Further Studies on the Gangliosidic Nature of the Cholinergic-Specific Antigen, Chol-1

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  The antigen designated as Chol-1β, detected by an antiserum specific for cholinergic neurons, has been purified to homogeneity from ganglioside mixtures extracted from *Torpedo* electric organ and pig brain. The final products from the two sources behaved identically in a wide range of tests and gave coincident immunopositive and Ehrlich-positive spots after thin layer chromatography in seven different solvent systems; they were thus considered to be identical and to constitute a single, pure chemical species. Gas-chromatographic analysis revealed the presence of long-chain bases, glucose, galactose, N-acetylgalactosamine, and sialic acid in integral molar ratios of 1:1:2:1:3; the compound's reactivity to cholera toxin after *Vibrio cholerae* sialidase treatment on thin layer chromatography and the recovery of GM1 as sole product of exhaustive sialidase treatment identified it as a member of the gangliotetrahexosyl series. From the products of partial enzymatic desialylation and treatment with β-galactosidase and a comparison of the compound's immunoreactivity to anti-Chol-1 antisera with that of other trisialogangliosides of defined molecular structure, we were able to assign a disialosyl residue α-Neu5Ac-(2 → 8)-α-Neu5Ac-(2 → 3)- to the inner galactose, and we suggest GalNAc as a possible site of linkage of the third sialic acid. © 1990 Academic Press, Inc.

This paper describes the partial characterization of a cholinergic-specific antigen (4–12), designated Chol-1β, from pig brain and *Torpedo* electric organ.

Mammalian cortical synaptosomes derived from cholinergic nerve terminals express on their surface anti-gens specific for this class of terminals; these cholinergic-specific antigens were discovered when antisera raised in sheep to isolated presynaptic plasma membranes of the cholinergic electromotor nerve terminals of *Torpedo marmorata* were found to possess the ability to induce the selective complement-mediated lysis of the cholinergic subpopulation of mammalian brain synaptosomes (4, 5). These antigens, designated generically Chol-1, were found to be present in large numbers in the ganglioside fraction extracted from *Torpedo* electric organ (5, 6) and as two species, designated Chol-1α and Chol-1β, in the ganglioside mixture from mammalian brain (7–9, 13). Furthermore, the anti-Chol-1 titer of the antiserum could be greatly increased by affinity purification on immobilized *Torpedo* electric organ gangliosides (8). This was considered as an indication of the gangliosidic nature of the Chol-1 antigens. The affinity-purified antiserum has been shown specifically to recognize cholinergic neurons in the central nervous system of the rat (9), and the cholinergic subpopulation of rat brain synaptosomes has been successfully affinity purified by means of an anti-Chol-1 antiserum (10, 11).

We have purified one of these antigens, Chol-1β, and partially characterized its structure by use of chemical and enzymatic methods.

MATERIALS AND METHODS

Materials. Commercial chemicals were of analytical grade or of the highest purity available. Solvents were distilled before use and water was freshly redistilled in a glass apparatus. Silica gel 100 (0.063–0.200 mm, 70–230 mesh, ASTM) and silica gel 60 (0.063–0.200 mm, 70–230 mesh, ASTM) for column chromatography and silica gel precoated thin layer plates (HPTLC,[2] Kieselgel 60, aluminium sheets) were pur-

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[2] Abbreviations used: Ganglioside nomenclature is in accordance with Svennerholm, 1980 (1) and IUPAC-IUB recommendations, 1977, 1982 (2, 3). Neu5Ac, N-acetylneuraminic acid; Cer, ceramide; GM2,

chased from Merck (FRG). DEAE–Sephadex A-25 for ion exchange column chromatography was from Pharmacia Fine Chemicals, Sweden. Bovine serum albumin, N-acetylneuraminic acid, and Jack bean β-galactosidase, type V, were from Sigma. Vibrio cholerae sialidase was from Behringwerke (FRG); Vibrio cholerae toxin B subunit conjugated to horseradish peroxidase was from List Biological Laboratories. Dialysis filters type WSVP, pore size 0.025 μm, were from Millipore. Sheep serum anti-Chol-1 was raised as previously described (5). Choline and [^{3}H]acetylCoA for the inhibition of the complement lysis test were purchased from Sigma. Peroxidase-labeled rabbit anti-sheep IgG for immunostaining was from Cappel Laboratories (FRG). Ganglioside standards GM2, GM1, GD1a, GD1b, GT1b, and GQ1b were prepared and characterized as previously described (15). GD1α, extracted and purified from rat ascites hepatoma AH 7974F cells, was a generous gift from Dr. Taki, Shizuoka College of Pharmacy, Shizuoka-Shi, Japan.

Whole pig and calf brains, removed at the slaughterhouse just after death, were freed of meninges and blood vessels, cut into thin slices, washed in a cold isotonic solution, frozen, and lyophilized. Specimens of the electric ray Torpedo marmorata were obtained at the Station Biologique d'Arcachon (France); electric organs were removed and stored in dry ice until extracted. The brains of domestic fowl and rat were removed after decapitating the animals and immediately submitted to ganglioside extraction. Human brain was obtained from the Department of Neurosurgery, University of Milan. The total ganglioside mixtures from fish brains were provided by Dr. N. Avrova, Leningrad, USSR.

Gangliosides were extracted from the various sources according to the tetrahydrofuran-phosphate buffer procedure (16).

Purification of Chol-1β antigen. Chol-1β antigen was extracted and purified from 100 kg of pig brain and from 40 kg of Torpedo electric organ. The crude gangliosides containing 800 mg of sialic acid were mixed with 10 g of silica gel 60 and applied as a dry powder to a silica gel 60 column (160 × 12 cm) then eluted with the solvent system propan-2-ol/32% aqueous NH$_4$OH, 7/3 by volume. The elution profile and subsequent chromatography fractions were monitored by TLC stained chemically and immunochemically (see below). Fractions containing the TLC immunopositive spot located immediately under GT1b and corresponding to Chol-1β as previously defined were collected, dried, and purified by four chromatographic runs on a silica gel 100 column (90 × 1 cm) equilibrated and eluted with chloroform/methanol/water, 60/35/8 by volume. Finally, the purified immunoreactive compound was applied to an ion exchange DEAE-Sephadex A-25 column (40 × 2 cm) and eluted as previously described (17).

II^{3}Neu5AcGgOse$_3$Cer, β-GalNAc-(1→4)-[α-Neu5Ac-(2→3)]-β-Gal-(1→4)-β-Glc-(1→1)-Cer; GM1, II^{3}Neu5AcGgOse$_4$Cer, β-Gal-(1→3)-β-GalNAc-(1→4)-[α-Neu5Ac-(2→3)]-β-Gal-(1→4)-β-Glc-(1→1)-Cer; GD1a, IV^{3}Neu5AcII^{3}Neu5AcGgOse$_4$Cer, α-Neu5Ac-(2→3)-β-Gal-(1→3)-β-GalNAc-(1→4)-[α-Neu5Ac-(2→3)]-β-Gal-(1→4)-β-Glc-(1→1)-Cer; GD1α, IV^{3}Neu5AcIII^{6}Neu5AcGgOse$_4$Cer, α-Neu5-Ac-(2→3)-β-Gal-(1→3)-[α-Neu5Ac-(2→6)]-β-GalNAc-(1→4)-β-Gal-(1→4)-β-Glc-(1→1)-Cer; GD1b, II^3(Neu5Ac)$_2$GgOse$_4$Cer, β-Gal-(1→3)β-GalNAc-(1→4)-[α-Neu5Ac-(2→8)-α-Neu5Ac-(2→3)]-β-Gal-(1→4)-β-Glc-(1→1)-Cer; GT1a, IV^3(Neu5Ac)$_2$II^{3}Neu5-AcGgOse$_4$Cer, α-Neu5Ac-(2→8)-α-Neu5Ac-(2→3)-β-Gal-(1→3)-β-GalNAc-(1→4)-[α-Neu5Ac-(2→3)]-β-Gal-(1→4)-β-Glc-(1→1)-Cer; GT1b, IV^{3}Neu5AcII^3(Neu5Ac)$_2$GgOse$_4$Cer, α-Neu5Ac-(2→3)-β-Gal-(1→3)-β-GalNAc-(1→4)-[α-Neu5Ac-(2→8)-α-Neu5Ac-(2→3)]-β-Gal-(1→4)-β-Glc-(1→1)-Cer; GT1c, II^3(Neu5Ac)$_3$GgOse$_4$Cer, β-Gal-(1→3)-β-GalNAc-(1→4)-[α-Neu5Ac-(2→8)-α-Neu5Ac-(2→8)-α-Neu5Ac-(2→3)]-β-Gal-(1→4)-β-Glc-(1→1)-Cer; GQ1b, IV^3(Neu5Ac)$_2$II^3(Neu5Ac)$_2$GgOse$_4$Cer, α-Neu5Ac-(2→8)-α-Neu5-Ac-(2→3)-β-Gal-(1→3)-β-GalNAc-(1→4)-[α-Neu5Ac-(2→8)-α-Neu5Ac-(2→3)]-β-Gal-(1→4)-β-Glc-(1→1)-Cer; TLC, thin-layer chromatography; HPTLC, high performance thin layer chromatography; POD, horseradish peroxidase; ChAT, choline acetyltransferase.

TLC and TLC staining. The ganglioside mixtures were separated on 8-cm long HPTLC plates developed at room temperature with the following solvent systems: chloroform/methanol/0.2% aqueous CaCl$_2$, (a) 50/42/11 and (b) 40/50/15 by vol; chloroform/methanol/water, (c) 25/55/20, (d) 25/55/25, and (e) 30/50/20 by vol; (f) propan-2-ol/32% NH$_4$OH/water, 6/2/1 by vol; and (g) chloroform/methanol/32% NH$_4$OH, 60/35/8 by vol. Ganglioside spots were made visible by treatment with a p-(dimethylamino)benzaldehyde spray reagent (Ehrlich's reagent), specific for sialic acid, followed by heating at 120°C for 10 min (18), or by immunostaining with anti-Chol-1 serum (8) or the B subunit of cholera toxin (19) before or after treating the TLC plates with sialidase.

Inhibition of complement lysis. Free antigen, by combining with antiserum, reduces the effectiveness of the latter in inducing the complement-mediated lysis of the mammalian cholinergic subpopulation of synaptosomes. The amount of ganglioside sialic acid, in micrograms required to bring about 40% inhibition of ChAT release under standard conditions (I_{40}) was used as a reciprocal measure of the purity of the antigen. I_{40} rather than I_{50} values were used because the more highly purified antigen preparations failed to cause more than 60% inhibition for reasons discussed below. The procedure was as previously described (8) except that synaptosomes from rat brain were used instead of those from guinea-pig brain.

Enzymatic hydrolysis of Chol-1β. Chol-1β (5 μg as sialic acid) in a final volume of 100 μl of 10 mM sodium acetate buffer, pH 5.5, was exhaustively hydrolyzed at 37°C for 10–15 h with 50 mU of sialidase. For partial hydrolysis, 5 μg of Chol-1β as sialic acid was treated with 1 to 3 mU of sialidase from 5 min to 15 h.

Treatment of Chol-1β with β-galactosidase was performed by incubating 5 μg of Chol-1 as sialic acid, or 15 μg of pure GM1 or GT1b with 0.3 units of Jack bean β-galactosidase in 0.05 M citrate buffer, pH 4.0, in the presence of 0.1% taurodeoxycholate, in a final volume of 10 μl, and for 10–15 h according to previously described conditions (20).

At the end of each enzymatic hydrolysis, the reaction mixture was dialyzed for 3 h on a 0.025-μm filter and analyzed by TLC using solvent system (a).

Gas–liquid chromatographic analysis. Chol-1β (20 μg as sialic acid) was treated at 80°C for 20 h in a screw-capped tube with 0.5 M anhydrous methanolic HCl. Fatty acid methyl esters were extracted with hexane and analyzed in a 25-m long S 30 capillary column whose temperature was raised 10°C·min^{-1} from 180 to 280°C, followed by 30 min at 280°C.

The O-methylglycosides and the long-chain bases released from Chol-1β, which were present in the methanolic phase, were analyzed together as trifluoroacetyl-derivatives on a 5% OV-210 glass column whose temperature was raised 2°C min^{-1} from 120 to 220°C followed by 30 min at 220°C. Under these experimental conditions, long-chain base derivatives were the last to leave the column. Saccharides and long-chain bases released by standard GM1 ganglioside were used as reference standards. Details have been previously reported (15).

Colorimetric procedures. Ganglioside-bound sialic acid was assayed by the resorcinol-HCl method (21, 22), pure Neu5Ac serving as the reference standard.

RESULTS

Figure 1 shows the immunoreactive pattern, after staining with the anti-Chol-1 serum, of gangliosides from the electric organ of Torpedo and from the brain of various animal species. The Torpedo electric organ ganglioside mixture shows several immunoreactive bands, but the most immunoreactive ones do not correspond to any of the major spots detectable by Ehrlich's reagent. With decreasing amounts of the Torpedo ganglioside mixture (down to 0.01 μg of sialic acid applied on a

GM2
GM1
Fuc-GM1
GD1a

GD1b
FucGD1b
GT1b
GQ1b

1 2 3 4 5 6 7   8 9
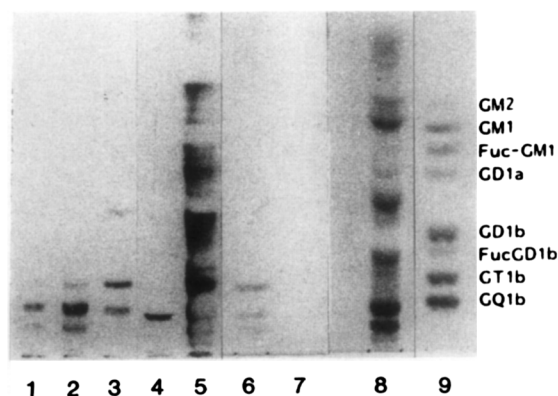
FIG. 1. TLC separation of ganglioside mixtures extracted from different animal species and tissues. Lanes 1–4, brains of 1 ox; 2, calf; 3, pig; 4, fowl; lanes 5 and 6, *Torpedo* electric organ: 5, 5 μg; 6, 0.01 μg as sialic acid; lane 7, standard gangliosides. Staining with anti-Chol-1 serum. Lane 8, *Torpedo* electric organ; lane 9, standard gangliosides. Staining with Ehrlich's reagent. The chromatograms were developed in solvent system (a).

4-mm line), only compounds having an R_f close to GT1b and GQ1b standard gangliosides remain reactive to the antiserum, in particular a spot located immediately under GT1b.

Compounds reacting with the anti-Chol-1 serum are also present, even if in much smaller amounts, in gangliosides from the brain of various animal species, such as ox, calf, pig, fowl, rat, human. Depending on the species under investigation, one, two, or three immunoreactive spots can be detected in the lower zone of the TLC where polysialogangliosides are usually located. By the contrary some fish species, like cod (*Gadus callarias*), dogfish (*Squalus acanthias*) and bass (*Dichentracus labrax*), tested for the immunoreactivity of their gangliosidic fraction, known to be rich in gangliosides of the "c" series, do not show any reactivity to the anti-Chol-1 serum, in the TLC area where polysialogangliosides are usually located.
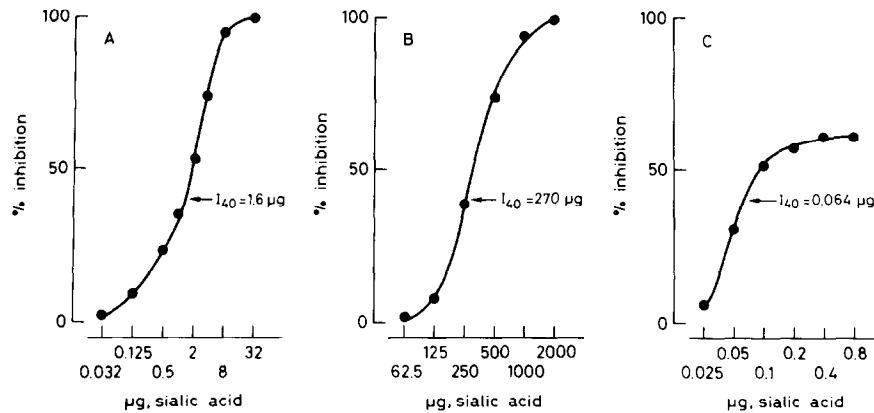
In the pig-brain ganglioside mixture, one immunoreactive band, present in such low quantity as not to be visible with Ehrlich's reagent, behaves chromatographically like the strongly immunoreactive spot migrating under GT1b in the ganglioside mixture from *Torpedo* electric organ (Fig. 1). This immunoreactive spot was coded as Chol-1β (9).

During the purification procedures Chol-1β displayed very unusual behavior in that it (a) was absorbed and retained by the silica gel of the chromatographic columns and (b) stuck to the glass flasks making it necessary to use large volumes of solvents to solubilize it. This caused a large loss of Chol-1β during the purification process and analytical procedures, decreasing the final yield of the Chol-1β. Nevertheless, after repeated chromatographic runs, we were able to obtain 100 and 40 μg (as sialic acid) of the purified antigen from the pig brain

and *Torpedo* electric organ ganglioside mixtures respectively. The total ganglioside mixture from pig brain and from *Torpedo* electric organ and the purified Chol-1β have been tested in the inhibition of complement lysis test (Fig. 2). It can be seen that using purified Chol-1β 100% inhibition is not achieved: since below 50% inhibition the inhibition was proportional to the logarithm of the antigen concentration, the I_{40} was considered to be a reliable measure of the inhibitory capacity of the ganglioside fractions. The I_{40} values are 270 and 1.6 μg for the total ganglioside mixture of pig brain and of *Torpedo* electric organ, respectively, and 0.064 μg for purified Chol-1β; thus the purified material was respectively over 4000 and 25 times more effective than a similar quantity of pig-brain and *Torpedo* electric organ gangliosides. The latter value is not surprising in that many other immunoreactive components able to inhibit the lysis of cholinergic synaptosomes are present in the total electric organ ganglioside mixture (6). The molar purification factor in *Torpedo* was separately determined by quantification of Chol-1β on the basis of the reactivity to POD-conjugated cholera toxin after neuraminidase treatment (see below). Analysis of the densitometric response of Chol-1β in the total ganglioside mixture of *Torpedo* electric organ allowed us to determine that Chol-1β is present in the total mixture in a proportion of about 1/1500.

The antigen purified from pig brain showed the same chemical and biochemical behavior, in a wide range of tests, as that from *Torpedo* electric organ. By TLC, using seven solvent systems, the two antigens showed the same chromatographic behavior, giving a single coincident Ehrlich's reagent positive, anti-Chol-1 immunoreactive and sialidase-cholera toxin reactive spot (Fig. 3). They had the same potency in inhibiting the complement lysis of synaptosomes and behaved identically under enzymatic treatment (see below). On the basis of these results, the products purified from pig brain and from *Torpedo* electric organ were considered to have the same oligosaccharide chemical structure and to be pure Chol-1β.

Gas-chromatographic analysis of Chol-1β revealed (Fig. 4) the presence of glucose, galactose, N-acetylgalactosamine, sialic acid, and long-chain bases in the molar ratio relative to glucose of 1.00:1.85:0.85:2.81:1.11, which should correspond to theoretical ratios of 1:2:1:3:1. Long-chain bases were present in the spectrum as a number of peaks corresponding to long-chain and O-methyl-long-chain base derivatives formed during methanolysis of Chol-1β, performed under the anhydrous conditions necessary for sugar analysis (23). The presence of the long-chain bases in the molecule identifies the gangliosidic chemical structure of Chol-1β. Fatty acids, separately analyzed, showed the following molar composition: $C_{18:0}$, 72%; $C_{16:0}$, 17%; $C_{20:0}$, 7%; $C_{18:1}$, 4%.

FIG. 2. Percentage inhibition of complement lysis of synaptosomes from rat brain in the presence of (A, B) total ganglioside mixture from (A) *Torpedo* electric organ, (B) pig brain, (C) purified Chol-1β from pig brain. Chol-1β from electric organ behaved similarly.

Exhaustive sialidase treatment (Fig. 5) of Chol-1β ganglioside produced only one product, G-1. Partial hydrolysis of Chol-1β ganglioside with sialidase (Fig. 5) gave three products, G-1, G-2, and G-3. G-1 was identified as GM1 from the chromatographic behavior on TLC and from its reactivity to cholera toxin without previous treatment with sialidase. G-2 migrated between GD1a and GD1b, and G-3 had the same R_f as GD1b. G-2 and G-3 obtained by mild sialidase treatment were converted into GM1 on the TLC plate by *Vibrio cholerae* sialidase, thus becoming positive to cholera toxin (data not shown). These data confirmed the gangliotetraosyl core structure of Chol-1β ganglioside.

Treatment of Chol-1β with β-galactosidase gave a product whose R_f on TLC using solvent system (a) in-

creased (Fig. 6). Under the same conditions, GM1 was transformed into GM2, while GT1b was not affected by the enzyme.

DISCUSSION

The cholinergic-specific antigen Chol-1β has been purified to TLC homogeneity, as an Ehrlich-positive, immunoreactive and sialidase-cholera toxin-positive band, from *Torpedo* electric organ and from pig brain, where it has to be considered a minor component of the ganglioside mixtures covering less than 0.1% of the total.

The gas-chromatographic analyses of the purified antigen revealed the presence of fatty acids, long-chain bases, neutral carbohydrates, and sialic acid. This definitely confirms the gangliosidic nature of Chol-1β. Chol-1β belongs to the gangliotetrahexosyl series as proved by the reactivity to cholera toxin after sialidase treatment on TLC and by the recovery of GM1 as final product after exhaustive treatment with sialidase.
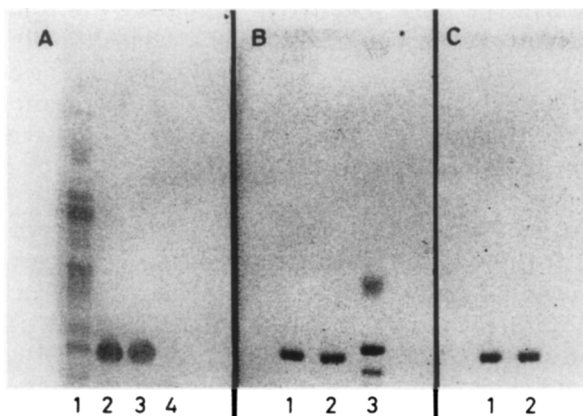
FIG. 3. Chromatographic behavior of Chol-1β from pig brain and from *Torpedo* electric organ. (A) Immunostaining of 1, *Torpedo* electric organ total ganglioside mixture; 2, 3, Chol-1β purified from: 2, pig brain; 3, *Torpedo* electric organ; 4, standard GQ1b, GT1b, GD1α; (B) Ehrlich's reagent staining Chol-1β purified from: 1, pig brain; 2, *Torpedo* electric organ; 3, standard GQ1b, GT1b, GD1α; (C) Sialidase-cholera toxin staining Chol-1β from: 1, pig brain, 2, *Torpedo* electric organ. The chromatograms were developed with solvent system (a).
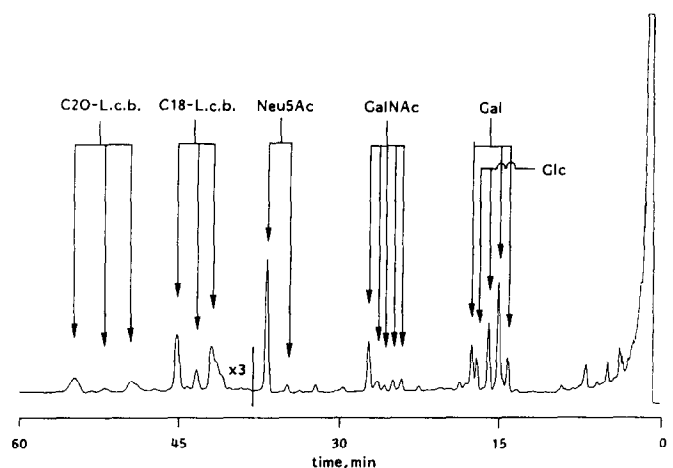

**FIG. 4.** Gas-chromatographic analysis of purified Chol-1β from pig brain. Chol-1β from electric organ behaved similarly.
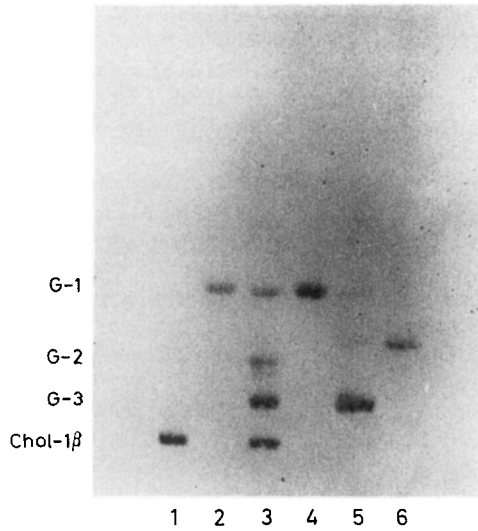
FIG. 5. Lanes 1–3, *Vibrio cholerae* sialidase treatment of Chol-1β from pig brain: 1, control, no treatment; 2, after treatment with sialidase in excess, 10–15 h; 3, after partial enzymatic desialylation; lanes 4–6, standards: 4, GM1, 5, GD1b; 6, GD1a. The TLC was developed with solvent system (a) and the products of enzymatic desialylation were revealed by Ehrlich's reagent.

The number of sialic acid residues detected by gas-chromatographic analysis is three. Chol-1β is then a trisialoganglioside of the gangliotetrahexosyl series which cannot be identified with any of the known trisialogangliosides. By TLC and with the solvent system (a), GT1a has a very different R_f value on TLC (24), while GT1b which is also distinguishable on account of its slightly higher R_f is not immunoreactive. Under the same chromatographic conditions GT1c has a R_f similar to that of Chol-1β but gangliosidic mixtures extracted from cod
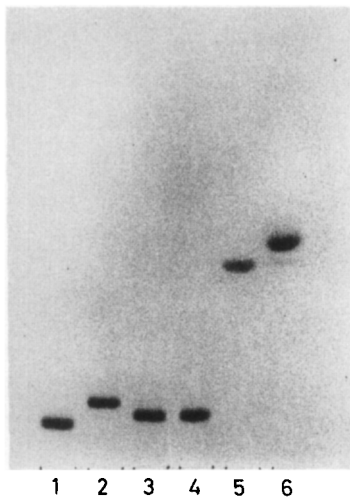
FIG. 6. Jack bean β-galactosidase treatment of, 1, 2, Chol-1β; 3–6, standards; 1, 3, 5, controls, no treatment; 2, 4, 6, after treatment; 3, 4, GT1b; 5, 6, GM1. The procedure was as in Fig. 5.
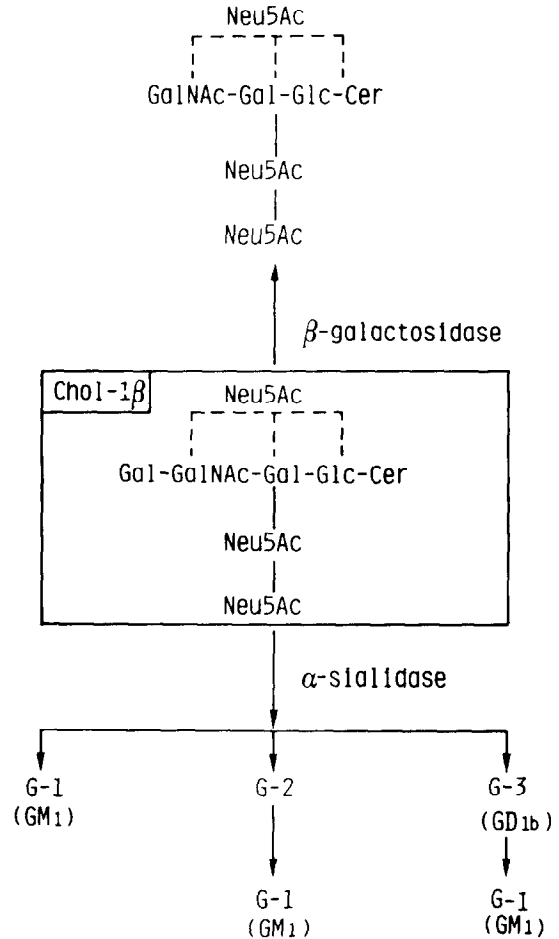
FIG. 7. Proposed scheme for enzymatic degradation of Chol-1β.

(*Gadus callarias*) and bass (*Dichentracus labrax*) brain, which are known to contain it (25, 26), are absolutely not immunoreactive, after TLC separation, in the region where Chol-1β migrates. Partial sialidase hydrolysis yielded two intermediate products: one, coded as G-3, behaves like GD1b and the other, G-2, is located between GD1a and GD1b. Moreover, Jack bean β-galactosidase, which under our experimental conditions works only on gangliosides with no sialic acid residues in position 3 of the external galactose residue, produced from Chol-1β a compound with slightly higher TLC mobility. This suggests that the terminal galactose is likely to be free of sialic acid. From these enzymatic results (see the scheme in Fig. 7), the position of two sialic acid residues can be assigned as in GD1b, which is one of the products of partial desialylation, while for the third we can at least exclude a linkage to the external galactose as in GT1b.

Compound G-2 should be a disialoganglioside, being formed by sialidase action on the trisialoganglioside Chol-1β and being transformed into GM1 by the action of sialidase directly on the plate. Therefore, one sialic acid is linked at position 3 of the inner galactose units

and the other to an unknown position of the ganglioside oligosaccharide portion.

Gangliosides bearing an α-glycosidic linkage between a sialic acid and N-acetylgalactosamine have been reported: GD1α from cancer cells (27) which has been used as reference standard in this work and, very recently, a trisialoganglioside extracted and purified from dogfish (*Squalus acanthias*) brain (28). A noteworthy point, a ganglioside mixture extracted from dogfish brain is not immunoreactive, excluding the possibility of identifying this ganglioside with Chol-1β.

Although GD1α migrates between GD1a and GD1b and thus resembles G-2 in chromatographic behavior, the two gangliosides cannot be identical since, apart from the difference in R_f, GD1α is hydrolyzed to gangliotetrahexosylceramide by the action of sialidase. The disialoganglioside obtained by enzymatic hydrolysis from the trisialoganglioside from *Squalus acanthias* retains the sialic acid residue linked to GalNAc of the parent compound and resembles G-2 chromatographically. These observations suggest that GalNAc could be a possible site for the third sialic acid of Chol-1β.

ACKNOWLEDGMENTS

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