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Natural Occurrence of Ganglioside Lactones

ISOLATION AND CHARACTERIZATION OF G_{D1b} INNER ESTER FROM ADULT HUMAN BRAIN*

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A new ganglioside containing an inner ester linkage was extracted from adult brain specimens, obtained at the time of surgery on 51–70-year-old subjects, purified, and analyzed. It contains glucose, galactose, *N*-acetylgalactosamine, an *N*-acetylneuraminic acid in the molar ratio 1:2:1:2, but, on ion-exchange chromatography, behaves as a monosialoganglioside.

Structural analyses showed its basic neutral glycosphingolipid core to be ganglio-*N*-tetraose ceramide, carrying a disialosyl residue on the 3-position of internal galactose. Sialidase degradation and chemical analysis of the products obtained after alkaline treatments suggested one sialic acid residue to be involved in an ester linkage.

Fast atom bombardment-mass spectrometry indicated the presence of an inner ester linkage between the carboxyl group of the external sialic acid residue and a hydroxyl group of the internal one.

On these bases, the new ganglioside can be assumed to be a G_{D1b} in lactonic form. This ganglioside is present only in trace amounts in the brain of infants, but its content increases with age, reaching a value of 3.5% of total sialic acid in 51–70-year-old subjects.

Gangliosides are sialic acid-containing glycosphingolipids that reside in the plasma membranes of vertebrate cells (1); their oligosaccharide chains, protruding from the cell surface, are assumed to be directly involved in membrane recognition phenomena and in the process of intercellular communication (2). The sialic acid residue(s) of gangliosides may play a fundamental role in these events. In fact, the sialic acid carboxyl groups are dissociated at physiological pHs, leading to the presence of negative charges on the membrane surface. The number of negative charges provided by gangliosides may vary either due to a change in number of sialic acids/ganglioside molecule or to the formation of inner ester linkages involving the carboxyl group, with the maintenance of the sialic acid residues.

The possible esterification of the carboxyl group of a sialic acid with one of the hydroxyl groups present in the ganglioside molecule, with the concomitant formation of a ganglioside lactone, was proposed by Kuhn and Muldner in 1964 (3) and investigated in the following years. Wiegandt (4) reported that gangliosides containing a sialic acid residue in $\alpha2\rightarrow8$ linkage with a second sialic acid could be expected to form lactone structures. Afterwards, Evans and McCluer (5) postulated the presence of an inner ester of G_{M3}[1] ganglioside in extracts from bovine adrenal glands. The same authors observed that some of the sialic acid residues present in mouse and rat brain gangliosides were labile in alkaline media and reducible with sodium borohydride (6). This led to the hypothesis of a natural occurrence of ganglioside derivatives, in which the sialic acid carboxyl group was involved in the formation of inner (lactones) or external esters. However, there are still no reports in the literature providing a definite proof of the existence of ganglioside lactones *in vivo*.

In this paper we produce definite evidence of the presence, in nature, of a ganglioside in lactonic form; this ganglioside, provisionally coded $G_{D1b\text{-}L}$, is a derivative of G_{D1b} and has been isolated and purified from specimens of adult human brain.

EXPERIMENTAL PROCEDURES[2]

RESULTS AND DISCUSSION

The two-dimensional TLC analysis of gangliosides from adult human cerebral cortex is reported in Fig. 1; the relative diagrammatic representation together with the codes recently introduced (7) are also given. The methodology allows the recognition of alkali-labile gangliosides which, after interme-

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[1] The abbreviations used are: G_{M3}, NeuAcα2→3Galβ1→4Glcβ1→1′Cer; G_{M2}, GalNAcβ1→4(NeuAcα2→3)Galβ1→4Glcβ1→1′Cer; G_{M1}, Galβ1→3GalNAcβ1→4(NeuAcα2→3)Galβ1→4Glcβ1→1′Cer; Fuc-G_{M1}; Fucα1→2Galβ1→3GalNAcβ1→4(NeuAcα2→3)Galβ1→4Glcβ1→1′Cer; GalNAc-G_{M1}; GalNAcβ1→4Galβ1→3GalNAcβ1→4(NeuAcα2→3)Galβ1→4Glcβ1→1′Cer; G_{D3}; NeuAcα2→8NeuAcα2→3Galβ1→4Glcβ1→1′Cer; G_{D1a}; NeuAcα2→3Galβ1→3GalNAcβ1→4(NeuAcα2→3)Galβ1→4Glcβ1→1′Cer; G_{D1b}; Galβ1→3GalNAcβ1→4(NeuAcα2→8NeuAcα2→3)Galβ1→4Glcβ1→1′Cer; Fuc-G_{D1b}; Fucα1→2Galβ1→3GalNAcβ1→4(NeuAcα2→8NeuAcα2→3)Galβ1→4Glcβ1→1′Cer; G_{T1b}; NeuAcα2→3Galβ1→3GalNAcβ1→4(NeuAcα2→8NeuAcα2→3)Galβ1→4Glcβ1→1′Cer; G_{Q1b}, NeuAcα2→8NeuAcα2→3Galβ1→3GalNAcβ1→4(NeuAcα2→8NeuAcα2→3)Galβ1→4Glcβ1→1′Cer; Cer, ceramide; HPTLC, high performance thin-layer chromatography; TLC, thin-layer chromatography; GLC, gas-liquid chromatography; GC-MS, gas chromatography-mass spectrometry; FAB-MS; fast atom bombardment-mass spectrometry.

[2] Portions of this paper (including "Experimental Procedures," Tables I–IV, and Figs. 1–6) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 85M-4185, cite the authors, and include a check or money order for $3.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

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diate ammonia treatment, originate spots lying far from the diagonal where the alkali-stable gangliosides are located. Among the major components one spot, coded F1, originates after ammonia treatment from an alkali-labile ganglioside, coded G_{D1b-L}. During the first chromatographic run G_{D1b-L} migrates in the region between G_{M1} and G_{D1a} and, therefore, can be easily confused with other gangliosides (like G_{D3}, Fuc-G_{M1} or GalNAc-G_{M1}) migrating in that area, when analyzed by conventional mono-dimensional TLC. The TLC R_F of F1, originated from G_{D1b-L}, does not correspond to any of putative parent gangliosides present in human brain, it being slightly lower than that of G_{D1a}. Moreover, a second spot, coded I1 (present in low amounts) and showing the same R_F of standard G_{D1b} in the second run, can be detected on the vertical corresponding to G_{D1b-L} after ammonia treatment. An identical chromatographic behavior was observed when G_{D1b} lactone (8), prepared semisynthetically, was submitted to the same two-dimensional TLC analysis. In that case, the first spot formed by ammoniolysis (and possibly corresponding to F1) was proven to be the amide derivative of G_{D1b} (G_{D1b}-amide), while the second one (which might correspond to I1) was the parent G_{D1b} originated by hydrolysis. On the basis of these behavioral similarities, we posed the working hypothesis that G_{D1b-L} might be G_{D1b} ganglioside in lactonic form occurring naturally in animal brain.

Following this hypothesis we began collecting evidence of the natural occurrence of this compound and then determined its chemical structure. Human brain ganglioside quantification was achieved by densitometric scanning of the two-dimensional TLC plates after ammonia treatment. Since in these conditions G_{D1b-L} is mainly (>90%) transformed into the F1 spot, G_{D1b-L} content values were obtained from F1 ones. G_{D1b-L} content was found to be strictly correlated with the age of the subject (see Table I) and increased from 0.16% in the 0–10-year group to 3.48% in the 51–70-year group. The differences in the age groups were statistically significant. When analyzed in parallel, G_{D1b} ganglioside increased from 7.85% in the 0–10-year group to 20.29% in the 11–30-year group, remaining nearly constant thereafter. These data are in good agreement with previous literature reports (9–11). As shown in Table II the presence of G_{D1b-L} (detected as F1) has already been reported in ganglioside mixtures extracted both from mammalian and avian brains (8, 38), where it ranged from 0.5 to 1.9% of total lipid-bound sialic acid. It should be noted that in all the investigated species, including human, no linear relationship between the content of G_{D1b-L} and that of G_{D1b} was present. In fact, in all the analyzed mixtures the ratio between G_{D1b-L} and G_{D1b} is quite variable (Tables I and II). All this makes unlikely the hypothesis of any artifactual and spontaneous transformation (possibly lactonization) of G_{D1b} into G_{D1b-L}; this process should originate constant G_{D1b-L}/G_{D1b} ratios, not depending on the G_{D1b} content in the tissue.

The possible artifactual formation of G_{D1b-L} throughout the procedure used for extraction and purification of gangliosides was checked. To this purpose specimens of human cerebral cortex were submitted in parallel to ganglioside extraction and purification by tetrahydrofuran/phosphate buffer or chloroform/methanol (12) as extracting solvent systems. F1 was present in the mixtures obtained by both procedures, thus suggesting the absence of the tetrahydrofuran/phosphate buffer extraction in originating G_{D1b-L} as a non-natural ganglioside derivative.

Moreover, samples of pure G_{D1b}, a mixture of standard gangliosides, and an alkali-treated sample (0.1 M NaOH in methanol, room temperature, overnight) of human brain ganglioside mixture were kept in tetrahydrofuran/phosphate buffer solution at room temperature for 24 h and then submitted to two-dimensional TLC analysis. In all cases neither F1 nor other alkali-labile gangliosides were shown to be present.

All this indicates that G_{D1b-L} is not artifactually originated but constitutes an authentic product of brain metabolism. To date, it cannot be stated if such a compound is formed via specific enzymatic action or by the establishment of chemically favorable physiological conditions. Since G_{D1b-L} content is higher in old rather than in young subjects, it was isolated from a pool of human brain ganglioside mixtures (8.2 mg, as lipid-bound sialic acid) from subjects aged 51–70 years. Fig. 2 shows the relative fractionation by silica gel column chromatography. After purification, 200 μg of G_{D1b-L} (as sialic acid) corresponding to a yield of 70% were obtained. The purity of the compound, assessed as described under "Experimental Procedures," was higher than 96%. Compositional analyses revealed that G_{D1b-L} contained glucose, galactose, *N*-acetylgalactosamine, *N*-acetylneuraminic acid, long chain base, and fatty acid in a molar ratio (referred to glucose) of 1.00:2.05:0.92:1.87:1.11:0.98 (theoretical, 1:2:1:2:1:1) (see Table III). Partial acid hydrolysis, permethylation, and chromium trioxide analyses indicated for G_{D1b-L} a structure with the neutral glycolipid core Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→1′Cer, in which the hydroxyl group at position 3 of the inner galactose unit is linked to a NeuAcα2→8NeuAcα2→ disialosyl residue. This structure corresponds to that of G_{D1b} ganglioside. Therefore, the structural difference between G_{D1b} and G_{D1b-L} has been cancelled by both the acidic conditions required for sugar and lipid analyses and the alkaline conditions used for permethylation analysis. In addition, when G_{D1b-L} was treated with NaOH, it changed its chromatographic behavior and migrated exactly as G_{D1b} ganglioside (Fig. 3); no difference was found in the chemical composition between G_{D1b-L}/NaOH and G_{D1b} (see Table III). Instead, when G_{D1b-L} was submitted to ammonia treatment, a compound with a TLC R_F intermediate between G_{D1b-L} and G_{D1b} was formed (Fig. 3). The lipid and neutral saccharide composition of G_{D1b-L}/NH_3 was the same as that of G_{D1b} (see Table III) while one of the two sialic acid residues had a chemical structure different from that of *N*-acetylneuraminic acid. In fact, when released by mild acid methanolysis and derivatized as Me_3Si, it displayed a GLC retention time (1.75) different from that corresponding to standard *N*-acetylneuraminic acid (stated as 1.00). The mass spectrometry spectrum of this particular sialic acid derivative is reported in Fig. 4. The spectrum, which shows a molecular ion M^+ minus CH_3 at m/z 595, and a series of ions as those at m/z 327, 405, and 283 differing 15 units from those obtained from the standard derivative of sialic acid (13) can be correlated to the amide derivative of *N*-acetylneuraminic acid, as proposed in the same figure. The only possibility for the formation of an amide derivative after ammonia treatment of G_{D1b-L} is the involvement of a sialic acid carboxyl group in an ester linkage. In fact, in the presence of ammonia, this linkage undergoes a process of ammoniolysis with the concurrent formation of the amide derivative. Of course, these results are consistent with the presence of either an inner or an external ester.

The behavior of G_{D1b-L} on DEAE-Sepharose column chromatography was the following. G_{D1b-L} was retained by the column in the course of the methanol-washing step, as expected for all gangliosides, and was eluted with 4 mM sodium acetate, the molarity being used to elute monosialogangliosides from the resin. Conversely, G_{D1b} as well as other disialogangliosides processed in parallel were eluted from the column using 50 mM sodium acetate (Fig. 5). This means that, in spite

of the presence of two sialic acid residues in the molecule, G_{D1b-L} behaves as a monosialoganglioside indicating that only one sialic acid carboxyl group is actually available for interacting with the resin.

Upon sialidase treatment, G_{D1b-L} was resistant to the enzyme action, in contrast with G_{D1b} and G_{D1b-L}/NaOH which were completely converted to G_{M1} under the same conditions. It is worth remembering that a prerequisite for sialidase action is the presence of a free carboxyl group on the sialic acid residue (14). Therefore, the resistance of G_{D1b-L} to sialidase, as opposed to the susceptibility of G_{D1b}, suggests that the chemical difference within the two substances concerns the external sialic acid residue.

The results hitherto reported unequivocally postulate the presence in the G_{D1b-L} molecule of an esterification of the sialic acid carboxyl group. The direct proof that G_{D1b-L} contains an ester linkage and that the ester linkage involves an intramolecular alcoholic function was provided by fast atom bombardment-mass spectrometry analysis. The fast atom bombardment-mass spectrometry spectrum of G_{D1b-L} is reported in Fig. 6, together with the scheme indicating the major pathways of fragmentation. The most significant ions were the pseudomolecular ions M-1, corresponding to the molecular species that contain C18 and C20 sphingosine, observed at m/z 1817 and 1845, 18 units below those of G_{D1b} measured under identical conditions (15) and the ions at m/z 581 and 537, deriving from the disialosyl residue minus 18 (H_2O) and to 581 minus CO_2, respectively. The loss of 18 units, corresponding to a molecule of water, in the G_{D1b-L} molecule with respect to G_{D1b}, indicates that the ester linkage involves one of the ganglioside hydroxyl groups. Moreover, the presence of ions corresponding to a sialosyl residue still lacking 18 units indicating that the lacton ring resides in the same disialosyl residue. As in the spectrum there are hardly any significant peaks at 308 and 290 m/z, corresponding to sialic acid, we can postulate that the ester linkage involves the carboxyl group of the external sialic acid and one of the hydroxyl groups of the internal sialic acid residue. By the use of molecular models it could be hypothesized that the hydroxyl groups in positions 7 or 9 are involved. On the basis of all the provided evidence it can be suggested that G_{D1b-L} is a G_{D1b} carrying an inner ester linkage between the carboxyl group of the external sialic acid residue and a hydroxyl group of the internal sialic acid residue.

The composition of human brain gangliosides has been extensively studied; however, to the best of our knowledge, the present study is the first to report the presence, in human brain, of a ganglioside in lactonic form. In a search for plausible explanations for such an omission the following considerations can be made. The inner ester of G_{D1b-L} is rather unstable under alkaline conditions; therefore, in both preparative and analytical steps, conservative methodologies must be used so as to avoid exposure to alkaline conditions. Moreover, the TLC detection of G_{D1b-L}, as well as of similar compounds, requires the use of proper solvent systems to avoid co-migrations with the major gangliosides. The 17 most relevant reports (9–11, 16–29) since 1968 on human brain ganglioside composition, together with the conditions employed for ganglioside extraction and analysis, are reported in Table IV. In nine of these (11, 17, 18, 20, 22, 24, 26, 28, 29) alkaline (NaOH and KOH) methanolysis or hydrolysis was used to get rid of contaminant phospholipids in the course of the ganglioside purification; under these conditions G_{D1b-L} is completely converted into G_{D1b}. In other reports (16, 19, 23, 27), the analysis of the ganglioside mixture was performed on TLC by the use of an ammonia-containing solvent system. This system converts the esterified carboxyl group partly into an amide and partly into a free carboxyl group, the ratio between the two derivatives depending on the ammonia molarity. G_{D1b}-amide migrates on TLC similarly to G_{D1a}, thus becoming indistinguishable. Vanier *et al.* (9) did not employ alkaline conditions; however, they missed the detection of G_{D1b-L} because of the use of a TLC solvent system (propanol/water), in which G_{D1b-L} co-migrates with G_{M1}. Finally, Fredman *et al.* (25) did not find G_{D1b-L} in their investigation on the brain of an infant (3 months) because of the very low G_{D1b-L} content at this age (Table I). According to the present survey, only Iwamori and Nagai (21) and Svennerholm and Fredman (10) could have detected the presence of lactonized ganglioside derivatives as the experimental conditions and the starting tissues were optimal for this purpose. In both papers the possible presence of ganglioside lactones was mentioned; however, the authors did not focus their attention on the natural occurrence of these compounds or their artifactual formation.

In conclusion, the present report provides conclusive evidence of the natural occurrence, in human brain, of a G_{D1b} ganglioside carrying an inner ester linkage. This ganglioside is also present in the brain of other animal species, and it is most likely that other ganglioside lactones occur in human as well as in other vertebrate brain tissue.

The biological significance of lactonized gangliosides is open to inspection. Of course the possibility of reducing the number of negative charges without changing the number of sialic acid residues exposed on the cell surface may be specifically important in some membrane recognition phenomena and intercellular events. It has been postulated, for instance, that there is involvement of the sialic acid negative charge with the binding of Ca^{2+} ions and the process of neurotransmitters release at nerve terminals (30, 31). In this regard it would be important to ascertain the existence of any enzymatically assisted process of both formation and hydrolysis of the lactone ring as a mechanism for prompt and reversible control of the negative charges provided by gangliosides on the cell surface.

REFERENCES

1. Wiegandt, H. (1982) *Adv. Neurochem.* **4,** 149–223
2. Brady, R. O. & Fishman, P. H. (1979) *Adv. Enzymol. Relat. Areas Mol. Biol.* **50,** 303–323
3. Kuhn, R. & Muldner, J. (1964) *Naturwissenschaften* **51,** 635–636
4. Wiegandt, H. (1966) *Ergeb. Physiol. Biol. Chem. Exp. Pharmakol.* **57,** 190–222
5. Evans, J. E. & McCluer, R. H. (1971) *Fed. Proc.* **30,** 1133 (Abstr.)
6. Gross, S. K., Williams, M. A. & McCluer, R. H. (1980) *J. Neurochem.* **34,** 1351–1361
7. Riboni, L., Malesci, A., Gaini, S. M., Sonnino, S., Ghidoni, R. & Tettamanti, G. (1984) *J. Biochem. (Tokyo)* **96,** 1943–1946
8. Sonnino, S., Ghidoni, R., Chigorno, V., Masserini, M. & Tettamanti, G. (1983) *Anal. Biochem.* **128,** 104–114
9. Vanier, M. T., Holm, M., Ohman, R. & Svennerholm, L. (1971) *J. Neurochem.* **18,** 581–592
10. Svennerholm, L. & Fredman, P. (1980) *Biochim. Biophys. Acta* **617,** 97–109
11. Segler-Stahl, K., Webster, J. L. & Brunngraber, E. G. (1983) *Gerontology* **29,** 161–168
12. Suzuki, K. (1965) *J. Neurochem.* **12,** 629–638
13. Kamerling, J. P. & Vliegenthart, J. F. G. (1982) in *Sialic Acid, Chemistry, Metabolism and Function* (Schauer, R., ed) pp. 95–125, Springer-Verlag, New York
14. Corfield, A. P. & Schauer, R. (1982) in *Sialic Acid, Chemistry, Metabolism and Function* (Schauer, R., ed) pp. 195–261, Springer-Verlag, New York
15. Egge, H., Katalinic, J. P., Reuter, G., Schauer, R., Ghidoni, R., Sonnino, S. & Tettamanti, G. (1985) *Chem. Phys. Lipids* **37,** 127–141
16. Siddiqui, B. & McCluer, R. H. (1968) *J. Lipid. Res.* **9,** 366–370
17. Vanier, M. T., Holm, M., Månsson, J. E. & Svennerholm, L.

(1973) *J. Neurochem.* **21,** 1375–1384
18. Ando, S. & Yu, R. K. (1977) *J. Biol. Chem.* **252,** 6247–6250
19. Yusuf, H. K., Merat, A. & Dickerson, J. W. T. (1977) *J. Neurochem.* **28,** 1299–1304
20. Ando, S., Chang, N.-C. & Yu, R. K. (1978) *Anal. Biochem.* **89,** 437–450
21. Iwamori, M. & Nagai, Y. (1978) *J. Biochem. (Tokyo)* **84,** 1601–1608
22. Ueno, K., Ando, S. & Yu, R. K. (1977) *J. Lipid Res.* **19,** 863–871
23. Iwamori, M. & Nagai, Y. (1979) *J. Neurochem.* **32,** 767–777
24. Yu, R. K. & Iqbal, K. (1979) *J. Neurochem.* **32,** 293–300
25. Fredman, P., Nilsson, O., Tayot, J.-L. & Svennerholm, L. (1980) *Biochim. Biophys. Acta* **618,** 42–52
26. Itoh, T., Li, Y.-T., Li, S.-C. & Yu, R. K. (1981) *J. Biol. Chem.* **256,** 165–169
27. Kraĉun, I., Rösner, H., Ćosović, C. & Stavljenić, A. (1984) *J. Neurochem.* **43,** 979–989
28. Traylor, T. D. & Hogan, E. L. (1980) *J. Neurochem.* **34,** 126–131
29. Ledeen, R. W., Yu, R. K. & Eng, L. F. (1973) *J. Neurochem.* **21,** 829–839
30. Svennerholm, L. (1980) *Adv. Exp. Med. Biol.* **125,** 533–544
31. Rahmann, H. (1983) *Neurochem. Int.* **5,** 539–547
32. Tettamanti, G., Bonali, F., Marchesini, S. & Zambotti, V. (1973) *Biochim. Biophys. Acta* **296,** 160–170
33. Ghidoni, R., Sonnino, S., Tettamanti, G., Baumann, N., Reuter, G. & Schauer, R. (1980) *J. Biol. Chem.* **255,** 6990–6995
34. Ghidoni, R., Sonnino, S., Wiegandt, H. & Zambotti, V. (1976) *J. Neurochem.* **27,** 511–515
35. Sonnino, S., Ghidoni, R., Galli, G. & Tettamanti, G. (1978) *J. Neurochem.* **31,** 947–956
36. Marusyk, R. & Sergeant, A. (1980) *Anal. Biochem.* **105,** 403–404
37. Yu, R. K. & Ledeen, R. W. (1970) *J. Lipid. Res.* **11,** 506–516
38. Chigorno, V., Sonnino, S., Ghidoni, R., Toffano, G., Venerando, B. & Tettamanti, G. (1980) *Neurochem. Int.* **6,** 191–197
39. Chigorno, V., Sonnino, S., Ghidoni, R. & Tettamanti, G. (1982) *Neurochem. Int.* **4,** 397–403
40. Svennerholm, L. (1957) *Biochim. Biophys. Acta* **24,** 604–611
41. Miettinen, J. & Takki Luukkainen, J. T. (1959) *Acta Chem. Scand.* **13,** 856–858

NATURAL OCCURRENCE OF GANGLIOSIDE LACTONES: ISOLATION AND CHARACTERIZATION OF GD1b INNER ESTER FROM ADULT HUMAN BRAIN

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EXPERIMENTAL PROCEDURES

Chemicals

Silica gel for column chromatography (Kieselgel 100, 0.063-0.2 mm, 70-230 mesh) and silica gel precoated thin layer plates (HPTLC, Kieselgel 60, 250 μm thick, 10x10 cm) were from Merck GmbH, Darmstadt, West Germany. DEAE-Sepharose CL-6B was from Pharmacia, Fine Chemicals, Uppsala, Sweden. Vibrio cholerae sialidase (acetylneuraminyl glycohydrolase, EC 3.2.1.18) from Behringwerke, Marburg, West Germany. N-acetylneuraminic acid (NeuAc) was from Sigma Chemical Co., St. Louis, MO, USA. Reagents for GLC were from C. Erba, Milano, Italy. VSWP filters, 0.025 μm pore size were from Millipore, Bedford, Ma, USA. Standard gangliosides, GM3, GM2, GM1, GD3, Fuc-GM1, GD1a, GD1b, Fuc-GD1b, GT1b and GQ1b were extracted according to Tettamanti et al. (32), purified and chemically characterized as described by Ghidoni et al. (33). GM3 was extracted from human spleen, GM2 from a Tay-Sachs brain, the others from pig brain. Semisynthetic GD1b lactone was prepared according to Sonnino et al. (8).

Human brain material

Samples of cerebral cortex weighing 0.3-1.0 g were obtained at the time of surgery from 44 patients aged from 3 months to 70 yr, and operated on for different intracranial pathologies. The tissue materials employed were those which had to be removed for that particular operation. In each case, a small specimen was taken from the area located near the pathologically involved tissue and ascertained to be free of either macroscopical or histological abnormalities.

Isolation and purification of GD1b-L

Extraction and purification of the crude ganglioside mixture from each sample of human cerebral cortex was attained as described by Tettamanti et al. (32). The ganglioside distribution was assessed in each individual sample by two dimensional TLC with intermediate ammonia treatment (see below). Afterwards, in order to isolate and purify the GD1b-L ganglioside, the ganglioside mixtures extracted from the cerebral cortex of 12 patients aging 51-70 yr. were pooled; a total of 8.2 mg of lipid bound sialic acid was obtained from 10 g of fresh tissue. The isolation was achieved by column chromatography on a Silica Gel 100 column (54.0 cm x 0.8 cm) equilibrated and eluted with the solvent system chloroform/methanol/water, 60:35:2, by vol at 18-20°C and at a flow rate of 40 ml/h. The total volume for a complete elution was about 1000 ml, 2 ml fractions being automatically collected. The elution pattern was monitored by TLC as specified below. The fractions containing GD1b-L were collected, evaporated under N_2 flow at 4°C, and re-chromatographed under the above conditions. The homogeneous material, thus obtained, was stored in chloroform/methanol, 1:1, by vol, at -20°C. The purity of the isolated ganglioside was assessed by chemical analyses (see below).

Chemical composition and structural analyses

The following compositional and structural analyses were performed: a)- determination of the carbohydrate and lipid composition; b)- partial acid hydrolysis followed by TLC recognition of the formed neutral glycosphingolipids; c)- permethylation analysis for the determination of sugar linkage position; d)- anomeric analysis. The experimental details of the above analytical methods have been given in previous papers (32,34,35).

Alkaline treatments

GD1b-L was submitted to alkaline treatment according to the following procedures: a)- ammonia vapors, exhaustively dried through soda line, were bubbled into a GD1b-L solution (10 μg/0.1 ml in dehydrated chloroform/methanol, 1:1 by vol) for an hour. At the end of the reaction, the solution was dried under a stream of nitrogen and the reaction products were assayed by TLC and GLC-MS analyses (see below). The product obtained after ammonia treatment of GD1b-L was coded GD1b-L/NH_3 ; b)- GD1b-L was dissolved in 0.5 M NaOH (10 μg/0.1 ml) and kept overnight at room temperature. At the end of the reaction the solution was dialyzed on VSWP filters (36) for 1 h against 200 ml of distilled water and the reaction products were then analyzed by TLC (see below). The product obtained after NaOH treatment of GD1b-L was coded GD1b-L/NaOH.

Ion exchange chromatography

GD1b-L (30 μg) was poured on a DEAE-Sepharose CL-6B column (3 cm x 0.3 cm, sedimented gel) (11) and the elution was carried out with methanol (10 ml), followed by 4 mM (10 ml) and 50 mM (10 ml) sodium acetate in methanol. One ml fractions were automatically collected and the elution profile was checked by TLC (see below).

Sialidase treatment

Sialidase treatment was performed as follows: the incubation mixture (50 μl final volume) containing 5 μg of GD1b, GD1b-L or GD1b-L/NaOH, 2 mU of enzyme, 10^{-3} M $CaCl_2$, 0.05 M sodium acetate buffer, pH 5.5, was maintained at 37°C for varying times (up to 1 h). At the end of the incubation the mixture was dialyzed (against 200 vol. of distilled water, 4°C, for 3 h) through VSWP filters and the reaction products analyzed by TLC (see below).

Gas chromatography - mass spectrometry (GC-MS)

GC-MS analysis of the Me_3Si derivatives of the sialic acids released by mild acid methanolysis (37) from GD1b-L, were performed on a Varian Mat 112 S (Varian, Bremen, West Germany) gas chromatograph-mass spectrometer coupled with a PDP 11/34 data system. The operating conditions were as follows: the GLC column was 200 cm x 0.2 cm, packed with OV1 on 100-200 mesh Gas Chrom P (Supelco, Bellefonte, Pa, USA); temperature: 230°C; helium flow rate: 20 ml/min; transfer line, separator and ion source temperature: 270°C; filament current: 60 μA; electron energy: 70 eV; accelerating voltage: 3.5 KV; resolution: 1000.

Fast atom bombardment-mass spectrometry (FAB-MS)

FAB-MS was performed on a ZAB HF reversed geometry mass spectrometer (V.G. Analytical, Manchester, UK). The sample (5 μg) dissolved in 1 μl of methanol/glacial acetic acid, 1:1 by vol was applied to a matrix of 1-mercapto-2,3-propanediol. The sample was ionized by bombardment with xenon atoms having a kinetic energy equivalent to 9 KeV. Spectra were recorded routinely on UV sensitive paper in a mass controlled down field scan of 200 s duration and evaluated by counting the spectral lines.

Thin layer chromatography and densitometric quantification of gangliosides

a)Human brain ganglioside mixture was analyzed for its ganglioside composition by the two dimensional TLC system, with intermediate ammonia treatment, previously reported (9,14,15). This system is capable of recognizing the presence of alkali labile gangliosides.
b) The monitoring of the chromatographic purification of GD1b-L, the assessment of GD1b-L homogeneity, and the products formed by the different treatments of GD1b-L were followed by monodimensional TLC on HPTLC plates using the solvent system of chloroform/methanol/0.2% aqueous $CaCl_2$, 50:42:11 by vol (20°C, run time 40 min).
Gangliosides were detected on the plate by spraying it with a p-dimethylaminobenzaldehyde reagent followed by heating at 120°C for 10 min and quantified by densitometry according to Chigorno et al. (39), with the improvement that the beam width and consequently the scan shift were reduced to 0.2 mm, thus providing better resolution.

Colorimetric methods

Ganglioside bound sialic acid was determined by the resorcinol-HCl method (40) as modified by Miettinen and Takki-Luukkainen (41).

TABLE I. Content of GD1b-L and GD1b in cerebral cortex of humans of different ages.

	Age groups			
	0-10 (3)[a]	11-30 (10)	31-50 (19)	51-70 (12)
GD1b-L[b]	0.16±0.06[c]	0.49±0.16	1.90±0.09	3.48±0.36
GD1b	7.85±0.34	20.29±1.43	23.28±1.40	20.94±1.87
GD1b-L/GD1b	0.020	0.024	0.082	0.166

[a] Number of cases in parentheses.
[b] GD1b-L has been quantified as F1 spot (see Fig. 1), the main derivative of GD1b-L after ammonia treatment.
[c] Ganglioside content is expressed as % ± S.D. of total lipid-bound sialic acid. Each sample was analyzed in duplicate.

TABLE II. Content of GD1b-L and GD1b in some mammalian and avian brain.

	Pig	Rabbit	Rat	Mouse	Pigeon
GD1b-L[a]	0.5	1.9	0.7	0.5	1.1
GD1b[a]	10.5	8.8	12.3	7.5	4.6
GD1b-L/GD1b	0.048	0.220	0.056	0.067	0.239

[a] Ganglioside content is expressed as % of total lipid-bound sialic acid.

TABLE III. Chemical composition of GD1b-L and of its derivatives after sodium hydroxide (GD1b-L/NaOH) and ammonia (GD1b-L/NH_3) treatments.

	Lipid and carbohydrate composition (molar ratio Glc=1.00)						
	Glc	Gal	GalNAc	NeuAc	NeuAc-NH_2[a]	l.c.b[b]	fatty acid
GD1b-L	1.00	2.05	0.92	1.87	-	1.11	0.98
GD1b-L/NaOH	1.00	1.98	0.91	1.94	-	0.96	1.00
GD1b-L/NH_3	1.00	1.94	0.99	1.01	0.92	1.03	1.10

	Long chain base composition (%)				Fatty acid composition (%)			
	e18:1	e18:0	e20:1	e20:1	16:0	18:0	18:1	20:0
GD1b-L	48.4	2.8	45.3	3.5	3.3	92.0	1.1	3.6

[a] NeuAc-NH_2; N-acetylneuraminamide.
[b] l.c.b.; long chain base.

Fig. 1. Two-dimensional TLC analysis of gangliosides from adult human cerebral cortex and relative diagramatic representation.
The sample refers to the temporal lobe of a 66-yr old subject. 5 μg of total lipid-bound sialic acid were spotted. At the plate edges, monodimensional TLC of standard gangliosides are displayed. In both directions, from the lowest polar gangliosides, the sequence corresponds to: GM3, GM2, GM1, GD3, GD1a, GD1b, Fuc-GD1b, GT1b, and GQ1b. For experimental details see the text

Table IV. Main literature reports on human brain gangliosides.

Source - Age	Alkaline hydrolysis during ganglioside purification	Presence of ammonia in the TLC solvent system	GD1b-L detection	Ref.
Human brain - adult (age not specified)	no	yes	not possible	16
Human cerebral cortex and frontal white matter - developmental study from prenatal life to 30 yr	no	no (propanol/water)	not possible	9
Human cerebral cortex and white matter - 2.2-27 mo	yes	no	not possible	17
Human brain - 57 yr	yes	no	not possible	18
Human forebrain, cerebellum and brain stem - developmental study from prenatal life to 26 mo	no	yes	not possible	19
Human grey and white matter - 57 yr	yes	no	not possible	20
Human whole brain - 35 yr	no	no	possible (detected)	21
Myelin from human white matter - age not reported	yes	no	not possible	22
Normal and Tay-Sachs human brain - 2.5 and 5 yr	no	yes	not possible	23
Oligodendroglia and myelin from human white matter, and neurons from human cerebral cortex - 25, 35 and 86 yr	yes	no	not possible	24
Human forebrain and cerebellum - 3 mo	no	no	absent in the source	25
Human forebrain - 2 mo-72 yr	no	no	possible (detected)	10
Tay-Sachs human brain - age not reported	yes	no	not possible	26
Human whole brain - developmental study from 25 to 85 yr	yes	yes	not possible	11
Several human brain regions - 40-50 yr	no	yes	not possible	27
Human brain - age not reported	yes	no	not possible	28
Human myelin, white and grey matter - 5.5-50 yr	yes	no	not possible	29

Fig. 2. Fractionation of human brain cerebral cortex on silica gel column.
In the first two lanes, standard ganglioside mixture (a) and human brain ganglioside mixture (b) were spo

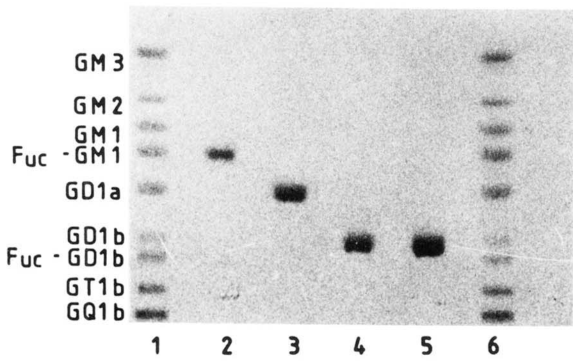

Fig. 3. Alkaline treatment of GD1b-L.
Lanes 1 and 6, standard ganglioside mixture; lane 2, purified GD1b-L; lane 3, GD1b-L after ammonia treatment ($GD1b-L/NH_3$); lane 4, GD1b-L after sodium hydroxide treatment (GD1b-L/NaOH); lane 5, standard GD1b. For experimental details see the text.

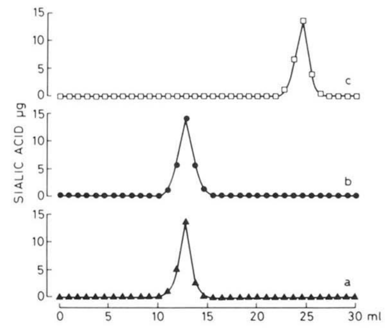

Fig. 5. Behavior of GD1b-L (a), standard GM1 (b) and standard GD1b (c) on an ion-exchange DEAE-Sepharose column chromatography.
For experimental details see the text.

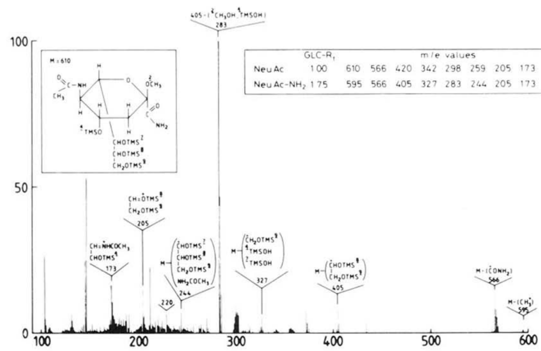

Fig. 4. Mass spectrum of the sialic acid derivative, released by mild acid methanolysis from $GD1b-L/NH_3$ and derivatized as Me_3Si.
The GLC retention times ($GLC-R_t$) and the significant fragmentation ions, referred to those of N-acetylneuraminic acid, are given in the insert.

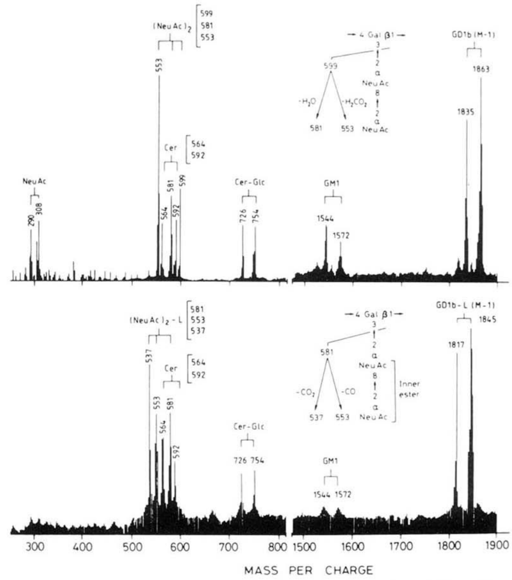

Fig. 6. Negative ion FAB-MS analysis of native standard GD1b (a) and GD1b-L (b).
Schemes indicating the major pathways of fragmentations are also given.