changes in micellar organisation. An analysis of variance (Table I) of the rates of sialic acid release at periods of low and of high rate showed significant differences between the graphically selected groups of rate measurements, but no significant differences between types of sialic acid derivative, including the erythrocyte material.

Further characterisation of this material is being carried out concurrently with a survey of its occurrence in erythrocytes from human subjects in normal and in a variety of pathological conditions.

This work is supported by a Research Fund from the Governors of the Bethlem Royal and Maudsley Hospitals.

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Received April 5th, 1963

Biochim. Biophys. Acta, 70 (1963) 486–487

PN 1266

Isolation and analysis of mono-, di-, and trisialogangliosides

The heterogeneity of ganglioside preparations in chromatographic systems has been well documented; however, the number of resolvable components and their composition reported by various investigators has varied (see refs. 1–5). This investigation sought to confirm or disprove some of the reports which were in conflict regarding the number of components and their composition. The resolution of 8 gangliosides by thin-layer chromatography has been noted, 5 of which are readily demonstrable. Homogeneous preparations of 4 of these 5 have been isolated, and their compositions closely correspond to theoretical values.

Gangliosides prepared from normal human gray matter as previously described[6,7] were subjected to thin-layer chromatography on Silica Gel G (E. Merck, A. G. Darmstadt, Germany) utilizing chloroform–methanol–water (60:35:8, v/v) (ref. 8). The dried plates were sprayed with resorcinol reagent[9] and the color developed in an HCl-saturated closed chamber at 110–120°. When minimal amounts of material were spotted, areas representing 4 major gangliosides were observed which were designated 1-G, 2-G, 3-G, and 4-G in order of decreasing chromatographic mobility. A fifth, faster-moving ganglioside designated FM was seen when larger amounts of gangliosides were chromatographed. 3 additional gangliosides with the fastest migration on

thin-layer chromatography were observed when very large amounts (700 μg) were chromatographed. Small amounts of two of these minor gangliosides were isolated by preparative thin-layer chromatography and appeared to contain 2 moles of hexose per mole of sialic acid[7].

Gangliosides (750 mg) were dissolved in chloroform–methanol and slurried with a small amount of Anasil S (Analytical Engineering Laboratories, Inc., Hamden, Conn.). The solvent was evaporated and the ganglioside–Anasil mixture was placed on a dry-packed column (30 × 3 cm) in the cold room. Elution was begun with chloroform–methanol–water (65 : 30 : 2, v/v), and 1-ml fractions were collected manually. When ganglioside ceased to be eluted from the column, the solvent was changed to chloroform–methanol–water (60:37:8, v/v), and 4-ml fractions were collected. All fractions were analyzed by thin-layer chromatography as above.

A faint yellow band of non-ganglioside material was first eluted from the column, closely followed by fractions containing a mixture of 4 resorcinol-positive spots each of which had a higher R_F than 1-G. Fractions were then obtained which contained only the slowest of these fast moving spots. This material was chromatographically identical to FM. Fractions were next eluted which contained only 1-G.

Fractions which contained only a single resorcinol-positive area corresponding consecutively to FM, 1-G, 3-G, and 4-G were eluted intermittently with the second solvent. It was noted that instead of 2-G being eluted after 1-G, a mixture of FM, 1-G, and 2-G was eluted. Fractions containing only 2-G were not detected.

Fractions were then pooled with care to assure homogeneous preparations, concentrated by rotary evaporation, and lyophilized. In order to remove contaminating silicic acid, the fractions were dissolved in minimal amounts of solvent, filtered, and lyophilized. 4-G did not readily dissolve in chloroform–methanol mixtures under these conditions, but was soluble in water. The converse was observed for FM. The 4-G material, which apparently still contained some silicic acid, was further purified by washing with acetone.

The 4 preparations appeared homogeneous by thin-layer chromatography with chloroform–methanol–water (60:35:8, v/v), and with *n*-propanol–water (7 : 3, v/v) (ref. 3), when approx. 75 μg were spotted. When larger quantities of FM were chromatographed, 1-G appeared as a contaminant.

N-Acetylneuraminic acid content was determined by the resorcinol assay[9,10]. Hexosamine analyses[11] were made after hydrolysis with 2 N HCl for 18 h at 100°, except for FM which was hydrolyzed with 5 N HCl for 7 h. Hexose values were determined with the phenol–H_2SO_4 assay[12] after hydrolysis with 2 N H_2SO_4 for 7 h at 100°; FM was hydrolyzed for 5 h with 3 N H_2SO_4. Galactose/glucose ratios were also determined. H_2SO_4 hydrolysates were neutralized with Dowex-1 (CO_3^{2-}), spotted on Whatman No. 1 paper, and irrigated with benzene–*n*-butanol–pyridine–water (1:5:3:3, v/v) (ref. 13) for 40–48 h. Areas corresponding to glucose and galactose of the reference strip were cut out, eluted with water, and the filtrates were analyzed for hexose. The percentage composition and molar ratios of carbohydrate components in the 4 compounds are shown in Table I. The presence of the aliphatic ceramide moiety in each of these gangliosides was demonstrated by a strong C–H absorption band at 3.4–3.5 μ.

The presence of three sialic acid residues in a ganglioside molecule first proposed by KUHN *et al.*[3] has been confirmed by JOHNSON[7] and by WOLFE AND LOWDEN[14].

TABLE I

CARBOHYDRATE COMPOSITION OF FOUR HUMAN BRAIN GANGLIOSIDES

	Gangliosides							
	FM		*1-G*		*3-G*		*4-G*	
	Theor.	*Detd.*	*Theor.*	*Detd.*	*Theor.*	*Detd.*	*Theor.*	*Detd.*
*Composition** (%)								
N-Acetylneuraminic acid	22.3	22.2	20.0	19.9	33.7	28.8	43.5	42.6
Hexosamine	12.9	10.9	11.6	12.0	9.7	9.9	8.4	7.9
Hexose	26.0	25.0	35.0	34.4	29.4	23.8	25.4	24.5
*Molar ratios***								
N-Acetylneuraminic acid	1	1.0	1	1.0	2	2.0	3	3.0
Hexosamine	1	0.85	1	1.1	1	1.2	1	0.96
Hexose	2	1.9	3	3.1	3	2.8	3	3.0
Galactose/glucose ratio	1	1.1	2	1.9	2	1.9	2	1.7

* The theoretical per cent composition was calculated on molecules containing a C_{18} sphingosine, stearic acid and the number of carbohydrate residues indicated in the theoretical molar ratios.

** The theoretical ratios are the lowest whole integers indicated from the per cent composition. The determined ratios were calculated relative to the moles of *N*-acetylneuraminic acid.

The 3 major gangliosides (1-G, 3-G, 4-G) isolated here correspond in composition and in chromatographic mobility to gangliosides G_1, G_3, and G_4 reported by KUHN *et al.*[3]. Although we were not able to isolate a homogeneous preparation of 2-G, the relative chromatographic mobility of this component suggests that 2-G is the second disialoganglioside (G_2) described by KUHN *et al.*[3]. Ganglioside FM, containing equal amounts of glucose and galactose, is apparently a fifth ganglioside not reported by KUHN *et al.*[3] and corresponds in composition to "Ganglioside A" of KLENK AND GIELEN[2] and the "Tay–Sachs' ganglioside" of SVENNERHOLM[4]. Hexosamine-free gangliosides were not detected.

It thus appears that the 4 major gangliosides of normal human brain (1-G, 2-G, 3-G and 4-G) consist of a mono-, 2 di-, and a trisialoganglioside each containing 2 moles of galactose. 1 of the 4 minor gangliosides (FM) is a monosialoganglioside containing 1 mole of galactose and may correspond to the major component of "Tay–Sachs' gangliosides". Preliminary data indicate that the remaining minor gangliosides contain 2 moles of hexose per mole of sialic acid.

This work was supported in part by Grants No. E-3887 and GF-17 338 from the U.S. Public Health Service.

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Received April 11th, 1963

PN 1282

Some observations on the mechanism of absorption of cholesterol in rats

Several aspects of the absorption of cholesterol have been actively studied for quite some time now, and yet very little was known about the actual mechanism of its absorption till GLOVER AND GREEN[1] and GANGULY *et al.*[2] put forward the hypothesis that cholesterol is probably absorbed by a process in which the sterol presented to the external side of the mucosal cell readily displaces a similar molecule already present in the lipoproteins of the cell membrane and thereby penetrates the cell. The above hypothesis was based upon the observation that no significant changes can be noticed in the sterol contents of the intestinal-mucosal cells of rats following either starvation, or feeding of cholesterol. A direct proof for the hypothesis was rendered difficult by the fact that the endogenous cholesterol is always present in large quantities in the mucosal cells, almost entirely in the free form[2], and that it is in this form that the sterol enters the cell[3]. However, the endogeneous cholesterol can readily be differentiated from the newly absorbed sterol by feeding ^{14}C-labelled cholesterol. Using such ^{14}C-labelled cholesterol it is now shown here that 74–77% of the newly absorbed sterol is recovered from the microsomal particles of the mucosal cell homogenate, the supernatant fraction being almost free of it.

[4-^{14}C]Cholesterol (0.4 μC), obtained from Radiochemical Centre, Amersham, after dilution with 3 mg of cholesterol, was dissolved in minimal amounts of diethyl ether. The other solution was poured on 500 mg of a normal stock diet and the solvent was allowed to evaporate off, after which the diet was thoroughly mixed. Normal male rats of this Institute strain weighing 120–130 g were starved for 18 h and individually offered 500 mg of the cholesterol-mixed diet. Usually the rats consumed the entire diet within 5 min of offering. Water was given *ad libitum*. At the given time intervals the rats were killed by direct heart puncture, while under ether anaesthesia. The small intestine was immediately removed and transferred into a beaker previously placed in crushed ice. It was then processed according to GANGULY *et al.*[2] with the difference that the microsomal fraction was collected by centrifuging the mitochondrial supernatant at 104000 $\times$ *g* for 60 min in a Spinco model L ultracentrifuge. The lipids of the sub-cellular particles of the mucosa and of the muscles of the small intestine were extracted by boiling for 10 min with 3 volumes of an alcohol–ether mixture (3:1, v/v), followed by two extractions with light petroleum