Gangliosides and sialosylglycoproteins in coated vesicles from bovine brain

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The content of gangliosides and sialosylglycoproteins was investigated in a coated-vesicle-enriched fraction prepared from bovine brain by the method of Pearse [(1975) *J. Mol. Biol.* **97**, 93–98] and further purified by g.p.c. (glass-permeation chromatography) [Pfeffer & Kelly (1981) *J. Cell Biol.* **91**, 385–391]. From morphological criteria and from the analysis of the polypeptide pattern on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis the coated-vesicle fraction (CV-fraction) appeared more than 95% pure. The ganglioside-NeuAc (*N*-acetylneuraminate), glycoprotein-NeuAc, phospholipid and cholesterol contents of CV-fraction were compared with those of bovine brain synaptic plasma membranes (SPM). The cholesterol to phospholipid molar ratio was 0.47 ± 0.07 in CV-fraction and 1.06 ± 0.08 in SPM. The ganglioside-NeuAc and glycoprotein-NeuAc to phospholipid molar ratios were 0.047 and 0.020 respectively in CV-fraction and 0.039 and 0.016 respectively in SPM. The ($\text{Na}^+ + \text{K}^+$)-dependent ATPase activity sensitive to ouabain (in μmol of P_i/h per nmol of phospholipid) was 1.04 in CV-fraction and 0.63 in SPM; the ratio between this activity and the activity resistant to ouabain was 2 in CV-fraction and 1.4 in SPM. A t.l.c. analysis of the ganglioside fractions showed that most of the ganglioside species present in SPM were present in CV-fraction. In a rat brain coated-vesicle preparation not subjected to g.p.c., the activities [as sugar-radioactivity (c.p.m.) transferred/h per μmol of phospholipid] of the enzymes CMP-NeuAc : sialosyl-lactosylceramide (GM_3) sialosyl-, UDP-Gal : *N*-acetylgalactosaminyl(sialosyl)lactosylceramide (GM_2) galactosyl- and UDP-GalNAc : sialosyl-lactosylceramide (GM_3) *N*-acetylgalactosaminyl-transferases, which were considered Golgi-apparatus markers, were about 19, 16 and 10% respectively of those determined in rat brain neuronal perikaryon-enriched fractions. Taken together, the results indicate that most of the major gangliosides are constituents of coated vesicles.

Previous work from this laboratory showed that, in quantitative terms, the most important site of synthesis of gangliosides was in the neuronal perikaryon (Maccioni *et al.*, 1978), in intracellular membrane fractions enriched in Golgi-apparatus membranes (Landa *et al.*, 1981). In these membranes, the oligosaccharide chains are synthe-

sized at the luminal side, protected from the action of exogenous neuraminidase (Maccioni *et al.*, 1974; Landa *et al.*, 1981). From this site of synthesis the gangliosides move to the neuronal plasma membrane, reaching the synaptic plasma membrane by fast axonal transport (Forman & Ledeen, 1972; Landa *et al.*, 1979). At the synaptic plasma membrane they lose their protection from neuraminidase attack, suggesting that some kind of inversion of their topographic disposition occurs at this level (Hansson *et al.*, 1977; Landa *et al.*, 1981). Results of experiments *in vivo* indicate that, in that position, ganglioside molecules turn over as units (Maccioni *et al.*, 1971; Holm & Svennerholm, 1972; Ledeen *et al.*, 1976).

The process by which these sialosylglycoconju-

Abbreviations used: Mes, 4-morpholine-ethanesulphonic acid; the nomenclature of the gangliosides (GM_1 etc.) is that of Svennerholm (1963); g.p.c., glass-permeation chromatography; SDS, sodium dodecyl sulphate; SPM, synaptic plasma membrane(s); NeuAc, *N*-acetylneuraminate.

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gates are translocated from the site of synthesis to the plasma membrane is poorly understood. Studies *in vivo* of axonal transport have shown that, at any time after injection of labelled precursor to rats (Landa *et al.*, 1977) or chicks (Landa *et al.*, 1979), the labelled gangliosides that are being transported to nerve endings are associated with membranous subcellular fractions, although the precise nature of these putative transporting membranes is unknown at present. As has been suggested for several glycoproteins, it is possible that, within the axon, gangliosides move in association with the intra-axonal smooth endoplasmic reticulum (Droz *et al.*, 1975). However, little is known about the pathway followed by sialosylglycoconjugates from the site of terminal glycosylation in the Golgi apparatus to the intra-axonal transporting structures and from these to the plasma membrane.

Coated vesicles constitute a kind of intracellular vesicle characterized by having a proteinaceous coat in which a single protein species named clathrin predominates (Pearse, 1975, 1976). They participate in the retrieval of plasma-membrane constituents and probably in the recovery of membrane itself, thus communicating the intracellular membrane system with the plasma-membrane compartment (Anderson *et al.*, 1977; Willingham & Pastan, 1980). Coated pits at the cytoplasmic surface of regions of the endoplasmic reticulum and Golgi apparatus have also been implicated in the transport of glycoconjugates from the endoplasmic reticulum to the Golgi apparatus and from this to the plasma membrane through the transient formation of transporting coated vesicles (Rothman & Fine, 1980; Kinnon & Owen, 1983).

In brain tissue, clathrin was shown to be abundant at the nerve endings (Cheng *et al.*, 1980) and morphological studies have raised the possibility that coated vesicles mediate synaptic vesicle membrane retrieval and recycling of synaptic membrane surface (Heuser & Reese, 1973; Fried & Blaustein, 1978). To discover whether coated vesicles are involved in the intracellular mobilization of neuronal gangliosides, we investigated in the present work the presence of sialosylglycoconjugates in these vesicles. The results indicate that gangliosides and sialosylglycoproteins are present in a highly purified coated-vesicle fraction from calf brain.

Materials and methods

Materials

Cholestane, ATP, Mes, controlled-pore glass (PG 3000-200), bovine serum albumin, sucrose and ouabain were from Sigma Chemical Co., St. Louis,

MO, U.S.A. Acrylamide and *NN*'-methylene-bisacrylamide were from BDH Chemicals Ltd., Poole, Dorset, U.K. Gangliosides GM₃, GM₂, GM₁, GD_{1a} and GT₁ and labelled CMP-[³H]NeuAc (sp. radioactivity 30000 c.p.m./nmol) were obtained as described previously (Maccioni *et al.*, 1974, 1977, 1978). UDP-[³H]galactose (UDP-Gal, sp. radioactivity 40000 c.p.m./nmol) and UDP-*N*-[¹⁴C]acetylgalactosamine (UDP-GalNAc, sp. radioactivity 45000 c.p.m./nmol) were from New England Nuclear Corp., Boston, MA, U.S.A. Precoated thin-layer silica-gel G plates were from E. Merck, Darmstadt, Germany.

Isolation of coated vesicles

The method followed was that of Pearse (1975), with minor modifications. Five bovine brains freshly obtained from the local slaughterhouse were processed in each experiment. Briefly, cerebella, brain stem and meninges were discarded and the brains were homogenized in 2 vol. of 50 mM-Mes buffer (pH 6.5)/1 mM-EGTA/0.5 mM-MgCl₂/3 mM-Na₂N₃ by two 15 s sessions at intermediate velocity in a Waring Blendor. The fraction of homogenate sedimented between 20000 *g* for 20 min and 55000 *g* for 60 min was layered on to a discontinuous gradient of sucrose from 5 to 60% (w/v) and centrifuged at 100000 *g* for 60 min. The material banding between 10% and 40% sucrose was collected, sedimented by centrifugation and applied to a second gradient made of sucrose from 20 to 60% and centrifuged at 55000 *g* for 16 h. Fractions from the gradient were monitored for coated vesicles by electron microscopy of negatively stained portions. The fractions containing the highest proportion of coated vesicles were pooled, sedimented by centrifugation and applied to a third discontinuous sucrose gradient (from 5 to 30%) and centrifuged at 100000 *g* for 1 h. The fractions floating between 20 and 30% sucrose were sedimented by centrifugation and constitute the 'coated-vesicle-enriched fraction'. At this step the amount of recovered protein was about 30 µg/g of fresh brain. Coated vesicles were also obtained from 17-day-old rat brains by the same procedure with essentially the same results. Unless otherwise stated, however, the experiments described were carried out in coated vesicle preparations from bovine brain.

G.p.c.

The 'coated-vesicle-enriched fraction' was further purified by chromatography in a column of controlled-pore glass (PG 3000-200) previously treated with 1% poly(ethylene glycol) (20000 *M_r*) (Pfeffer & Kelly, 1981). The column (1.2 cm × 90 cm) was packed under constant vibration and was equilibrated at 4°C with 50 mM-Mes

buffer (pH 6.5)/0.2 M-sucrose/0.3 M-NaCl/10 mM-EGTA/3 mM-Na₂N₃. A portion of the coated-vesicle-enriched fraction suspended in the same buffer was homogenized with the aid of a Teflon-pestle homogenizer and applied to the column. The column was eluted with the same buffer and the elution pattern was monitored by measuring the A_{280} of the eluate.

Electron microscopy

For negative staining of fractions, a drop of membrane suspension was left to stand on a Formvar carbon-coated grid for 30 s. The grid was washed with several drops of distilled water and finally negatively stained with a drop of 1% uranyl acetate. For ultrathin sections, membrane pellets were fixed in glutaraldehyde, postfixed in OsO₄, dehydrated in graded ethanol and acetone and embedded in Araldite. Sections were stained with lead citrate and uranyl acetate. The fractions were observed in a Hitachi HU-11B electron microscope operating at 75 kV.

Isolation of gangliosides, phospholipids and sialosylglycoproteins

Fractions were treated with 3.6 vol. of methanol and 1.8 vol. of chloroform at 60°C. After centrifugation the pellet was suspended in 0.2 ml of water and treated with 4 vol. of chloroform/methanol (1:2, v/v) and centrifuged again. The combined extracts were dried under a stream of N₂, resuspended in an adequate volume of chloroform/methanol/water (40:20:3, by vol.) and partitioned against 0.2 vol. of water. The lower phase was washed once with theoretical upper phase to obtain the gangliosides in the upper phase and the phospholipids and cholesterol in the lower phase. The delipidated protein residue was used for determination of glycoprotein-bound NeuAc.

SDS/polyacrylamide-gel electrophoresis

Delipidated protein residues were solubilized by heating at 100°C for 3 min in 125 mM-Tris/HCl buffer (pH 6.8)/1.25% SDS/2 mM-EDTA/3% (v/v) 2-mercaptoethanol/12.5% (v/v) glycerol (Laemmli, 1970). Slab gels [7.5% (w/v) acrylamide and 0.2% (w/v) *NN'*-methylenebisacrylamide] were developed and stained essentially as described by Fairbanks *et al.* (1971). Bromphenol Blue was used as tracking dye.

Chemical determinations

N-Acetylneuraminic acid from gangliosides and sialosylglycoproteins was determined by the thio-barbituric acid procedure of Aminoff (1961) after hydrolysis of the samples in 50 mM-H₂SO₄ at 80°C for 60 min (glycoproteins) or 90 min (gangliosides). Lipid phosphorus was determined as described by

Fiske & SubbaRow, as modified by Bartlett (1959), cholesterol by g.l.c. (with cholestane as internal standard), and protein by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Chromatographic analysis of gangliosides

Gangliosides in the upper phase were freed from water-soluble contaminants by passage through a Sephadex G-25 column equilibrated in chloroform/methanol/water (30:20:3, by vol.) (Arce *et al.*, 1966). Chromatography was performed on Merck precoated thin-layer silica-gel G plates. The solvent used was chloroform/methanol/0.25% CaCl₂ (60:35:8, by vol.). Gangliosides were revealed after spraying with resorcinol reagent and heating.

Enzyme determinations

ATPases (EC 3.6.13) were determined under the conditions given by Hosie (1965). Ouabain (1 mM) was used to calculate the contribution of (Na⁺ + K⁺)-dependent ATPase. The rate of ATP hydrolysis was measured by the formation of P_i (Berenblum & Chain, as described by Lindberg & Ernster, 1956). Ganglioside glycosyltransferases were determined under the conditions previously described (Landa *et al.*, 1979, 1981; Panzetta *et al.*, 1980).

Results

Purification of coated vesicles

The 'coated-vesicle-enriched fraction' obtained after the third sucrose-gradient step (Pearse, 1975) contained about 60% of coated vesicles and 40% of smooth vesicles. The purification of coated vesicles was increased after a chromatographic step through controlled-pore glass (Pfeffer & Kelly, 1981), which removed the large smooth-surfaced vesicles. Fig. 1 shows the pattern of elution in a typical experiment, which was highly reproducible. Two broad peaks of material absorbing at 280 nm were obtained. The first peak (tubes 20–28) contained about 37% of the recovered protein (Fraction I); the second peak (tubes 29–40) contained the rest of the protein. The recovery was about 70–80% of the protein applied to the column. When examined under the electron microscope, the material in the first peak contained few coated vesicles and abundant smooth-surfaced vesicles. The second peak was monitored every two tubes under the electron microscope; according to the percentage of coated vesicles roughly estimated by counting their profiles over the total vesicle profiles in several fields of the grid, it was divided into two fractions, II and III. Fraction II (tubes 32–35) showed a considerable amount of coated vesicles (approx. 80%), and Fraction III (tubes 36–

Fig. 1. Controlled-pore g.p.c. of the coated-vesicle-enriched fraction

The preparation obtained after the third sucrose-gradient-centrifugation step was subjected to g.p.c. as indicated in the Materials and methods section. The protein and coated-vesicle elution patterns were monitored by measuring the A_{280} and by counting the coated-vesicle profiles in negatively stained samples respectively. See the Materials and methods section for details.

40) contained the highest concentration (more than 95%) (Fig. 2). Fraction III was named 'CV-fraction' and selected for further analysis. The inset in Fig. 2 shows the lattice-like coat structure at higher magnification as well as the vesicle in the interior as viewed in ultrathin sections of CV-fraction.

Protein of coated vesicles

Fraction II, CV-fraction and fraction I were analysed electrophoretically for their relative content of clathrin, the major protein of coated vesicles. Fig. 3 is a picture of an overloaded gel; as expected, in CV-fraction the most intensely stained band was clathrin (M_r 180000). Prominent peptides of M_r 100000, 55000, 36000 and 33000 were also present. Consistent with the morphological data, the peptides of M_r 67000, 62000 and 45000 associated with the material excluded from the column (Fraction I) were almost absent from the pattern of CV-fraction, those of M_r 100000, 74000, 60000 and 36000 present in CV-fraction were in minor proportion in the pattern of fraction I, and those in fraction II were essentially the sum of the patterns of CV-fraction and fraction I. Fig. 3 also shows the peptide pattern of a preparation enriched in synaptic plasma membranes (SPM) prepared from bovine brain as described by Whittaker (1964). SPM constitute an abundant

and glycoconjugate-rich membrane fraction in brain (Seminario *et al.*, 1964; Lapetina *et al.*, 1967; Landa *et al.*, 1977) which was considered the most probable potential contaminant of CV-fraction that could invalidate the analysis for the presence of gangliosides and sialosylglycoproteins. Since the SPM-associated peptides of M_r 55000–45000 and of M_r between 33000 and the final position of the tracking dye were undetectable in the pattern of CV-fraction, we concluded that this fraction was not contaminated with membranes representative of the bulk of SPM.

Ganglioside-NeuAc, glycoprotein-NeuAc, cholesterol and phospholipids

Table 1 shows that the concentrations per mg of protein of ganglioside-NeuAc and glycoprotein-NeuAc were, in CV-fraction, about 25% of that determined in SPM; accordingly the ratios between these glycoconjugates were essentially the same in CV-fraction and SPM. On the other hand, the phospholipid and cholesterol concentrations were about 20% and 10% respectively in CV-fraction relative to SPM, which gives a cholesterol-to-phospholipid molar ratio in CV-fraction about 50% of that in SPM. These concentration values should not be directly compared with those in SPM, since approx. 60% of the protein in CV-fraction corresponds to the extrinsic protein clathrin, whereas most of the protein in SPM is most probably membrane-associated. In addition, empty coats may comprise 16–65% of the coated-vesicle preparations obtained by current methods (Woodward & Roth, 1978), thus increasing the fraction of non-membrane protein in CV-fraction. A more realistic and comparable expression of the sialosylglycoconjugate concentrations in the different fractions is to refer them to the phospholipid content of the fractions [ratios (a)/(c) and (b)/(c) in Table 1]. On this basis the concentrations of the sialosylglycoconjugates in CV-fraction and in SPM were essentially the same, which indicates that gangliosides and sialosylglycoproteins are constituents of CV-fraction. Concerning our value for phospholipid/mg of protein in CV-fraction (170 nmol/mg): it was lower than that reported by Pearse (1975), but in close agreement with that reported by Rubenstein *et al.* (1981) after purification of coated vesicles by agarose-gel electrophoresis, an alternative procedure for the removal of contaminant smooth vesicles.

Chromatographic analysis of gangliosides

Fig. 4 shows a thin-layer chromatogram of gangliosides isolated from CV-fraction, Fraction I, Fraction II and SPM. Most of the gangliosides species present in SPM were present in Fraction I, II and CV-fraction. A densitometric analysis

Fig. 2. *Electron micrographs of negatively stained samples of CV-fraction*
CV-fraction was obtained after g.p.c. of the coated-vesicle-enriched fraction as indicated in Fig. 1 (pool of tubes 36–40). The inset shows the vesicular content of a coated vesicle as viewed in ultrathin sections. See the Materials and methods section for details. The bar represents 200nm.

Table 1. *Ganglioside-NeuAc, glycoprotein-NeuAc, phospholipids and cholesterol in CV-fraction and in SPM*

The coated-vesicle-enriched fraction obtained after the third-sucrose-gradient centrifugation described by Pearse (1975), was subjected to controlled-pore g.p.c. as described by Pfeffer & Kelly (1981) to obtain a highly purified CV-fraction as indicated in the Materials and methods section. A fraction enriched in SPM was prepared from bovine brain essentially by the method of Whittaker (1964). The fractions were analysed for their contents of phospholipids, cholesterol and NeuAc bound to gangliosides and to glycoproteins. For methodological details, see the Materials and methods section. Values are means $\pm$ S.E.M. for the number of experiments indicated in parentheses. In each experiment determinations were carried out in duplicate. Ratios without S.E.M. were calculated from the corresponding mean values.

Constituent	Fraction . . .	Content (nmol/mg of protein)	
		CV	SPM
(a) Ganglioside-NeuAc		8.0 $\pm$ 1.0 (6)	34.5 $\pm$ 3.0 (8)
(b) Glycoprotein-NeuAc		3.5 $\pm$ 0.1 (6)	14.1 $\pm$ 0.7 (8)
(c) Phospholipid		170.0 $\pm$ 15 (6)	887.0 $\pm$ 68 (8)
(d) Cholesterol		82.0 $\pm$ 2.6 (6)	954.0 $\pm$ 97 (8)
Ratio			
(a)/(b)		2.3 $\pm$ 0.1 (6)	2.4 $\pm$ 0.1 (8)
(d)/(c)		0.47 $\pm$ 0.07 (6)	1.06 $\pm$ 0.08 (8)
(a)/(c)		0.047	0.039
(b)/(c)		0.020	0.016

Fig. 3. SDS/polyacrylamide-gel electrophoresis of fractions after g.p.c. and of SPM

G.p.c. fractions obtained as indicated in Fig. 1 and the SPM fraction prepared as indicated in the Materials and methods section were analysed electrophoretically (see the text for details). Track 1, fraction II (100 µg); 2, CV-fraction (150 µg); 3, fraction I (50 µg); 4, CV-fraction (50 µg); 5, SPM (120 µg). M_r standards used (not shown) were: bovine brain clathrin, 180000; bovine serum albumin, 67000; bovine brain tubulin, 55000; porcine stomach pepsin, 34700; human α -globulin 43000 (heavy chain) and 23000 (light chain).

showed that the percentage contribution of each one of the major gangliosides was similar among these fractions (an average of about 5, 15, 45, 10 and 25% for GD_3 , GM_1 , GD_{1a} , GD_{1b} and GT_1 respectively); perhaps a difference between SPM and CV-fraction or fractions I and II was that in SPM the percentage ratio of GD_3 to GM_1 was about 0.3 and in the other fractions it was about 0.85.

ATPase activity

It was considered worthwhile to determine whether, in addition to sialosylglycoconjugates, other SPM markers, such as $(Na^+ + K^+)$ -dependent ATPase, were present in CV-fraction. A previous search revealed a Ca^{2+} -dependent ATPase in coated-vesicle preparations (Blitz *et al.*, 1977), but it was subsequently reported that this activity belongs to contaminating smooth membranes (Rubenstein *et al.*, 1981). The last-mentioned authors, however, did not include Na^+ ions

Fig. 4. T.l.c. of gangliosides isolated from fractions after g.p.c. and from SPM

Gangliosides were isolated from g.p.c. fractions obtained as indicated in Fig. 1. For details on the isolation of gangliosides and on the chromatographic procedure see the Materials and methods section. Track 1, CV-fraction; 2, fraction I; 3, fraction II; 4, SPM. The position of the different gangliosides was established by comparison with the mobility of standard gangliosides run simultaneously (not shown).

in their assays, which according to Hosie (1965) are necessary for enzyme activity. Table 2 shows that, relative to SPM, and on a protein or phospholipid basis, CV-fraction showed activity values of about 30 or 150% respectively for the ouabain-sensitive $(Na^+ + K^+)$ -dependent ATPase and of about 20 or 100% respectively for the ouabain-resistant Mg^{2+} -dependent ATPase. Our value for the ouabain-sensitive activity in CV-fraction [approx. 60% of the $(Mg^{2+} + Na^+ + K^+)$ -dependent ATPase activity] was higher than that of approx. 25% reported recently by Forgac *et al.* (1983) in calf brain coated vesicles, which, in addition, contain an ATP-dependent ouabain-resistant proton pump.

Ganglioside glycosyltransferase activities

An experiment was carried out to assess whether CV-fraction has intrinsic ganglioside glycosyltransferase activities or are contaminated by Golgi-

Table 2. *ATPase activity in CV-fraction and in SPM*

Fractions obtained as indicated in Table 1 were analysed for their ATPase activity in the presence and in the absence of ouabain under the conditions indicated in the Materials and methods section. Values for activity expressed per mg of protein are means $\pm$ S.E.M. for two independent experiments, each one carried out in duplicate. Values for activity expressed per nmol of phospholipid resulted from the ratio between the mean values of activity per mg of protein and the mean value for phospholipid per mg of protein given in Table 1.

Fraction	$10^{-2} \times$ ATPase activity ($\mu\text{mol of P}_i/\text{h}$ per nmol of phospholipid)		ATPase activity ($\mu\text{mol of P}_i/\text{h}$ per mg of protein)	
	Ouabain-sensitive	Ouabain-resistant	Ouabain-sensitive	Ouabain-resistant
CV	1.04	0.50	1.78 ± 0.12	0.86 ± 0.08
SPM	0.63	0.44	5.60 ± 0.90	3.90 ± 0.17

Table 3. *Ganglioside glycosyltransferase activities in the coated-vesicle-enriched fraction after the third sucrose-gradient centrifugation in SPM and in neuronal perikarya*

Brain cortices from 17-day-old rats were processed separately to obtain coated vesicles (Pearse, 1975), synaptic plasma membranes (Whittaker, 1964) and neuronal perikarya (Sellinger *et al.*, 1971). The enzymic assays and determination of radioactivity of sugar transferred to the corresponding acceptors were as previously described in preparations of rat brain SPM and neuronal perikarya (Maccioni *et al.*, 1978; Landa *et al.*, 1979, 1981). SPM and neuronal perikarya were assayed 3 days after they had been prepared, the time required for the preparation of the coated-vesicle-enriched fraction. Values for activity expressed per mg of protein are means $\pm$ S.E.M. for one experiment carried out in duplicate; those expressed per nmol of phospholipid resulted from the ratio between the mean values of activity per mg of protein and the mean value for phospholipid content per mg of protein given in Table 1. Abbreviation used: N.D., not detected.

Glycosyltransferase	Activity		
	Neuronal perikarya	SPM	Coated-vesicle-enriched fraction
$\text{GM}_3 \xrightarrow{\text{CMP-NeuAc}} \text{GD}_3$			
(c.p.m./h per mg of protein)	593 ± 18	208 ± 15	200 ± 1.7
(c.p.m./h per nmol of phospholipid)	5.98	0.236	1.17
$\text{GM}_2 \xrightarrow{\text{UDP-Gal}} \text{GM}_1$			
(c.p.m./h per mg of protein)	415 ± 35	N.D.	117 ± 38
(c.p.m./h per nmol of phospholipid)	4.09	0.01	0.68
$\text{GM}_3 \xrightarrow{\text{UDP-GalNAc}} \text{GM}_2$			
(c.p.m./h per mg of protein)	1166 ± 246	139 ± 51	210 ± 110
(c.p.m./h per nmol of phospholipid)	11.70	0.15	1.23

derived membranes. This is a difficult problem in brain, owing to the lack of procedures to obtain a highly purified Golgi fraction as a reference. We compared the activities in the 'coated vesicle-enriched fraction' with those of rat brain SPM and neuronal perikarya, whose activities we have already established were low and high respectively (Maccioni *et al.*, 1977, 1978; Landa *et al.*, 1979, 1981). Table 3 shows that, on a protein basis, the activities in the 'coated vesicle-enriched fraction' were as low as in SPM, with values ranging between 12 and 35% of those in the neuronal perikaryon fraction. In terms of the phospholipid content of each fraction under comparison, the activities in the 'coated vesicle-enriched fraction' were between 10 and 20% and those of SPM between 1

and 4% of those in neuronal perikarya. These values should be lowered by a factor of about 3 if the comparison is made with a Golgi-enriched fraction prepared from neuronal perikarya as described previously (Landa *et al.*, 1981).

Discussion

This is to our knowledge the first attempt to investigate the presence of gangliosides and sialosylglycoproteins in coated vesicles from brain. The coated-vesicle fraction we obtained appeared pure either by morphological or electrophoretic criteria. Although this preparation contained gangliosides and sialosylglycoproteins, their concentration in the vesicles of CV-fraction was

difficult to ascertain. Expression on the basis of protein, although useful for most purification procedures, has some limitations when fractions of quite different characteristics are compared. Expressing the concentration of the well-known membrane components we are investigating in terms of phospholipids, an ubiquitous and quantitatively important membrane component, gave concentration values for gangliosides and sialosylglycoproteins in CV-fraction similar to those in SPM; most SPM gangliosides were present in CV-fraction, although the relative concentration of GD₃ to GM₁ was higher in CV-fraction than in SPM.

Since CV-fraction was highly purified, the comparable concentration of sialosylglycoconjugates precludes the possibility that all of them belong to contaminating SPM. It may be considered that CV-fraction is composed entirely of empty coats, and that the lipid components measured in the fraction belong to contaminating plasma membrane; theoretically, with about 25% of contaminating plasma membrane, it would result in a ganglioside and sialosylglycoprotein concentration in CV-fraction similar to that quoted in Table 1. This is not likely, however, for the following reasons: first, the presence of coated vesicles in the fraction was shown by electron-microscopic examination of ultrathin sections, and was also recognized by the presence of coated structures with electron-transparent cores in negatively stained samples; second, the cholesterol-to-phospholipid ratio was lower in CV-fraction (about 50%) than in SPM, which in addition argues for the intracellular origin of CV-fraction membranes; and third, the presence of peptides of M_r 100000 and 55000, which are considered to be vesicle-associated (Moskowitz *et al.*, 1983), argues for the presence of coated vesicles in CV-fraction. The choice of ouabain-sensitive ($\text{Na}^+ + \text{K}^+$)-dependent ATPase as a marker for SPM did not prove helpful in answering this question. This activity was present in CV-fraction in a proportion relative to SPM similar to that of sialosylglycoconjugates, so that the same arguments apply to the distribution of this activity, which would seem intrinsic to coated vesicles. Another possibility was that the glycoconjugates belonged to smooth-surfaced membranes co-sedimenting with coated vesicles through the sucrose-gradient-purification steps and not totally removed by g.p.c. The likelihood of uncomplete removal of these membranes by the chromatographic step was discarded, however, since the protein electrophoretic pattern differs in fraction I and CV-fraction.

On the basis of current information, it is possible that most coated vesicles of a brain preparation come from nerve endings. However, the possibility

of a contribution, to some extent, by coated vesicles originating from coated regions of the endoplasmic reticulum and Golgi cisternae, or already present in the cell bodies, is worthy of consideration, since we know that Golgi-derived vesicles contain occluded gangliosides (Maccioni *et al.*, 1974, 1977; Landa *et al.*, 1981). The analysis of glycosyltransferase activities showed minor activities of these Golgi-apparatus marker enzymes in the coated-vesicle-enriched-fraction. This indicates that coated pits from Golgi apparatus do not massively contribute coated vesicles to the CV-fraction of brain, or, conversely, that glycosyltransferases are segregated from Golgi-coated regions.

Taken together, our results indicate that coated vesicles share gangliosides and sialosylglycoproteins with the SPM. These results constitute a first attempt to answer the question as to which, if any, SPM constituents are excluded from the microdomains from which coated vesicles originate, probably during recycling coupled to the process of neurotransmitter release (Heuser & Reese, 1973) or any other membrane-associated events involving exchange between plasma and intracellular membranes.

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