Muscle glycolipids in inherited muscular dystrophy of chickens

Samar K. Kundu*, Lalit K. Misra[+,•] and Madan G. Luthra*,[+]

*Departments of *Medicine, [+]Physiology and •Pediatrics, Baylor College of Medicine, Houston, TX 77030, USA*

Received 14 October 1982

Gangliosides and neutral glycolipids of muscles from normal and dystrophic chickens were studied. Total glycolipid content of the degenerating muscles was higher than the normal muscles. In addition, the myopathic muscles contained a ganglioside which was absent in the unaffected muscles from normal and dystrophic chickens. Based on the thin-layer chromatographic mobility, treatment with neuraminidases from *Vibrio cholerae* and *Arthrobacter ureafaciens*, and reactivity of the asialo-derivative towards anti-ganglio-*N*-triaosylceramide antibody, the dystrophic-specific ganglioside was tentatively identified as G_{M2}. Data obtained from young and old dystrophic chickens suggested a direct relationship of this ganglioside to muscular dystrophy.

Ganglioside *Neutral glycolipid* *Chicken muscular dystrophy* *Dystrophic-specific ganglioside* *G_{M2}* *Degenerating muscle*

1. INTRODUCTION

Increasing evidence indicates that muscular dystrophy is a generalized membrane defect [1]. Glycolipids are vital membrane components and are involved in many biological functions [2–4]. A number of studies concerning the identification of glycolipids in normal muscles have been reported [5–7]. However, involvement of glycolipids in myopathic conditions is not fully explored. Because of the histopathological similarities to human muscular dystrophy, chickens with inherited muscular dystrophy serve as an excellent experimental model [8,9]. In [10] a reduction in total neutral glycolipids was reported, especially Forssman glycolipid in pectoral and bicep muscles of newly-hatched dystrophic chicks compared to their normal counterparts. However, no data on the relationship between these changes to the severity and/or progression of muscular dystrophy was reported. Moreover, no information is available on the sialic acid containing glycolipids, gangliosides, which have been shown to influence myogenesis [11], formation of neuromuscular junctions [12] and nerve generation [13]. To understand the role of gangliosides in muscular dystrophy, we studied their distribution and content in the affected and unaffected synergistic flight muscles of dystrophic chickens. We found that muscles undergoing degeneration contained a ganglioside which was absent in both normal and unaffected muscles. A tentative identification of this dystrophic ganglioside is discussed.

Abbreviations: CMH, galactosyl or glucosylceramide; CDH, lactosylceramide; CTH, globotriaosylceramide; LT, lactotriaosylceramide GlcNAc(β1–3)Gal(β1–4)-Glc-Cer; As G_{M2}, ganglio-*N*-triaosylceramide; PG, lactoneotetraosylceramide Gal(β1–4)GlcNAc(β1–3)Gal-(β1–4)Glc-Cer; As G_{M1}, ganglio-*N*-tetraosylceramide; Forss, Forssman glycolipid; SPG, sialosyllactoneotetraosylceramide NeuAc(α2–3)Gal(β1–4)GlcNAc(β1–3)-Gal(β1–4)Glc-Cer; SNH, sialosyllacto-*N*-*nor*-hexaosylceramide NeuAc(α2–3)[Gal(β1–4)GlcNAc(β1–3)]$_2$Gal-(β1–4)Glc-Cer

The abbreviations for gangliosides of the ganglio series are as in [31]; for ganglioside structures see [32]

Published by Elsevier Biomedical Press
00145793/82/0000—0000/$2.75

2. MATERIALS AND METHODS

2.1. *Animals*

New Hampshire chickens of genetic lines 412 and 413 were obtained from the Department of Avian Sciences, University of California at Davis. These lines were developed from the F_2 generation of a cross between the dystrophic line 304 and normal New Hampshire chickens. Because of common origin, line 412 birds serve as genetically related normal controls to the homozygous dystrophic chickens of line 413. This genetic uniformity between the lines 412 and 413 is important in minimizing the variation due to the background genes, which are known to modify the expression of the single dystrophic gene [14].

Here, chickens at the early (5-week-old) and the advanced (2.5-year-old) stages of muscular dystrophy were used. Righting ability of all birds was evaluated by the exhaustion score method in [15]. In this method, the consecutive number of times a bird could rise from supine position in rapid succession during a single trial is counted. This method is very sensitive and highly reproducible in evaluating the dystrophic condition [16]. The anterior latissimus dorsi (ALD) and posterior latissimus dorsi (PLD) muscles were collected immediately after killing of animals. The PLD (fast muscle) is affected by muscular dystrophy. The ALD (slow muscle) is not affected by muscular dystrophy until 2 years of age [17].

2.2. *Enzymes and antibodies*

Neuraminidase of *Vibrio cholerae* was obtained from Behring Diagnostics (Sommerville NJ) and neuraminidase of *Arthrobacter ureafaciens* was from Boeringer Mannheim Biochemicals (Indianapolis IN). β-Galactosidase and β-*N*-acetylhexosaminidase from jack bean meal were kindly donated by Dr Y.-T. Li of Tulane University. Purified rabbit IgG antibody to lactotriaosylceramide (LT), globotriaosylceramide (CTH), ganglio-*N*-triaosylceramide (As G_{M2}), globotetraosylceramide, ganglio-*N*-tetraosylceramide (As G_{M1}), lactoneotetraosylceramide (PG) and Forssman glycolipid (Forss) have been prepared in this laboratory and well characterized [18,19]. Mouse monoclonal anti-As G_{M2} was a gift from Dr S. Hakomori. Peroxidase-conjugated goat, anti-rabbit and anti-mouse Ig antibodies were obtained from Cappel Laboratories (Cochranville PA).

2.3. *Isolation and quantification of gangliosides and neutral glycolipids*

The details of our procedure for isolation of glycolipids have been described in [20]. In brief, lipids were extracted with chloroform–methanol, 1:1 (v/v), and neutral and acidic lipids were separated on a column of DEAE–Sephadex A-25 (Pharmacia Fine Chemicals, Piscataway NJ). The neutral glycolipids present in neutral lipid fraction were acetylated as in [21] and the ganglioside present in acidic lipid fraction was subjected to alkaline hydrolysis. Salts and other non-lipid contaminants were removed by reversed-phase chromatography on a C_{18} Sep-Pak cartridge developed in [22].

The yield of gangliosides in each preparation was determined by gas–liquid chromatographic (GLC) analysis of the lipid-bound sialic acid [23]. Thin-layer chromatography was performed on precoated silica gel 60 plates (E. Merck Labs., Darmstadt). The thin-layer chromatography of gangliosides was performed with either chloroform–methanol–water, 55:45:10 (by vol.) containing 0.02% (w/v) of $CaCl_2 \cdot 2\,H_2O$ or chloroform–methanol–2.5-N ammonia solution, 60:40:9 (by vol.). The thin-layer chromatography of neutral glycolipids was performed with chloroform–methanol–water mixtures (60:35:4) or (60:35:8) by vol.

2.4. *Enzymatic hydrolysis*

In order to selectively cleave the terminal sialic acid, gangliosides (25–50 μg) were dissolved in 200 μl 50 mM acetate buffer (pH 5.0) containing 2 mM $CaCl_2$ and incubated with 10 units of *V. cholerae* neuraminidase for 24 h at 37°C [24]. Neuraminidase from *A. ureafaciens* was used for the hydrolysis of all sialic acid residues [24,25]. Similar hydrolysis conditions were used except that the acetate buffer contained 1 mg/ml sodium cholate and 100 mU enzyme. The hydrolysis of asialoglycolipids by β-galactosidase (200 mU for 25–50 μg glycolipid) and β-*N*-acetylhexosaminidase (300 mU for 25 μg glycolipid) was carried out in 50 mM citrate buffer (pH 4.4) containing 1 mg/ml sodium taurodeoxycholate for 24 h at 37°C. After incubation with appropriate enzymes, 1 ml chloroform–methanol 1:1 was added and the reaction mixture was dried under nitrogen. The dried sample dissolved in 5 ml water and passed

through a C_{18} Sep-Pak cartridge. The glycolipid sample was eluted with chloroform–methanol 1:2 as in [22] and examined by thin-layer chromatography.

2.5. *Immunoblot procedure for identification of glycolipids*

The procedure used was a modified technique of [26]. In our procedure, glycolipid samples (0.05–0.5 μg) were subjected to thin-layer chromatography on a high-performance aluminium backing plate (E. Merck) in a suitable solvent system as described above. After air drying, the plate was immersed in hexane containing 0.05% poly(isobutylmethylacrylate)-beads (Polysciences, Warrington PA) for 30 s. The plate was then sprayed with 0.02 M phosphate-buffered saline (PBS) (pH 7.3) and soaked in chilled PBS containing 1% bovine serum albumin (BSA) at 4°C for 30 min. Excess buffer was removed from the plate. The purified antibody diluted with PBS–1% BSA (10–50 μg/ml IgG) was then overlayered on the plate and incubated overnight at 4°C. After washing the plate with PBS, the plate was overlayered as before with peroxidase-conjugated goat anti-rabbit Ig antibody (1/300 dilution with PBS–1% BSA) and allowed to incubate for 4 h at 4°C. The plates were washed with PBS, and overlayered with freshly prepared substrate solution (3,3′-diaminobenzidine tetrahydrochloride, 1 mg/ml, in water containing 1 mg/ml imidazole to which 1 μl/ml hydrogen peroxide (30%) had been added) and allowed to react at room temperature. The positive bands appear after 20 min of exposure to the substrate.

3. RESULTS AND DISCUSSION

Total ganglioside content of muscles from normal and dystrophic chickens is summarized in table 1. An increase of ~1.7-fold in the total ganglioside content was seen in PLD muscle of 5-week-old dystrophic chicks as compared to PLD from age-matched normal chicks. In contrast to PLD muscle, unaffected ALD muscle in dystrophic chicks did not show a significant difference in their ganglioside content. Total ganglioside content of PLD muscle of 2.5-year-old chickens was 2.2-times greater than that of normal chickens. The increase in ganglioside content of

Table 1

Concentration of gangliosides in normal and dystrophic chicken muscles[a]

Age	Type of muscles[b]	Normal (μg lipid-bound sialic acid/g wet muscle)	Dystrophic
5 weeks	ALD	8.85	9.05
	PLD	5.4	9.2
2.5 years	ALD	9.55	11.32
	PLD	4.70	10.60

[a]Determined by gas-liquid chromatography [23]
[b]Muscles were pooled from 2–4 chickens in each group

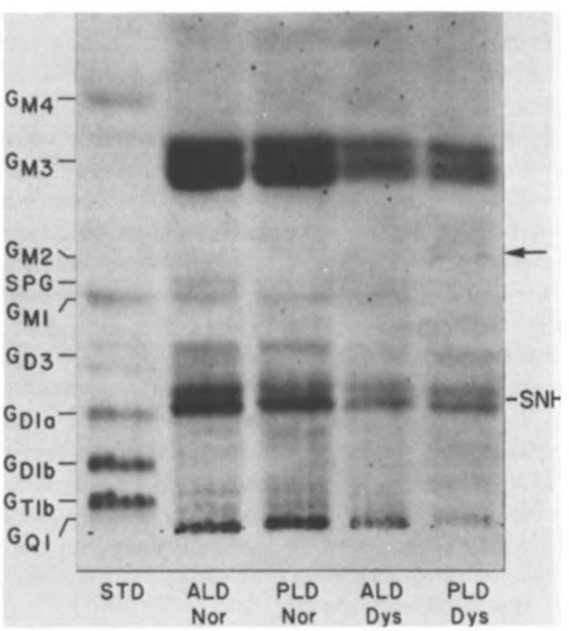

Fig. 1. Thin-layer chromatogram of 5-week-old chicken muscle gangliosides. The solvent was chloroform–methanol–water (60:40:9) containing 0.02% $CaCl_2 \cdot 2\,H_2O$ (w/v) and gangliosides were detected with resorcinol reagent. Lanes ALD-nor contained gangliosides from 0.6 g, PLD-nor from 0.9 g, ALD-dys from 0.19 g and PLD-dys from 0.29 g wet muscles. Lane STD contained standard ganglioside mixture. The arrow in Lane PLD-dys indicates the dystrophic ganglioside; nor, normal; dys, dystrophic. The appearance of 2 bands for the dystrophic ganglioside is due to the heterogeneity in the ceramide moiety. Note that dystrophic ganglioside is absent in normal muscles in spite of a large amount of sample used for thin-layer chromatography separation.

dystrophic PLD muscles is greater in 2.5-year-old chickens compared to young birds. This difference in muscle ganglioside content between young and old dystrophic birds is attributed to the severity of muscle degeneration, which correlated well with their righting ability. A slight increase in total ganglioside content of ALD muscles from 2.5-year-old birds was also observed (table 1). This increase coincides with the degenerative process which begins to affect the ALD muscles by 2 years of age [17]. Furthermore, ganglioside content of dystrophic PLD (fast) increased to a level of ALD (slow) muscles (table 1). Failure in the transition from slow to fast muscle types during embryonic development [27] may explain the similarities observed in ALD and PLD ganglioside content of dystrophic chickens.

Thin-layer chromatography of gangliosides isolated from muscles of 5-week-old chicks and 2.5-year-old chickens are shown in fig. 1 and fig. 2, respectively. The major gangliosides identified are G_{M3}, G_{D3}, SPG and SNH. The identification of G_{M3} and G_{D3} was based on the thin-layer chromatography mobilities of the native gangliosides and their *V. cholerae* neuraminidase hydrolysis product, lactosylceramide (CDH). SPG was identified by comparing its thin-layer chromatography mobility with that of a known standard [20]. The asialoglycolipid obtained from SPG after *V. cholerae* neuraminidase treatment

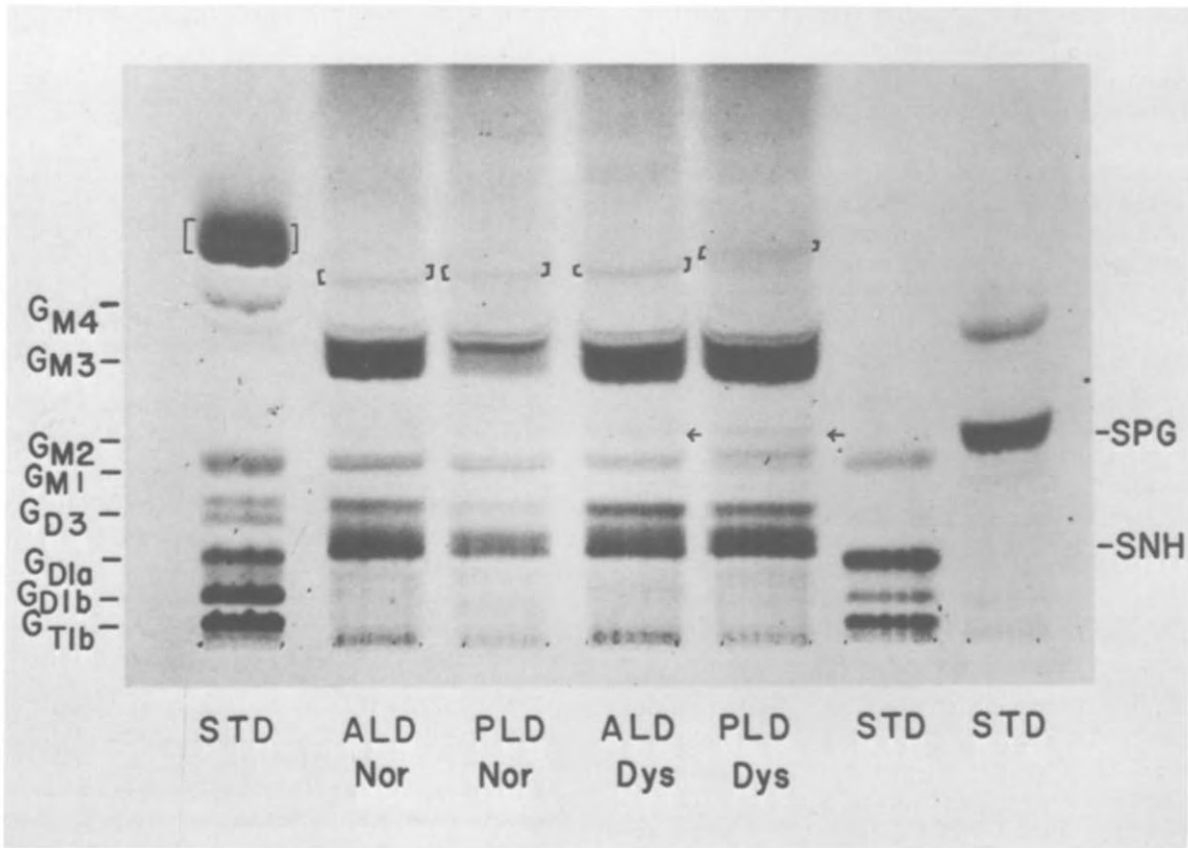

Fig. 2. Thin-layer chromatogram of 2.5-year-old chicken muscle gangliosides. The solvent was chloroform–methanol–water (60:40:9) containing 0.02% $CaCl_2 \cdot 2\ H_2O$ (w/v) and the gangliosides were detected with resorcinol reagent. Bands enclosed in square brackets were non-ganglioside impurities. Lanes ALD-nor, PLD-nor, ALD-dys and PLD-dys each contained gangliosides from 1 g of wet muscles; nor, normal; dys, dystrophic. STD lanes contained standard ganglioside mixtures. The arrows in lanes PLD-dys and ALD-dys indicate the new ganglioside which is absent in normal ALD and PLD muscles.

was identified as PG by the immunoblot procedure with purified antibody. The thin-layer chromatography mobilities of the two SNH bands obtained from muscles were identical to those of human erythrocytes SNH bands [29,30]. These two bands had the same carbohydrate chain but differed from each other in their ceramide moiety. The principal fatty acid of the upper band was $C_{16:0}$, whereas the lower band contained a predominance of $C_{22:0}$, $C_{24:0}$ and $C_{24:1}$. Successive treatments of the muscle SNH bands with *V. cholerae* neuraminidase, β-galactosidase and β-*N*-acetylhexosaminidase gave 2 bands which were identified as lactoneotetraosylceramide by the immunoblot procedure.

The most significant finding was the presence of a new ganglioside band (→, fig. 1) in dystrophic PLD muscles of 5-week-old chicks. This ganglioside had an apparent chromatographic mobility similar to G_{M2}. The same ganglioside was also seen in PLD muscles from 2.5-year-old chickens (fig. 2). However, this ganglioside was absent in PLD and ALD muscles of all normal chickens (fig. 1,2). In contrast to 5-week-old dystrophic chicks, ALD muscles of 2.5-year-old dystrophic chickens contained this ganglioside. The presence of this ganglioside in ALD muscles of old dystrophic chickens is consistent with observations that ALD muscles undergo degenerative changes at the late stage of disease progression [17]. Therefore we postulate a direct involvement of this ganglioside in the dystrophic process.

The dystrophic-specific ganglioside was characterized by its susceptibility to neuraminidase treatment. *V. cholerae* neuraminidase failed to cleave sialic acid residue from this ganglioside. However, it was possible to cleave sialic acid by *A. ureafaciens* neuraminidase in the presence of bile salt. These data suggested that sialic acid was present on the internal sugar residue of the dystrophic-specific ganglioside molecule. Asialoglycolipid produced by *A. ureafaciens* neuraminidase hydrolysis was identified as As G_{M2} by immunoblot procedure, using either mouse monoclonal or rabbit anti-As G_{M2} antibodies. The thin-layer chromatography mobility, the susceptibility to neuraminidase treatment and the reactivity of asialo-derivative towards anti-As G_{M2}-ceramide antibody suggest that the dystrophic-specific ganglioside has a carbohydrate structure similar to that of G_{M2}.

The neutral glycolipid patterns of ALD and PLD muscles of 5-week- and 2.5-year-old normal and dystrophic chickens are shown in fig. 3. An overall increase in neutral glycolipids was observed in dystrophic chickens of both age groups. The glycolipids tentatively identified on the basis of their reactivities towards specific antibodies by the immunoblot procedure are: LT, CTH, globotetraosylceramide, PG, As G_{M1} and Forss. The slow migrating bands seen below As G_{M1} were not identified (fig. 3).

Preliminary results on glycolipid distribution in other affected muscles, namely pectoralis, showed a significant increase in both ganglioside and total neutral glycolipid content (not shown). Our results on neutral glycolipid content of pectoralis showed a trend opposite to that in [10]. This difference could possibly be due to the fact that they used one-day-old white leghorn dystrophic chicks instead of 5-week-old and 2.5-year-old New Hampshire chickens used here.

We have found that both neutral glycolipids and ganglioside contents are elevated in the affected muscles of dystrophic chickens. More significant-

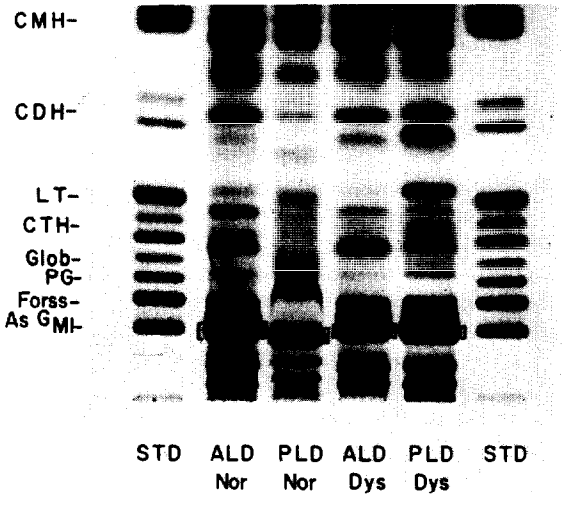

Fig. 3. Thin-layer chromatogram of 2.5-year-old chicken muscle neutral glycolipids. The solvent was chloroform–methanol–water (60:30:5, by vol.) and glycolipids were detected with α-naphthol reagent. Bands enclosed in square brackets contain non-glycolipid impurities. Lanes ALD-nor, PLD-nor, ALD-dys and PLD-dys each contained glycolipids from 0.5 g wet muscles. STD lanes contained standard mixtures.

ly, we have demonstrated the presence of a dystrophic-specific ganglioside, tentatively identified as G_{M2}, in the affected muscles. These data suggest that this ganglioside may play a role in muscular dystrophy. Currently we are investigating the distribution, localization and metabolism of this ganglioside in various muscles of dystrophic chickens during embryonic development and postnatal growth.

ACKNOWLEDGEMENTS

Authors wish to acknowledge Dr R.C. Fanguy for providing some chickens used in this study, Dr D.M. Marcus for his helpful discussions and Arlene Terry for typing the manuscript. This work was supported in part by grants from Muscular Dystrophy Association and American Heart Association, Texas Affiliate to M.G.L., USPHS grant AI05336 to S.K.K. and USPHS grant RP-05424 to L.K.M.

REFERENCES

[1] Rowland, L.P. (1976) Arch. Neurol. 33, 315–321.
[2] Kohn, L.D., Consiglio, E., DeWolf, M.J.S., Grollman, E.F., Ledley, F.D., Lee, G. and Morris, N.P. (1980) Adv. Exp. Biol. 125, 321–326.
[3] Marcus, D.M. (1981) in: Blood Group Immunochemistry and Genetics (Miescher, P.A. and Jaffe, E.R. eds) Seminars in Hematology vol. XVIII, no. 1, pp. 1–77, Grunne and Stratton, New York.
[4] Yamakawa, T. and Nagai, Y. (1978) Trends Biochem. Sci. 3, 128–131.
[5] Chien, J.L. and Hogan, E.L. (1980) in: Cell Surface Glycolipids (Sweeley, C.C. ed) Amer. Chem. Soc. vol. 128, pp. 135–148.
[6] Svennerholm, L., Bruce, A., Mansson, J.E., Rynmark, B.M. and Vanier, M.T. (1972) Biochim. Biophys. Acta 280, 626–636.
[7] Saito, M. and Rosenberg, A. (1982) J. Lipid Res. 23, 3–8.
[8] Mendel, J.R., Higgins, R., Sahenk, Z., Cosmos, E. (1979) Ann. NY Acad. Sci. 317, 409–430.
[9] Shafiq, S.A., Adiedw, S., Ryan, D. and Milhorat, A.T. (1974) in: Effect of Denervation and Hereditary Muscular Dystrophy on the Differentiation of Chicken Fiber Types with Some Observations on Early Changes in Muscular Dystrophy and Related Neuromuscular Diseases (Milhorat, A.T. ed) pp. 230–242, Elsevier/Excerpta Medica, Amsterdam, New York.
[10] Happel, R.D., Chien, J.L. and Hogan, E.L. (1980) Fed. Proc. FASEB 39, 417.
[11] Whatley, R., Ng, K.D., Rogers, J., McMurray, W.C. and Sanyal, B.D. (1976) Biochem. Biophys. Res. Commun. 70, 180–185.
[12] Obata, K., Oide, M. and Handa, S. (1977) Nature 266, 369–371.
[13] Ceccarelli, B., Aporti, F. and Finesso, M. (1976) in: Ganglioside Function: Biochemical and Pharmacological Implications (Porcellati, G. et al. eds) Plenum, New York.
[14] Wilson, B.W., Randall, W.R., Patterson, G.T. and Entrikin, R.K. (1979) Ann. NY Acad. Sci. 317, 224–246.
[15] Chung, C.S., Morton, N.E. and Peters, H.A. (1960) Am. J. Hum. Genet. 12, 52–66.
[16] Entrikin, R.K., Patterson, G.T. and Wilson, B.W. (1982) Muscle Nerve 5, 321–327.
[17] Barnard, E.A. and Barnard, P.J. (1979) Ann. NY Acad. Sci. 317, 374–399.
[18] Marcus, D.M. and Schwarting, G.A. (1976) Adv. Immunol. 23, 203–240.
[19] Karol, R.A., Kundu, S.K., Suzuki, A. and Marcus, D.M. (1980) Blood Transfus. Immunohaemat. 23, 589–598.
[20] Kundu, S.K. (1981) Methods Enzymol. 72, 174–185.
[21] Saito, T. and Hakomori, S. (1971) J. Lipid Res. 12, 257–259.
[22] Kundu, S.K. and Suzuki, A. (1981) J. Chromatogr. 224, 249–256.
[23] Ledeen, R.W., Yu, R.K. and Eng, L.F. (1973) J. Neurochem. 21, 829–839.
[24] Li, Y.-T., King, M.J. and Li, S.-C. (1980) in: Enzyme Degradation of Gangliosides (Svennerholm, L., Mandel, P., Drefus, H. and Urban, P.F. eds) Structure and Function of Gangliosides, pp. 93–104, Plenum, New York.
[25] Saito, M., Sugano, K. and Nagai, Y. (1979) J. Biol. Chem. 254, 7845–7854.
[26] Magnani, J.L., Smith, D.F. and Ginsberg, V. (1980) Anal. Biochem. 109, 399–402.
[27] Cosmos, E., Butler, J., Allard, E.P. and Mazliah, J. (1979) Ann. NY Acad. Sci. 317, 571–593.
[28] Kundu, S.K., Marcus, D.M., Pascher, I. and Samuelsson, B.E. (1981) Fed. Proc. FASEB 40, 1545.
[29] Kundu, S.K., Marcus, D.M. and Roelcke, D. (1982) Immunol. Lett. 4, 263–267.
[30] Kundu, S.K. and Scott, D.D. (1982) J. Chromatogr. 232, 19–27.
[31] Svennerholm, L. (1963) J. Neurochem. 10, 613–623.
[32] Ledeen, R.W. and Yu, R.K. (1982) Methods Enzymol. 83, 139–191.