notes on methodology

A rapid and quantitative method for the isolation of gangliosides and neutral glycosphingolipids by DEAE–silica gel chromatography

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Summary DEAE–silica gel has been shown to be an improvement over DEAE–Sephadex for the quantitative isolation of gangliosides and neutral glycosphingolipids from animal tissues or cells. Preliminary results indicated that it can also be used for protein separation. Direct comparative studies of DEAE–silica gel with DEAE–Sephadex showed preferences for the former for the following reasons: *i*) faster flow rate; *ii*) more rapid equilibration with the starting buffer; *iii*) easier regeneration; *iv*) more economical; and *v*) a lesser susceptibility to microbial attack.

Supplementary key word DEAE–Sephadex

In recent years, DEAE–Sephadex has been routinely used for the quantitative separation of gangliosides and neutral GSL from animal tissues or cells (1–5). The present report describes the preparation of a new ion exchanger, DEAE–silica gel, from commercially available materials and its application as an ion exchanger for the quantitative isolation of gangliosides and neutral GSL in organic solvents. A comparative study between DEAE–silica gel and DEAE–Sephadex was made; the results indicated that DEAE–silica gel was preferable.

Materials and Methods

Porous silica gel (pore diameter, 200 nm; 120–200 mesh; surface area, 150 m^2/g; pore volume, 1.68 ml/g) was obtained as a gift from Separation Laboratory, Bloomfield, NJ[2]. γ-Glycidoxypropyltrimethoxysilane was purchased from Polyscience, Inc., Warrington, PA; *N,N*-diethylethanolamine was from Aldrich Chemical Company, Milwaukee, WI; DEAE–Sephadex A-25 was from Pharmacia Fine Chemicals, Piscataway, NJ; Florisil (60–100 mesh) was from Fisher Scientific Company, Fairlawn, NJ, and Unisil (200–325 mesh) was from Clarkson Chemical Company, Williamsport, PA. Beef brain was obtained from Max Cohen Butchers, Newark, NJ. The blood samples were obtained in acid–citrate–dextrose anticoagulant and was worked up immediately. The purified gangliosides and neutral GSL used as standard components have been described previously (5, 6).

Ganglioside yield from each isolation was determined by GLC analysis of the lipid-bound sialic acid (7). Quantification of individual GSL was carried out as described previously (5, 8). TLC was carried out on precoated silica gel 60 plates (E. Merck Laboratories, Germany) with the following solvent systems: chloroform–methanol–2.5 N NH_4OH 60:40:9 (v/v) for gangliosides and chloroform–methanol–water 60:30:5 (v/v) for neutral GSL. α-Naphthol and resorcinol reagents (9, 10) were utilized to detect neutral GSL and gangliosides, respectively.

Preparation of DEAE-silica gel (***Scheme 1***). Silica gel (10 g) was deaerated under vacuum for 30 min and then heated at 45°C for 20 hr with a mixture containing 100 ml of 10% γ-glycidoxypropyltrimethoxysilane in toluene and 10 ml of *N,N*-diethylethanolamine (11). The reaction mixture was allowed to cool to room temperature. Filtration through a sintered glass funnel of coarse porosity and exhaustive washing with methanol removed toluene, unbound silane, and other by-products. Final purification of the gel was achieved by washing with water followed by conversion to the chloride form with dilute hydrochloric acid. After drying under vacuum, the gel was stored at room temperature.

Total capacity of the DEAE–silica gel (found to be 140 μmol/g dry wt) was determined by titrating the base with 0.1 N perchloric acid in a nonaqueous solvent (12). Hemoglobin capacity (found to be 120 mg/g dry wt) was determined as follows. Gel (0.5 g) was washed several times with 0.01 M sodium phosphate buffer, pH 8.0, and treated with 100 mg of hemoglobin in 10 ml of the same buffer. The mixture was stirred for 30 min and then filtered through a sintered glass funnel. The gel was then washed with

Abbreviations: DEAE, diethylaminoethyl; BBG, beef brain ganglioside mixture; GSL, glycosphingolipid (s); GLC, gas–liquid chromatography; TLC, thin-layer chromatography; NANA, *N*-acetylneuraminic acid; PBS, 0.02 M potassium phosphate (pH 7.3)-0.15 M sodium chloride.

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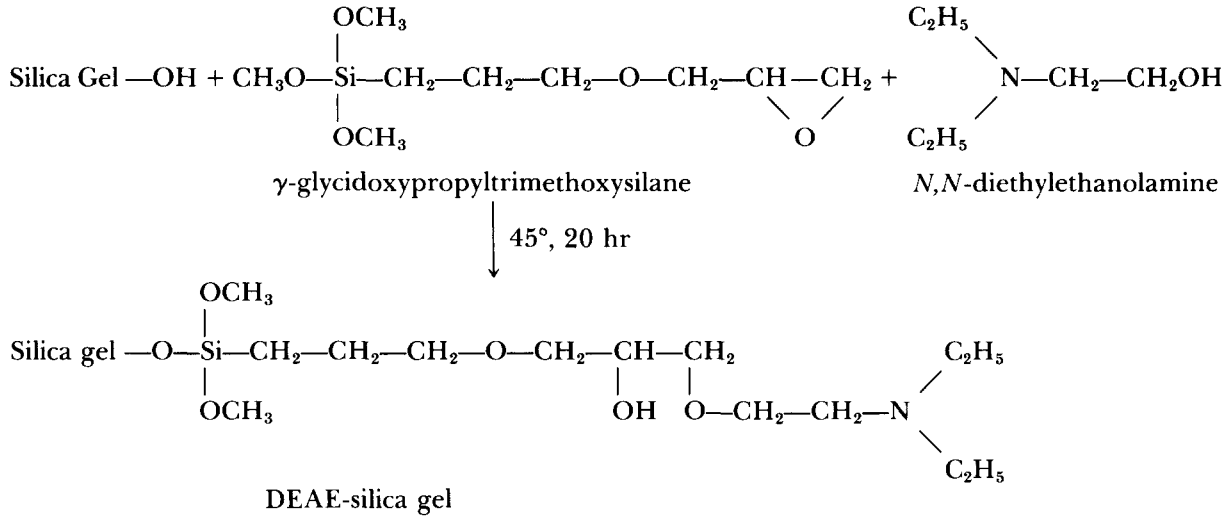
[2] Porous silica gel (pore diameter, 200 nm) was obtained commercially from the Separation Laboratory, P. O. Box 1744, Bloomfield, NJ 07003 and from E. Merck Laboratories, Germany.



Scheme 1. Preparation of diethylaminoethyl (DEAE)-silica gel.

0.01 M sodium phosphate buffer, pH 8.0 (3 × 5 ml), to remove all the unbound protein. Desorption of the adsorbed hemoglobin from the gel was then accomplished by washing the gel with 0.5 M sodium phosphate buffer, pH 4.0 (4 × 5 ml). The washings were combined and the concentration of hemoglobin was determined by reading the absorbance at 410 nm.

Conversion of DEAE–silica gel (chloride form) to acetate form. DEAE–silica gel (10 g, chloride form) was equilibrated by shaking with 100 ml of chloroform–methanol–0.8 M sodium acetate 30:60:8 (v/v) for 5 min; after allowing the suspension to settle for an additional 5 min, the clear supernatant solution was removed with a pipet. The DEAE–silica gel was treated in this manner three times more and finally washed with 4 100-ml volumes of chloroform–methanol–water 30:60:8 (v/v) and packed in a column (1.5 cm ID and 30 cm length). Elution with 100 ml of the same solvent mixture ensured complete removal of sodium acetate.

Conversion of DEAE–silica gel (chloride form) to the acetate form was also achieved by equilibration with 1 M acetic acid or 0.2 M sodium acetate in methanol. Acetic acid or sodium acetate was completely removed by exhaustive washing with water or methanol, respectively. The gel must be washed with at least three bed volumes of chloroform–methanol–water 30:60:8 (v/v) before use.

Regeneration of DEAE–silica gel. Regeneration of used DEAE–silica gel was achieved by washing the column with chloroform–methanol–0.8 M sodium acetate 30:60:8 (v/v) or 0.2 M sodium acetate in methanol followed by complete removal of sodium acetate by washing with chloroform–methanol–water 30:60:8 (v/v) or methanol, respectively. After using the same gel a number of times, complete regeneration was carried out as follows. *i*) The gel was washed with a mixture of 0.1 M aqueous sodium bicarbonate and 0.2 M sodium chloride to remove proteins; *ii*) the gel was washed with water until neutral; and *iii*) it was converted to the acetate form as described above.

Applications

Isolation of gangliosides and neutral GSL from beef brain. A portion of beef brain (30 g, wet wt)[3] was extracted three times with 10 volumes of chloroform–methanol 2:1 (v/v) at room temperature (13). The total lipid obtained after evaporation was dissolved in 300 ml of chloroform–methanol–water 30:60:8 (v/v). Ten ml of this extract was used in each experiment as described below. Aliquots of the same beef brain total lipid extract were used in all the experiments for direct comparison.

Method A. DEAE–silica gel (10 g, acetate form, dry wt) was used. Ten ml of beef brain total lipid extract was diluted to 50 ml with chloroform–methanol–water 30:60:8 (v/v) and was allowed to flow on to the column under gravity. After the 50 ml had been added, the column was eluted with 150 ml of the same solvent mixture. The combined eluates comprised the first fraction (Fr. 1) containing the uncharged and zweitterionic lipids. The second fraction (Fr. 2) containing the acidic lipids was eluted with 200

[3] This contains a mixture of gray and white matter. Gray matter contains about 0.785 mg of lipid-bound sialic acid per g of wet weight of brain, whereas white matter contains about 0.232 mg of sialic acid per g of wet weight of brain (14). Therefore, the amount of lipid-bound sialic acid would depend on the ratio of gray and white matters in this portion of the whole brain.

ml of chloroform–methanol–0.8 M sodium acetate 30:60:8 (v/v). Total elution time was approximately 5 hr.

Method B. DEAE–silica gel (10 g, acetate form, dry wt) was used. Ten ml of beef brain total lipid extract was diluted to 100 ml with chloroform–methanol–water 30:60:8 (v/v) and was put on to the column as described in method A. After the 100 ml had been added, the column was eluted with 150 ml of methanol. The combined eluates comprised Fr. 1. Fr. 2 was then eluted with 200 ml of 0.2 M sodium acetate in methanol. Total elution time was approximately 6 hr.

Method C. DEAE–Sephadex (2.2 g, acetate form, dry wt) was used with 10 ml of beef brain total lipid extract according to the procedure described by Ledeen, Yu, and Eng (3). Total elution time was more than 10 hr.

Method D. DEAE–Sephadex (2.2 g, acetate form, dry wt) was used. An aliquot of 10 ml of beef brain total lipid extract was diluted to 200 ml with chloroform–methanol–water 30:60:8 (v/v) and was allowed to flow on to the column. The flow rate was adjusted to approximately 0.7 ml/min as described by Ledeen et al. (3). After the 200 ml had been added, the column was more rapidly eluted with 100 ml of methanol. The combined eluates comprised Fr. 1. Fr. 2 was then eluted rapidly with 200 ml of 0.2 M sodium acetate in methanol. Total elution time was more than 10 hr.

The acidic lipid fractions (Fr. 2) from each of the above four methods were treated according to the procedure of Ledeen et al. (3) for the isolation of gangliosides. The yields of the lipid-bound sialic acid of the beef brain ganglioside mixtures are presented in **Table 1.** The TLC of the ganglioside mixtures isolated by method A (DEAE–silica gel) and method C (DEAE–Sephadex) is shown in **Fig. 1**. The ganglioside mixtures isolated by methods B and D (not shown) showed identical patterns.

For the isolation of neutral GSL, the first fractions (Fr. 1) containing the uncharged and zweitterionic lipids from each of the four methods were worked up according to the procedure outlined by Saito and Hakomori (18). The amounts of the total hexose in the neutral GSL fractions are presented in Table 1. The TLC of the neutral GSL fractions isolated by methods A and B (DEAE–silica gel) and method

TABLE 1. Lipid-bound sialic acid and total hexose in gangliosides and neutral glycosphingolipids of whole beef brain and normal human erythrocytes[a]

	DEAE–Silica Gel[b]		DEAE–Sephadex[b]	
	Method A	Method B	Method C	Method D
mg of sialic acid/g of fresh weight of beef brain[c]	0.634 ± 0.014	0.643 ± 0.008	0.638 ± 0.011	0.639 ± 0.018
mg of sialic acid recovered from 1 g of fresh weight of beef brain[c] plus 0.32 mg of added beef brain ganglioside sialic acid[c]	0.946 ± 0.011 (96%)[d]	0.948 ± 0.014 (96%)[d]	0.940 ± 0.006 (96%)[d]	0.951 ± 0.024 (97%)[d]
mg of sialic acid/20 ml of packed human erythrocytes	0.150 ± 0.018[e] 0.151 ± 0.016[f]	0.152 ± 0.014[e] 0.156 ± 0.012[f]	0.149 ± 0.018[e] 0.146 ± 0.016[f]	0.150 ± 0.008[e] 0.152 ± 0.006[f]
mg of sialic acid recovered from 20 ml of packed human erythrocytes plus 0.100 mg of added human erythrocyte ganglioside sialic acid[c]	0.244 ± 0.016[e] (94%)[d]	0.245 ± 0.011[e] (93%)[d]	0.242 ± 0.008[e] (93%)[d]	0.244 ± 0.014[e] (94%)[d]
mg of total hexose in neutral GSL/g of fresh weight of beef brain	2.08 ± 0.014	2.13 ± 0.024	2.11 ± 0.022	2.05 ± 0.016
mg of total hexose in neutral GSL/20 ml of packed human erythrocytes	2.23 ± 0.008[e] 2.12 ± 0.020[f]	2.20 ± 0.026[e] 2.02 ± 0.015[f]	2.26 ± 0.016[e] 2.16 ± 0.022[f]	2.22 ± 0.014[e] 2.05 ± 0.016[f]

[a] Expressed as mg of sialic acid and total hexose ± confidence limits. The confidence limits are 95% and were calculated using the formula $\left[\frac{SD}{\sqrt{n}}\right] t_{(n-1),0.05}$, where SD is the standard deviation and t is the Student's t for $(n-1)$ degrees of freedom. Each method described here was carried out at least four times. Sialic acid was determined by gas–liquid chromatography (7) and total hexose by phenol–sulfuric acid reaction (15).

[b] DEAE–silica gel (10 g, acetate form, dry wt) and DEAE–Sephadex (2.2 g, acetate form, dry wt) were used in all the experiments described in this table. We have found that up to 4 g of fresh wt of beef brain can be loaded on the same size DEAE–silica gel column and still give a quantitative yield of total ganglioside.

[c] Known amounts of beef brain ganglioside mixture (0.32 mg sialic acid) and human erythrocyte ganglioside mixture (0.100 mg sialic acid) were added to the beef brain and human erythrocyte total lipid extracts, respectively, to check the recovery of gangliosides by DEAE–chromatography.

[d] The numbers within parentheses indicate the percentage of recovery of gangliosides.

[e] From a male donor, age 25 yr.

[f] From a male donor, age 30 yr.

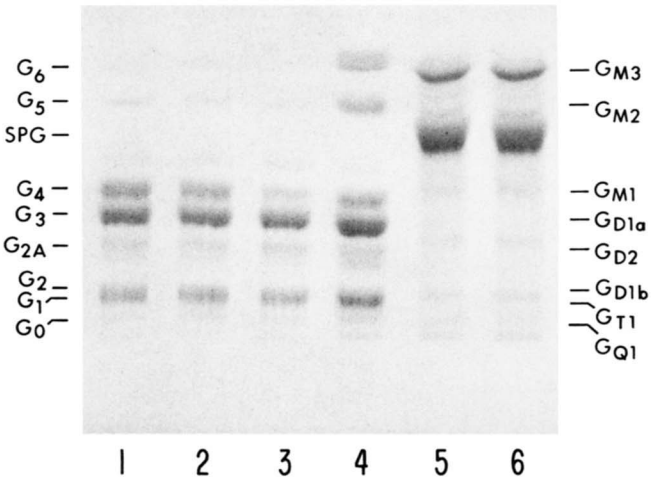


Fig. 1. TLC of gangliosides from beef brain (BBG) and human erythrocytes. *1*) BBG by DEAE–Sephadex (method C); *2*) BBG by DEAE–silica gel (method A); *3*) BBG standards; *4*) BBG, G_5 (= G_{M2}), and G_6 (= G_{M3}) standards; *5*) human erythrocyte gangliosides by DEAE–Sephadex (method C); *6*) human erythrocyte gangliosides by DEAE–silica gel (method A). Each of the lanes contained 9–11 μg of sialic acid. Solvent system was chloroform–methanol–2.5 N NH_4OH 60:40:9 (v/v) (see Materials and Methods for experimental conditions). All bands were purple after detection by resorcinol spray. Ganglioside nomenclatures of Korey and Gonatas (16) and Svennerholm (17)[5] are depicted on left and right, respectively. SPG denotes sialosylparagloboside.

C (DEAE–Sephadex) are shown in **Fig. 2**. The GSL fraction isolated by method D (not shown) showed an identical pattern. The neutral GSL fractions from each of the four methods were also tested for gangliosides by spraying the TLC plates with resorcinol reagent (10). No purple color, attributed to ganglioside, was observed in any of the neutral GSL fractions, thereby indicating complete separation of the gangliosides in the acidic fractions (Fr. 2).

Isolation of gangliosides and neutral GSL from human erythrocytes. Four hundred ml of freshly drawn human blood in acid–citrate–dextrose anticoagulant was washed with PBS and the packed erythrocytes (ca. 200 ml)[4] were extracted with chloroform–methanol as described previously (5). The total lipid was redissolved in 200 ml of chloroform–methanol–water 30:60:8 (v/v). Twenty ml of this extract was used on DEAE–silica gel (10 g, acetate form, dry wt) and also on DEAE–Sephadex (2.2 g, acetate form, dry wt). An aliquot of the same erythrocyte total lipid extract was used in each of the experiments for direct comparison. The gangliosides and the neutral GSL were isolated by each of the four methods described for beef brain. The yields of the lipid-bound sialic acid of two male donors, ages 25 and 30 years, are presented in Table 1. The TLC of the total ganglioside mixtures from both donors by the four methods showed identical patterns. The TLC patterns of the total gangliosides by method A (DEAE–silica gel) and method C (DEAE–Sephadex) are shown in Fig. 2. The amounts of the total hexose in the neutral GSL fractions are also presented in Table 1.

The individual oligoglycosyl ceramides from the total erythrocyte neutral GSL fractions were also isolated as their acetates by preparative TLC and analyzed by GLC (5, 8). The concentration of the neutral GSL in normal human erythrocytes of the two donors is presented in **Table 2**. The bands that are more polar than paragloboside, constituting less

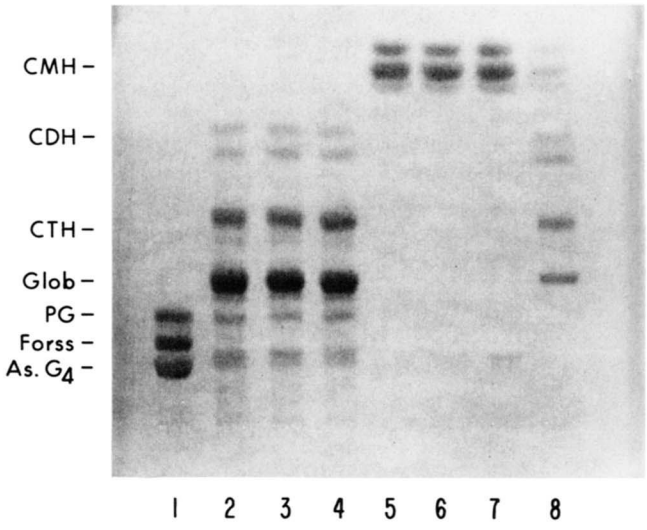


Fig. 2. TLC of neutral glycosphingolipids (GSL) from human erythrocytes and beef brain. *1*) Standards containing paragloboside (PG), Forssman glycolipid (Forss) and asialo G_4 (= asialo G_{M1}); *2*) neutral erythrocyte GSL by DEAE–Sephadex (method C); *3*) and *4*) neutral erythrocyte GSL by DEAE–silica gel (methods A and B, respectively); *5*) neutral beef brain GSL by DEAE–Sephadex (method C); *6*) and *7*) neutral beef brain GSL by DEAE–silica gel (methods A and B, respectively); *8*) standards containing glucosyl ceramide (CMH), lactosyl ceramide (CDH), trihexosyl ceramide (CTH), and globoside (Glob). Lanes 2–4 each contained 100–110 μg of GSL and lanes 5–7 each had 60–70 μg of GSL. Solvent system was chloroform–methanol–water 60:30:5 (v/v) (see Materials and Methods for experimental conditions). All bands were purple after detection by α-naphthol spray.

[4] The amount of neutral GSL and gangliosides from erythrocytes would depend on how tightly they are packed. We centrifuged at 3000 rpm for 25 min for packing the erythrocytes, whereas Vance and Sweeley (19) used a speed of approximately 1600 rpm. Accordingly, the concentration of oligoglycosyl ceramides in normal human erythrocytes cited by these authors (19), expressed in terms of packed erythrocytes, would be expected to give much lower values than those shown in Table 2. The differences in values may also be due to the differences in the methodologies. However, the present data agree well with our previous results (5) and more recent data (20).

[5] The symbols used for gangliosides are those of Korey and Gonatas (16) which are correlated with the symbols of Svennerholm (17) as follows. $G_6 = G_{M3}$; $G_5 = G_{M2}$; $G_4 = G_{M1}$; $G_3 = G_{D1a}$; $G_{2A} = G_{D2}$; $G_2 = G_{D1b}$; $G_1 = G_{T1}$; $G_0 = G_{Q1}$.

TABLE 2. Concentration of neutral glycosphingolipids in normal human erythrocytes[a]

Phenotype	Method	CMH	CDH	CTH	Globoside	Paragloboside
Normal, age 25 yr	DEAE–silica gel (method A)	0.42	2.40	6.34	18.16	1.21
	DEAE–silica gel (method B)	0.36	2.51	6.28	18.06	0.95
	DEAE–Sephadex (method C)	0.34	2.39	6.16	17.84	0.88
	DEAE–Sephadex (method D)	0.35	2.50	6.20	18.11	0.85
Normal, age 30 yr	DEAE–silica gel (method A)	0.32	2.70	5.34	17.32	0.26
	DEAE–silica gel (method B)	0.41	2.62	5.27	17.63	0.32
	DEAE–Sephadex (method C)	0.36	2.72	5.18	17.56	0.27
	DEAE–Sephadex (method D)	0.38	2.60	5.22	19.00	0.26

[a] Expressed as μmol per 100 ml of packed erythrocytes[4] of two male donors ages 25 and 30 yr, respectively. The concentration of α-naphthol positive minor bands more polar than paragloboside were not determined. Values were determined by gas–liquid chromatography (5, 8) and quantified as described by Vance and Sweeley (19). The values presented in this table are the mean of two separate experiments.

than 5% of the total erythrocyte GSL, were ignored in the present study to simplify quantification.

All the neutral GSL fractions from human erythrocytes were also tested for gangliosides by spraying the TLC plates with resorcinol reagent (10). No purple color, attributed to ganglioside, was observed in any of the preparations, thereby indicating complete separation of the gangliosides from the neutral GSL fractions.

Recovery test of gangliosides. To 10 ml of beef brain total lipid extract, 0.32 mg of beef brain ganglioside sialic acid was added. Similarly, to 20 ml of human erythrocyte total lipid extract, 0.100 mg of human erythrocyte ganglioside sialic acid was added. An aliquot of the same beef brain or human erythrocyte total lipid extract was used in each of the experiments for direct comparison. The total gangliosides were then isolated according to the four methods described above. The same amounts of DEAE–silica gel and DEAE–Sephadex were used as described in methods A–D. The recovery of total gangliosides from beef brain and human erythrocytes are presented in Table 1.

Discussion

The above results indicate that DEAE–silica gel is an improvement over DEAE–Sephadex for the quantitative separation of acidic and neutral lipids for the following reasons. *i*) The flow rate of DEAE–silica gel was at least twice as fast as that of DEAE–Sephadex. *ii*) DEAE–silica gel can be more rapidly equilibrated with the starting buffer; the chloride form of DEAE–silica gel is converted to the acetate form by simply shaking the gel with the acetate solution for a few minutes whereas, for DEAE–Sephadex, overnight swelling with the acetate solution is a necessary step (3). We have also found that complete conversion to the acetate form could not be achieved until DEAE–Sephadex was soaked with the acetate solution for at least 8–10 hr. *iii*) It can be more easily regenerated; though DEAE–silica gel and DEAE–Sephadex both can be regenerated while the gel is in the column, regeneration of the former takes less than half the time needed for DEAE–Sephadex because of a faster flow rate. *iv*) It is more economical; commercially available silica gel is not expensive and the preparation of DEAE–silica gel is a simple method which can be adopted in any laboratory. *v*) It is less susceptible to microbial attack; for storage of DEAE–Sephadex, preservatives are usually added to prevent microbial growth, whereas for DEAE–silica gel, no special treatment is necessary for storage.

The applications of DEAE–silica gel for the quantitative isolation of gangliosides and neutral GSL from beef brain and human erythrocytes illustrate its usefulness. Each of the experiments was tested with the same beef brain total lipid extract and also with the same human erythrocyte extract at least four times. The lipid-bound sialic acid of beef brain and human erythrocyte gangliosides of two donors as measured by DEAE–silica gel and DEAE–Sephadex methods gave similar values (Table 1). Their TLC patterns were also identical (Fig. 1) and agree well with the published data (5). The total hexose analysis of the neutral GSL fractions from beef brain and human erythrocytes of two donors by DEAE–silica gel and DEAE–Sephadex methods are also in excellent agreement (Table 1). The TLC of the neutral GSL from beef brain showed mostly cerebrosides as expected (21), whereas the GSL from human erythrocytes showed increased amounts of higher oligoglycosyl ceramides (Fig. 2) (22). Analysis of individual GSL, isolated as their acetates by preparative TLC of the acetylated erythrocyte GSL fractions showed similar pattern of distribution (Table 2). A similar distribution pattern of normal human erythrocytes was also noted by us (5, 20).

The question of recovery of gangliosides was ascertained by adding known quantities of total ganglioside mixtures to the total lipid extracts and then utilizing the four methods described in this report. In the case of beef brain gangliosides, we obtained a recovery of 96–97% of total ganglioside mixture, whereas for human erythrocyte gangliosides, a recovery of 93–94% was noted for the four methods (Table 1). These nearly quantitative yields of the total gangliosides agree well with the data cited by Ledeen et al. (3) who obtained a recovery of 93% for total gangliosides from human white matter using DEAE–Sephadex and method C, which was also used in this study for direct comparison. A report on some aspects of this work was presented as a preliminary communication (23).

We have also utilized DEAE–silica gel for the separation of gangliosides and neutral GSL from spleen, kidney, and other extraneural tissues and obtained excellent results. Rabbit serum proteins have also been fractionated using DEAE–silica gel and Tris–HCl–NaCl gradient. Using DEAE–silica gel and a linear gradient of ammonium acetate in methanol (24), we were able to fractionate the beef brain ganglioside mixture into mono-, di-, tri-, and tetrasialo species (25). Using a similar gradient, we also separated the human erythrocyte ganglioside mixture into mono and disialo species. Further details of these procedures and the protein separation technique will be forthcoming.

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REFERENCES

1. Rouser, G., G. Kritchevsky, A. Yamamoto, G. Simon, C. Galli, and A. J. Bauman. 1969. Diethylaminoethyl cellulose column chromatographic procedures for phospholipids, glycolipids, and pigments. *In* Methods in Enzymology. J. M. Lowenstein, editor. Academic Press, New York. **14:** 272–317.
2. Yu, R. K., and R. W. Ledeen. 1972. Gangliosides of human, bovine, and rabbit plasma. *J. Lipid Res.* **13:** 680–686.
3. Ledeen, R. W., R. K. Yu, and L. F. Eng. 1973. Gangliosides of human myelin: sialosylgalactosylceramide (G_7) as a major component. *J. Neurochem.* **21:** 829–839.
4. Price, H. C., S. K. Kundu, and R. W. Ledeen. 1975. Structures of gangliosides from bovine adrenal medulla. *Biochemistry.* **14:** 1512–1518.
5. Marcus, D. M., M. Naiki, and S. K. Kundu. 1976. Abnormalities in the glycosphingolipid content of human p^k and p erythrocytes. *Proc. Natl. Acad. Sci. USA.* **73:** 3263–3267.
6. Kundu, S. K., S. K. Chakravarty, N. Bhaduri, and H. K. Saha. 1977. A novel reagent for phospholipid detection. *J. Lipid Res.* **18:** 128–130.
7. Yu, R. K., and R. W. Ledeen. 1970. Gas–liquid chromatographic assay of lipid-bound sialic acids: measurement of gangliosides in brain of several species. *J. Lipid Res.* **11:** 506–516.
8. Fong, J. W., R. W. Ledeen, S. K. Kundu, and S. W. Brostoff. 1976. Gangliosides of peripheral nerve myelin. *J. Neurochem.* **26:** 157–162.
9. Siakotos, A. N., and G. Rouser. 1965. Analytical separation of non-lipid water soluble substances and gangliosides from other lipids by dextran gel column chromatography, *J. Amer. Oil Chem. Soc.* **42:** 913–919.
10. Svennerholm, L. 1957. Quantitative estimation of sialic acids. II. A colorimetric resorcinol–hydrochloric acid method. *Biochim. Biophys. Acta.* **24:** 604–611.
11. Lewis, C., and W. H. Scouten. 1976. Photooxidation of methionine. *J. Chem. Ed.* **53:** 395–397.
12. Pifer, C. W., and E. C. Wollish. 1952. Titrations in non-aqueous solutions. Potentiometric titration of salts of organic bases in acetic acid. *Anal. Chem.* **24:** 300–306.
13. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simplified method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **226:** 497–509.
14. Ledeen, R., K. Salsman, and M. Cabrera. 1968. Gangliosides in subacute sclerosing leukoencephalitis: isolation and fatty acid composition of nine fractions. *J. Lipid Res.* **9:** 129–135.
15. Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **28:** 350–356.
16. Korey, S. R., and J. Gonatas. 1963. Separation of human brain gangliosides. *Life Sci.* **2:** 296–302.
17. Svennerholm, L. 1963. Chromatographic separation of human brain gangliosides. *J. Neurochem.* **10:** 613–623.
18. Saito, T., and S. Hakomori. 1971. Quantitative isolation of total glycosphingolipid from animal cells. *J. Lipid Res.* **12:** 257–259.
19. Vance, D. E., and C. C. Sweeley. 1967. Quantitative determination of neutral glycosyl ceramides in human blood. *J. Lipid Res.* **8:** 621–630.
20. Kundu, S. K., S. M. Steane, J. E. C. Bloom, and D. M. Marcus. 1978. Abnormal glycolipid composition of erythrocytes with a weak P antigen. *Vox. Sang.* (in press).
21. Ledeen, R. W., and R. K. Yu. 1973. Structure and enzymatic degradation of sphingolipids. *In* Lysosomes and Storage Diseases. H. G. Hers and F. Van Hoof, editors. Academic Press, New York. 105–144.
22. Hakomori, S., and B. Siddiqui. 1974. Isolation and characterization of glycosphingolipid from animal cells and their membranes. *In* Methods in Enzymology, Biomembranes. S. Fleischer and L. Pascher, editors. Academic Press, New York. **32:** 345–369.
23. Kundu, S. K., and S. K. Roy. 1977. DEAE–silica gel as ion-exchanger for isolation of glycolipids. *Trans. Amer. Soc. Neurochem.* **8:** 128.
24. Momoi, T., S. Ando, and Y. Nagai. 1976. High resolution preparative column chromatographic system for gangliosides using DEAE–Sephadex and a new porous silica, iatrobeads. *Biochim. Biophys. Acta.* **441:** 488–497.
25. Kundu, S. K. 1977. Studies on glycosphingolipid structure and organic molecules of potential interest. D. Sc. thesis, University of Calcutta.