The Isolation and Partial Characterization of the Glycolipids of BP8/C3H Ascites-Sarcoma Cells

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1. The total lipid was extracted from BP8/C3H ascites-sarcoma cells with acetone, light petroleum, pyridine and chloroform-methanol successively. Each extract was treated with mild alkali. The alkali-stable lipids from the pyridine and chloroform-methanol extracts, which included the glycolipids, were fractionated on silicic acid and silica gel G columns.
2. The total yield of glycolipid was about 60 mg./100 g. dry wt. of tumour cells, about 0.4% of the total lipid. Four classes of glycolipid were isolated and characterized as ceramide monohexoside (G1), ceramide dihexoside (G2), ceramide trihexoside (G3) and ceramide hexosaminyltrihexoside (G4).
3. G1, G2, G3 and G4 constituted 55, 21, 9 and 15% of the total glycolipid respectively.
4. G1 was a mixture of ceramide glucoside (70%) and ceramide galactoside.
5. The general structures of the oligosaccharide moieties of G2, G3 and G4 were elucidated by partial acid hydrolysis of the glycolipids with water-soluble polystyrenesulphonic acid. G2 was mostly ceramide galactoside with about 10% of ceramide galactosylgalactoside. G3 and G4 were probably a ceramide digalactosylglucoside and a ceramide *N*-acetylgalactosaminylgalactosylgalactosylglucoside respectively.
6. The fatty acid compositions of the glycolipids were very similar; lignoceric acid and nervonic acid were the major components and all contained monohydroxy acids in proportions varying from 10 to 25% of the total acids.

The main glycolipid found in tissues of the central nervous system is composed of sphingosine, fatty acid and hexose in the molar proportions 1:1:1. This ceramide monohexoside is a ceramide galactoside (also called galactocerebroside). The corresponding ceramide glucoside is the most common glycolipid in other tissues (Makita & Yamakawa, 1962; Svennerholm & Svennerholm, 1963*a,b*). A variety of more complex glycolipids, in minor quantities, have also been found in several tissues (Rapport, Graf & Alonzo, 1960; Yamakawa, Yokoyama & Handa, 1963; Martensson, 1963). Abnormal quantities of some complex glycolipids have been isolated from the brain lipids of persons with Tay-Sachs disease (Gatt & Berman, 1963) and from the kidneys of a person with Fabry's disease (Sweeley & Klionsky, 1963).

Minor amounts of some glycolipids have been found in the lipid extracts of Landschutz ascites-carcinoma cells and of BP8/C3H ascites-sarcoma cells (Gray, 1963). One of the glycolipids was a ceramide glucoside and its relative amount appeared to differ in the two types of tumour cell. Studies on the lipid composition of these mouse tumour cells showed that the distribution of the major lipids, the phospholipids, was not significantly different from

that in most normal tissues, but the presence of even minor quantities of unusual components such as glycolipids might in itself be a sign of some metabolic or functional difference between the tumour cells and normal cells. With this in mind a more detailed study of the glycolipid composition was made, and in the present work four classes of glycolipid were isolated from BP8/C3H ascites-sarcoma cells and partially characterized.

MATERIALS AND METHODS

Analytical methods. The estimation of P, amino N, aldehyde and fatty acid ester groups was carried out as indicated by Gray & Macfarlane (1958). The micro-assay of P was carried out essentially as described by Bartlett (1959). Sphingosine in the isolated glycolipids was determined as described by Long & Staples (1961) and Gray (1963); the fatty acids in the glycolipids were identified by gas-liquid chromatography (Gray, 1960).

Carbohydrate in the glycolipid was estimated as follows. The glycolipid (2-3 mg.) was hydrolysed with 1.0 ml. of 3 *N*-hydrochloric acid at 100° for 2-3 hr. in a stoppered tube. The hydrolysate was extracted with chloroform-methanol [1 vol. of 2:1 (v/v) mixture, followed by two lots of 1 vol. of 17:3 (v/v) mixture] to remove sphingosine and fatty acids, and the aqueous acid extract was neutralized with sodium

hydroxide. Total hexose in the aqueous hydrolysate was determined by the method of Dubois, Gilles, Hamilton, Roben & Smith (1956). The sub-micro method of Park & Johnson (1949) for the estimation of reducing sugar was found to be reliable when used on the aqueous hydrolysates of isolated glycolipids. Total glucose was determined by the method of Dahlqvist (1961), with glucose oxidase. Hexosamine determinations were carried out by the method of Rundle & Morgan (1955) on these hydrolysates, and also on samples of glycolipid hydrolysed with 2 N-hydrochloric acid for 18 hr. at 100° in a sealed tube.

Chromatography of phospholipids and glycolipids on silicic acid-impregnated paper was done in di-isobutyl ketone-acetic acid-water (40:20:3, by vol.) at 2° (Marinetti, Erbland & Koehen, 1957). Sugars were identified in the aqueous extracts after hydrolysis of the lipid fractions with 3 N-hydrochloric acid for 2 hr. at 100° and removal of the hydrochloric acid with AG11A8 'retardion' resin (California Corp. for Biochemical Research, Los Angeles, U.S.A.), by chromatography on Whatman no. 1 acid-washed paper in pyridine-butan-1-ol-water (4:6:3, by vol.) and ethyl acetate-pyridine-water (10:4:3, by vol.), followed by spraying with silver nitrate-sodium hydroxide reagent (Trevelyan, Procter & Harrison, 1950). Hexosamines were also identified by chromatography in butan-1-ol-acetic acid-water (4:1:5, by vol.) and spraying with ninhydrin.

Fractions from chromatographic columns were monitored by thin-layer chromatography on silica gel G or H (E. Merck A.-G., Darmstadt, Germany), with a solvent system of chloroform-methanol-water (70:30:3, by vol.). Compounds were detected on the plates by spraying with acid-molybdate reagent (Skipski, Peterson & Barclay, 1962) or with sulphuric acid (30%, v/v) and heating. Compounds containing carbohydrate were often distinguishable from other compounds when sprayed with sulphuric acid by the deep purple colour produced before complete charring.

Materials. The BP8/C3H ascites-sarcoma cells (benzopyrene-induced) were grown in mice at the Microbiological Research Establishment, Porton, Wilts. The cells were collected in 0.9% sodium chloride and freeze-dried. I am indebted to Dr D. A. L. Davies for a generous supply of freeze-dried cells.

Extraction. As the glycolipids are a very small proportion of the total lipids in the tumour cells (Gray, 1963), the extraction and preliminary fractionation was devised to remove as much neutral lipid and phospholipid as possible before attempting isolation.

The freeze-dried tumour cells (2 kg.) were first extracted three times with 6 l. of acetone (I), and then successively with the same volume of light petroleum (b.p. 60–80°) (II), pyridine (III) and chloroform-methanol (2:1, v/v) (IV). The acetone extract was dialysed through a rubber membrane (Van Beers, de Iongh & Boldingh, 1958) to remove the neutral lipid present (approx. 100 g.). All the extracts were then separately submitted to mild alkaline hydrolysis according to the method of Hübscher, Hawthorne & Kemp (1960) as follows. The extracts were evaporated to dryness and redissolved in chloroform (0.5 ml./mg. of P). Methanol (1 ml./mg. of P) was added and the solutions were made alkaline with methanolic N-sodium hydroxide to give a concentration of 0.05 N-sodium hydroxide. The solutions were kept at room temperature (20°) for 30 min. and then were neutralized with hydrochloric acid. Chloroform (0.5 ml./mg. of P) and water (2 ml./mg. of P) were added to the

Table 1. *Distribution of total lipid phosphorus, alkali-stable lipid phosphorus and total hexose in the solvent extracts from freeze-dried BP8/C3H ascites-sarcoma cells*

Experimental details are given in the text. Total hexose was determined as glucose. The starting material was 2 kg. of freeze-dried tumour cells.

Extracting solvent	Total lipid P (mg.)	Alkali-stable lipid P (mg.)	Total hexose (mg.)
Acetone (I)	358	13	6
Light petroleum (b.p. 60–80°) (II)	559	84	17
Pyridine (III)	2210	140	244
Chloroform-methanol (2:1, v/v) (IV)	1697	340	136

solutions, and the upper aqueous-methanol layers containing the water-soluble phosphoric esters formed from hydrolysis of the acylglycerophosphatides were removed. The chloroform layers were washed twice with 0.1 M-potassium chloride (0.2 vol.) and dried over anhydrous sodium sulphate. The chloroform was removed by evaporation under vacuum, and the lipid residues were redissolved in light petroleum (b.p. 40–60°) and dialysed through rubber membranes to remove the fatty acids. The dialysis residues (Ia, IIa, IIIa and IVa) were analysed for P and carbohydrate (Table 1).

EXPERIMENTAL AND RESULTS

Paper chromatography (Marinetti *et al.* 1957) showed that the acetone extract (I) contained most of the neutral lipid in the tumour cells. The light-petroleum extract (II) contained the remainder. The phospholipids (4854 mg. of P) were mainly in extracts III and IV (Table 1). Mild alkaline hydrolyses of the four extracts produced 4177 mg. of water-soluble P (88% of total P). Analysis of the alkali-stable lipid in each of the extracts Ia, IIa, IIIa and IVa showed that Ia and IIa together contained only 5% of the total hexose but 17% of the total alkali-stable P compounds, and were discarded in order to simplify the subsequent isolation and purification of the glycolipids.

Chromatography on silicic acid-impregnated paper showed that the phospholipids in fractions IIIa and IVa were mainly sphingomyelin and lysoplasmalogen. There were some minor compounds including a little unhydrolysed acylglycerophosphatide. Gray (1963) found that the glycolipids in the tumour cells were completely eluted from a silicic acid (Mallinckrodt) column with methanol concentrations in chloroform up to 35%, and so the sphingomyelin in fractions IIIa and IVa was next separated from the glycolipids by silicic acid chromatography.

Scheme 1. Flow sheet for the isolation of glycolipids from a lipid extract of BP8/C3H ascites-carcinoma cells.

Chromatographic purification of glycolipids

Fraction IIIa (see Scheme 1) dissolved in chloroform-methanol (19:1, v/v) was chromatographed on a silicic acid (Mallinckrodt) column (120 g.) in the same solvent, and the eluted fractions were monitored by thin-layer chromatography and analysed for P (Bartlett, 1959). A stepwise increase in the concentration of methanol in chloroform was made from 5, 15, 25, 35 and finally to 50%. Fractions were combined, according to their content as indicated by thin-layer chromatography and analysis, into two fractions, A and B (Scheme 1). Fraction A contained glucose, galactose and a trace of mannose, and fraction B contained glucose, galactose, galactosamine and traces of mannose. Fraction IVa (Scheme 1) dissolved in chloroform-methanol (19:1, v/v) was chromatographed on a silicic acid (Mallinckrodt) column (220 g.) in the same solvent, and the procedure for development and analysis of the eluted fractions was as described for IIIa. The fractions were pooled to give two fractions, A₁ and B₁, that were qualitatively similar to A and B respectively. Fractions A and A₁ (AA₁) and B and B₁ (BB₁) were combined for further fractionation and purification of the glycolipids (Scheme 1).

The P compounds remaining in the fractions eluted from the silicic acid columns were not effectively removed by chromatography on Florisil (Radin, Brown & Lavin, 1956), though this procedure had proved satisfactory in the purification of glycolipids from other tissues (Rapport *et al.* 1960; Yamakawa *et al.* 1963). Chromatography on an alumina (Woelm) column (120 g.) by the method of Long & Staples (1961) partially removed P compounds from fraction AA₁ (Scheme 1); it did not remove the P compounds from fraction BB₁. A good separation of most of the P compounds from the glycolipids and the glycolipids from each other was observed when samples of AA₁ and BB₁ were chromatographed on thin-layer plates of silica gel G in the solvent system chloroform-methanol-water (70:30:3, by vol.), and so it was expected that a satisfactory purification and separation of the glycolipids in AA₁ and BB₁ would be obtained by chromatography on columns of silica gel G.

Fraction AA₁ dissolved in chloroform was loaded on to a column (80 g.) of silica gel G (Merck) in chloroform, and development was carried out with chloroform-methanol-water (90:10:1, by vol.). Two cleanly separated carbohydrate-containing compounds were eluted from the column (Scheme 1). The first, G₁, was free from P, but the second, G₂, contained a small amount of P (less than 1 mg.) that was removed by passing a solution of the material in chloroform-ethanol-water (2:5:2, by vol.) through Bio-Deminrolit (The Permutit Co.

Ltd., London, W.4) (mixed bed resin). Fraction BB₁ in chloroform was loaded on to another column (80 g.) of silica gel G (Merck) in chloroform. Development of the column was carried out with chloroform-methanol-water (70:30:3, by vol.), and analysis of the fractions from the column showed that BB₁ was separated into three carbohydrate-containing compounds (Scheme 1), G₃, G₄ and G₅. They were still contaminated with P compounds and they were treated with Bio-Deminrolit as for G₂. G₃ and G₄ were quantitatively recovered free from P, but G₅ was not eluted from the column of Bio-Deminrolit. A small amount was recovered from the resin after eluting with 0.1 N-hydrochloric acid, but this also eluted P. Most of the fraction was still strongly adsorbed and no further attempt at recovery was made.

The four glycolipid fractions G₁, G₂, G₃ and G₄ were each reprecipitated twice from chloroform or chloroform-methanol (4:1, v/v) with acetone. The recovery of glycolipid was 90–95%. They gave colourless solutions in chloroform-methanol (9:1 or 4:1, v/v), though on standing for long periods (e.g. 7 days) at 0° crystal aggregates in the form of spherulids gradually formed on the walls of the tubes. Each fraction when chromatographed on silicic acid-impregnated paper in di-isobutyl ketone-acetic acid-water (40:20:3, by vol.) for 18 hr. at 2° gave only one clear yellow spot, viewed under ultraviolet light, after staining with rhodamine G. The *R_F* values under these conditions were: G₁ 0.66; G₂ 0.43; G₃ 0.26; G₄ 0.09. Thin-layer chromatography on silica gel G (or H) with chloroform-methanol-water (70:30:3, by vol.; or 65:25:4, by vol., according to the procedure of Wagner, Hörhammer & Wolff, 1961) showed that each fraction was free from contaminants. Each fraction, however, appeared as a double spot, which according to Svennerholm & Svennerholm (1963b) indicated the presence of hydroxy fatty acids (slower-moving spot) in the glycolipid as well as normal saturated fatty acids.

Identification of the glycolipids

Fraction G₁. The chromatographic properties of G₁ on silicic acid-impregnated paper, thin-layer plates of silica gel G (or H) and alumina in various solvents were similar to those of an authentic sample of ceramide galactoside from human brain. The molar ratio of sphingosine to hexose (Table 2) was 1:0.93. The only hexoses detected were glucose, galactose and a trace of mannose. It was concluded from the results (Table 2) that G₁ was mainly ceramide glucoside (70%) and ceramide galactoside. Monohydroxy fatty acids and normal fatty acids were identified after chromatography (Mangold & Malins, 1960) of the ether extract of an

Table 2. *Analyses of the glycolipids isolated from BP8/C3H ascites-sarcoma cells*

Experimental details are given in the text. Glucose was determined with glucose oxidase (Dahlqvist, 1961). The values in parentheses are calculated values based on the structures lignocerylphingosylglucoside (for G1), lignocerylphingosylgalactosylglucoside (for G2), lignocerylphingosylgalactosylgalactosylglucoside (for G3) and lignocerylphingosyl-*N*-acetylgalactosaminylgalactosylgalactosylglucoside (for G4).

	Composition of G1		Composition of G2		Composition of G3		Composition of G4	
Glycolipid (mg./100 g. of freeze-dried cells)	...	...25		9.5		3.8		6.4
Glycolipid (% by wt. of total lipid)	...	... 0.2		0.07		0.03		0.05
Glycolipid (% by wt. of total glycolipid)	...	...55		21		9		15
	Percentage based on dry wt.		Percentage based on dry wt.		Percentage based on dry wt.		Percentage based on dry wt.	
	Molar proportions		Molar proportions		Molar proportions		Molar proportions	
Sphingosine	33 (35)	1	29 (29)	1	25 (24.8)	1	22 (21)	1
Hexose	19.5 (21)	0.93	32.5 (35)	1.87	39 (44.8)	2.65	40 (38)	3
Glucose	13.6 (21)	0.65	12.5 (17.5)	0.72	9.1 (14.9)	0.62	11.6 (12.6)	0.87
Hexosamine	—	—	—	—	—	—	12.1 (15.5)	0.86

Table 3. *Fatty acid composition of the glycolipids from BP8/C3H ascites-sarcoma cells*

Experimental details are given in the text. Values are expressed as percentages of total methyl esters. Mono-hydroxy acids are not included.

Fatty acid and designation	Fatty acid composition			
	G1	G2	G3	G4
Palmitic acid (16:0)	19	18	16	18
Stearic acid (18:0)	2	2	4	3
Octadecenoic acid (18:1)	2	—	—	—
Arachidic acid (20:0)	1	1	2	2
Behenic acid (22:0)	14	15	17	15
Docosamonoenoic acid (22:1)	—	Trace	—	—
Tricosanoic acid (23:0) (?)	2	2	2	2
Lignoceric acid (24:0)	27	29	29	29
Nervonic acid (24:1)	32	31	27	29
Tetracosadienoic acid (24:2)	1	2	4	2

aqueous acid hydrolysis of G1. A visual estimation of the hydroxy fatty acids on the chromatogram indicated a value of about 20% of the total fatty acids. Major components of the normal fatty acids were lignoceric acid (27%) and nervonic acid (32%) (Table 3). No attempt was made to identify the exact quantity and composition of the hydroxy acids at this stage, because of the inherent difficulties of the quantitative separation of the hydroxy acids from the normal fatty acids and of the preparation of suitable derivatives for analysis by gas-liquid chromatography, when only a few milligrams of material were available. G1 was the major component of the isolated glycolipids, but its absolute amount (0.2%) in terms of total lipid in the tumour cells was very low (Table 2).

Fraction G2. The glycolipid G2 accounted for 21% by weight of the total glycolipid isolated from the tumour cells. The molar ratio of sphingosine to hexose was 1:1.87. Glucose, galactose and a trace of mannose were identified. The total hexose accounted for 32.5% by weight of the glycolipid (Table 2); the carbohydrate in the intact glycolipid did not contain a free reducing group. The analysis showed that G2 was a ceramide dihexoside and the structure of the disaccharide(s) was determined by partial hydrolysis with polystyrenesulphonic acid (Painter, 1960). This water-soluble acid has selective catalytic properties (Painter, 1961), and the yields of oligosaccharides formed by the hydrolysis of a polysaccharide with polystyrenesulphonic acid are much higher than the maximum yields obtainable by mineral acid (Painter, 1962).

The glycolipid (3.6 mg.) was suspended in 0.4 ml. of water, 0.4 ml. of 0.4 *N*-polystyrenesulphonic acid was added, and the tube was half-immersed in a water bath at 90° and stoppered. Samples (0.05 ml.) were removed at intervals and spotted on Whatman no. 1 chromatography paper. The spots were neutralized on the paper with 0.05 ml. of aq. 10% (v/v) pyridine. Chromatography was carried out in ethyl acetate-pyridine-water (10:4:3, by vol.) for 24 hr. Chromatograms were sprayed with silver nitrate-sodium hydroxide reagent and the intensities of the spots were a visual measure of the amounts of saccharides present during the course of hydrolysis. The results summarized in Table 4 showed that only two disaccharides, lactose and a substance tentatively identified as 6-*O*- β -galactosyl-galactose (R_{lactose} 0.71), were present in G2. The slow liberation of glucose compared with that of galactose indicated that the lactose was linked through glucose

Table 4. *Products of the partial hydrolyses of the glycolipids with polystyrenesulphonic acid*

Experimental details are given in the text. All samples were chromatographed on paper and the products identified with reference to authentic compounds. Authentic markers of 6-*O*- α - and 6-*O*- β -galactosylgalactose were gifts from Dr T. J. Painter, Lister Institute, London, S.W. 1. The first sample from the hydrolysate was taken after 15 min.

Glycolipid	Identity of products and time of detection	Comment
G2	Galactose (15 min.)	Concentration increased steadily with time of hydrolysis
	Lactose (30 min.)	Low concentration, reaching a maximum over the period 1-4 hr.; small amount still present after 18 hr.
	Glucose (1 hr.)	Liberated at much slower rate than galactose but concentration increased steadily with time
G3	6- <i>O</i> - β -Galactosylgalactose (1 hr.)	Present at very low concentration (R_{Lactose} 0.71)
	Galactose (15 min.)	Concentration increased steadily with time
	Glucose (30 min.)	Liberated at much slower rate than galactose; concentration increased steadily with time.
	Lactose (1 hr.)	Low concentrations, reaching a maximum over the period 2-6 hr.; small amounts still present after 30 hr.
G4	6- <i>O</i> - β -Galactosylgalactose (1 hr.)	Very low concentration
	Trisaccharide? (1 hr.)	Concentration increased steadily with time
	Galactose (15 min.)	As for G3
	Glucose (1 hr.)	Low concentrations; less lactose than galactosylgalactose
	Lactose (2 hr.)	Concentration higher than that of either lactose or galactosylgalactose, reaching a maximum over the period 3-6 hr. (R_{Lactose} 1.24)
	6- <i>O</i> - β -Galactosylgalactose (2 hr.)	Concentration increased with time
	Disaccharide (<i>N</i> -acetylgalactosaminylgalactose?) (2 hr.)	Very low concentration (R_{Lactose} 0.56)
	Galactosamine (3 hr.)	Very low concentration (R_{Lactose} 0.32)
	Trisaccharide (a)*? (2 hr.)	
	Trisaccharide (b)? (2 hr.)	

* Alternatively, galactosaminylgalactose.

to the ceramide moiety. The absolute amount of glucose (Table 2) indicated that G2 was mainly a ceramide lactoside similar to cytolipin H (Rapport *et al.* 1960), with a minor component (approx. 12% of G2) identified as ceramide galactosylgalactoside.

G2 contained both monohydroxy and normal fatty acids. The normal fatty acids, which accounted for at least 80% of the total acids, had a composition very similar to that of G1, with lignoceric acid and nervonic acid predominating (Table 3).

Fraction G3. The glycolipid G3 was the smallest component of the isolated glycolipids (Table 2). It contained galactose, glucose and a trace of mannose. The molar ratio of sphingosine to hexose was 1:2.65. Analysis and chromatography showed that G3 was a ceramide trihexoside, and partial hydrolysis with polystyrenesulphonic acid showed that two disaccharides, lactose and the compound tentatively identified as 6-*O*- β -galactosylgalactose, were amongst the hydrolysis products (Table 4). The slow release of glucose and rapid release of galactose indicated that the trisaccharide was linked through glucose to the ceramide moiety. It was concluded that G3 was probably a ceramide digalactosylglucoside. The ratio of glucose to galactose (Table 2) suggested that a small amount of the glycolipid (about 10%) had a trisaccharide moiety containing

only galactose, but it is possible that the hydrolysis was incomplete, which would account for the low hexose value (Table 2) and the low glucose value, since glucose was liberated much more slowly than galactose. The composition of the normal fatty acids was very similar to those of G1 and G2. About 10% of the total fatty acids were monohydroxy acids.

Fraction G4. The glycolipid G4 was the only compound isolated that contained hexosamine. The only water-soluble products of an acid hydrolysis of the glycolipid were galactose, glucose and galactosamine. The total reducing sugar, with a galactose-glucose (1:1) mixture as the standard, was 49% of the dry weight of glycolipid. The hexosamine amino group gave no reaction with ninhydrin before acid hydrolysis of the glycolipid, which indicated that the galactosamine was probably *N*-acetylated. The results (Table 2) showed that G4 was a ceramide hexosaminyltrihexoside.

Partial acid hydrolysis of G4 under the same conditions as for G2 and G3 gave a number of products (Table 4). Several products were formed during the course of hydrolysis that were not identified because no reference compounds were available for comparison, but their R_{Lactose} values

served as guides to probable structure in general terms (Table 4). The results were consistent with a characterization of G4 as a ceramide *N*-acetylgalactosaminylgalactosylgalactosylglucoside. The glycolipid contained both normal and monohydroxy fatty acids. The normal fatty acids accounted for about 75% of the total acids, and their composition was similar to those of G1, G2 and G3 (Table 3).

Lack of material prevented the identification of the linkages between the monosaccharide units in the glycolipids by the usual chemical methods, but the chromatography of the products of partial hydrolysis provided some evidence of structure. The presence of lactose is indicative of an α -(1 $\rightarrow$ 4)-link between glucose and galactose. Another product of hydrolysis was chromatographically similar to 6-*O*- β -galactosylgalactose. The glycolipids G3 and G4 did not show blood-group substance A or B activity in inhibition tests carried out by Dr W. M. Watkins. This means that 3-*O*- α -galactosylgalactose (B activity) and 3-*O*-*N*-acetyl- α -galactosaminylgalactose (A activity) disaccharide units are probably not present in the molecules.

DISCUSSION

The glycolipids accounted for only about 0.5% of the total lipids in the tumour cells, and their complete separation from other lipids without losses was a major practical problem. A systematic extraction with acetone and light petroleum removed 60% of the total lipid, including all the neutral lipids, with a loss of 5% of the total glycolipid. The simplicity and quickness of this extraction, compared with more refined chromatographic techniques, recommends it for work where large amounts (more than 50 g.) of neutral lipid are to be separated from extracts containing glycolipids. A subsequent extraction of the tumour cells with pyridine (Rosenheim, 1913) is of little practical value in separating phospholipid from glycolipid, and the extraction is simplified if, after treatment with acetone and light petroleum, chloroform-methanol (2:1, v/v) is used to remove all the remaining lipid.

It was difficult to remove certain phospholipids from the glycolipids in the alkali-treated extracts IIIa and IVa by Florisil or alumina or silicic acid chromatography. Other workers using similar techniques for isolating glycolipids from other tissues have not reported difficulty of this nature (Gatt & Berman, 1963; Svennerholm & Svennerholm, 1963b; Yamakawa *et al.* 1963), and it is possible that the ascites-tumour tissue contains small amounts of one or more unique phospholipids (possibly artifacts).

Four different glycolipids were isolated and the

recovery based on total hexose values of the crude lipid extracts III and IV was about 76%. This is regarded as a satisfactory recovery considering the complicated isolation procedure. The ceramide monohexoside (G1) contained both glucose and galactose, though the glycolipid present in tissues other than those of the central nervous system is reported to contain only glucose (Svennerholm & Svennerholm, 1963b). It is likely that the ceramide monohexoside reflects a lack of selectivity, also found in the phospholipid fatty acids (Gray, 1963), which appears to be a property of the functional systems in tumour cells (Greenstein, 1954). Rapport, Graf & Yariv (1961) showed that the ceramide dihexoside (cytolipin H) isolated from human epidermoid tumour tissue grown in conditioned rats and also from spleens of normal animals contained exclusively the disaccharide lactose. It also appeared to be the only disaccharide present in the ceramide dihexoside from blood serum (Svennerholm & Svennerholm, 1963b). G2 was mostly ceramide lactoside, but a minor fraction was identified as a ceramide galactosylgalactoside and was similar to a compound isolated from Tay-Sachs brain lipids (Gatt & Berman, 1963). The ceramide trihexoside was similar to compounds isolated from spleen, blood serum and kidney (Yamakawa *et al.* 1963; Svennerholm & Svennerholm, 1963b; Martensson, 1963), and in Fabry's disease it appears to accumulate in the kidney (Sweeley & Klionsky, 1963). The galactosamine-containing glycolipid (G4) was similar to a lipid hapten (cytolipin K) isolated from human kidney by Rapport, Graf & Schneider (1963), and also to compounds isolated from blood serum (Svennerholm & Svennerholm, 1963b) and erythrocyte stroma (Yamakawa *et al.* 1963).

The work described in the present paper and in the others quoted tends to show that a tissue is likely to contain not one class of glycolipid but several classes containing oligosaccharides of increasing size. There is a very close similarity in the fatty acid composition of each of the four glycolipids isolated from the tumour cells, and the question arises whether or not a close metabolic relationship exists between them. For instance, if *N*-acetylneuraminic acid were linked to the *N*-acetylgalactosamine of the glycolipid G4 the resulting compound would be a ganglioside (a compound of this particular composition was isolated from ox brain by Kuhn, Wiegandt & Egge, 1961), and a systematic enzymic degradation of the ganglioside could produce the glycolipids G4, G3, G2 and G1. On the other hand each glycolipid might arise by the separate synthesis of each oligosaccharide moiety followed by condensation with the same ceramide, and the fatty acid composition of each glycolipid would then be similar. A

third possibility is the stepwise addition of saccharide residues to a basic unit, i.e. a ceramide monohexoside, to yield successively G2, G3 and G4 compounds, but at present there is no experimental evidence to indicate any particular pathway for the biosynthesis of the glycolipids.

One of the main problems emphasized by studies in the phospholipid field is the difficulty of relating structure to function and, in the animal cell, conclusive evidence of specific functions for most of the phospholipids has not been obtained. The situation seems more promising with respect to the glycolipids. It has been shown that certain glycolipids are lipid haptens and it is probable that all glycolipids have haptenic function (Rapport, Graf, Skipski & Alonzo, 1959; Rapport *et al.* 1963; Joffe, Rapport & Graf, 1963). Recent work on the blood-group substances (Côté & Morgan, 1956; Painter, Watkins & Morgan, 1962; Rege, Painter, Watkins & Morgan, 1963) has shown that certain small oligosaccharide sub-units exhibit the serologically specific activity of the whole substance, albeit with a much lower order of magnitude. The glycolipids might be considered therefore as lipid carriers for a variety of oligosaccharides, each with a different immunological function.

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