosyltransferase in heavy-myelin and membrane fractions in 16-day-old animals could not be due to contamination by microsomal membranes, since at that age the microsomal fraction had considerably lower specific activity. We have also observed a similar developmental profile for the enzyme in myelin from the spinal cord and hindbrain (Koul & Jungalwala, 1978).

Calculations on the basis of recovery of myelin subfractions and microsomal membranes would suggest that although myelin in young animals had considerably higher specific activity of galactosyltransferase, it represents only about 25% of the total amount of galactosyltransferase. The total amount of the enzyme in the microsomal membranes was still considerably higher than that in the myelin membranes.

The functional role of the galactosyltransferase in early myelin is not known. However, it can be speculated that the specific localization of only cerebroside-synthesizing enzyme in early myelin may have some role in the myelination process.

It is suggested that the function of the enzyme galactosyltransferase, which is probably present in the oligodendroglial plasma membrane and in early myelin, is to make enough cerebrosides to form an early core of primordial myelin membrane. Later, as myelination progresses, other lipids as well as cerebrosides and proteins arrive by transport mechanisms to become incorporated into this template to form myelin. The lower activity of the enzyme in myelin at later stages of myelination may indicate its redundancy. The functional role of galactosyltransferase in myelinogenesis is supported by the observations that antibodies against galactocerebrosides from rabbits are known to produce inhibition of myelination in newborn-mouse cerebellar tissue cultures (Hruby *et al.*, 1977) and that serum from rabbits with experimental allergic encephalomyelitis, which produced demyelination in rat cerebellar organ cultures, also inhibited specifically the activity of the enzyme galactosyltransferase of glial cells and inhibited further myelin formation (Latovitzki & Silberberg, 1975). When such cultures were replaced with normal serum the inhibition was reversed and normal myelination occurred. It is known that the factor in experimental-allergic-encephalomyelitis serum responsible for inhibition of myelination is anticerebroside antibody (Fry *et al.*, 1974). It has been suggested that antibody–antigen binding somehow acts to decrease the enzymic activity or prevent induction of myelination.

Several investigators have noted that isolated myelin can be separated into several subfractions on density-gradient centrifugations (Benjamins *et al.*, 1973; Matthieu *et al.*, 1973; Agrawal *et al.*, 1974; Zimmerman *et al.*, 1975). Recently, more interest has been developed to evaluate if these subfractions have any physiological significance or if they

represent mere artefacts of homogenization and differential stages of development in different areas of brain. Various investigators have used slightly different compositions of density gradients to subfractionate osmotically-shocked myelin into different fractions. Thus individual subfractions prepared by different investigators cannot be strictly compared. However, generally it has been observed that myelin could be separated into essentially three to four subfractions. Unlike most of the other investigators, we have separated myelin into basically four subfractions by isopycnic gradients. Three of the subfractions, i.e. the light-myelin, heavy-myelin and membrane fractions, had about equal 2′:3′-cyclic nucleotide 3′-phosphohydrolase specific activity, a myelin enzyme marker, the pellet having a somewhat lower activity of this enzyme (Table 2). There was no significant difference in the activity of this enzyme in different subfractions within the age groups studied. The relative amounts of the three membrane subfractions remained practically the same with age. Morphologically the three myelin subfractions also appeared similar. However, the chemical composition of the membrane subfractions, especially the heavy-myelin and membrane fractions was different and it varied with age. The heavy-myelin fraction contained relatively less protein (18–30%) and more lipids compared with the membrane fraction (Table 4), which had about 40–45% protein by weight.

The protein profile and relative amounts of the proteins in myelin subfractions appeared to be different. Similarly the lipid composition of the heavy-myelin and membrane fractions was slightly different. Thus, the membrane fraction appeared to be similar to the heavy-myelin fraction with respect to its enzymic activity, e.g. the activities of galactosyltransferase and 2′:3′-cyclic nucleotide 3′-phosphohydrolase, but appeared to be different in its overall chemical composition. It has been postulated by others that the membrane fraction, containing relatively more protein and less lipid, may be a precursor of the mature myelin membrane (Agrawal *et al.*, 1974). However, the definite physiological significance of this membrane in the process of myelination still remains to be established.

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