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Sphingolipids in Blood Platelets of the Pig

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Summary: Sphingolipids isolated from platelets of the pig were identified as ceramides, glucosyl- and galactosylceramides, lactosylceramides, sulfatides and galactosyl-galactosyl-glucosyl ceramides. Bis-(*N*-glycoloylneuraminosyl)-lactosylceramide represents the major component out of at least eight different gangliosides. Analytical evidence is presented for the occurrence of a bis(*N*-glycoloylneuraminosyl)glucosyl-glucosylceramide in this ganglioside fraction.

The long chain base, fatty acid and carbohydrate composition was determined for all sphingolipids except the minor gangliosides. Ceramides comprised 45% of the neutral glycolipid and sulfatide fractions. Ceramide trihexosides occur as the main neutral glycolipid fraction. 4*t*-Sphingenine is the predominant long chain base, the saturated and unsaturated C_{16}- and C_{20}-homologues account for 4% of the total bases. Greater amounts of sphinganine were present in one of the three different ceramide classes and in the main ganglioside fraction. 4D-Hydroxysphinganine was found in one of the ceramide fractions and hydroxy fatty acids were detected in high amounts in another ceramide fraction. The latter were also present in smaller amounts in the glycosylceramides and the sulfatide fraction.

Sphingolipoide in Schweinethrombozyten

Zusammenfassung: Die Sphingolipoide von Schweinethrombozyten wurden isoliert und als Ceramide, Glucosyl- und Galaktosylceramide, Lactosylceramide, Sulfatide und Galaktosyl-galaktosyl-glucosylceramide identifiziert. Bis(*N*-glykoloylneuraminosyl)-lactosylceramide bilden die Hauptfraktion von wenigstens acht Gangliosiden. Aufgrund der analytischen Daten enthält diese Gangliosidfraktion ein Bis(*N*-Glycoloyl-neuraminosyl)glucosyl-glucosylceramid.

Die langkettigen Basen, Fettsäuren und die Kohlenhydratbausteine aller Sphingolipoide, ausgenommen die der in geringer Menge vorkommenden Ganglioside, wurden bestimmt. 45% der

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Enzymes:

α-Galactosidase, α-D-galactoside galactohydrolase (EC 3.2.1.22)
Neuraminidase, glycoprotein *N*-acetylneuraminylhydrolase (EC 3.2.1.18)
Phospholipase C, phosphatidylcholine cholinephosphohydrolase (EC 3.1.4.3).

Abbreviations:

TMS = trimethylsilyl; ECNSS-M = mixed polymer of ethylen glycol succinate and cyanoethylsilicone (Applied Science Laboratories Inc.).

Gangliosides:

$A_1(G_{M2})$ = *N*-acetylgalactosaminyl-(sialosyl-)lactosylceramide;
$A_2(G_{M1})$ = galactosyl-*N*-acetylgalactosaminyl-(sialosyl-)-lactosylceramide;
$B_1(G_{D1a})$ = sialosyl-galactosyl-*N*-acetylgalactosaminyl-(sialosyl)-lactosylceramide;
B_3 = *N*-acetylgalactosaminyl-(disialosyl)-lactosylceramide;
$C_1(G_{D1b})$ = galactosyl-*N*-acetylgalactosaminyl-(disialosyl)-lactosylceramide;
C_2 = sialosyl-galactosyl-*N*-acetylgalactosaminyl-(sialosyl)-lactosylceramide;
$C_3(G_{T1})$ = sialosyl-galactosyl-*N*-acetylgalactosaminyl-(disialosyl)-lactosylceramide;
C_4 = disialosyl-galactosyl-*N*-acetylgalactosaminyl-(disialosyl)-lactosylceramide.

neutralen Glykolipoid- und Sulfatidfraktion sind Ceramide. Die Ceramidtrihexoside bilden die Hauptfraktion der Ceramidglykoside. Überwiegend *4t-Sphingenin kommt als langkettige Base* in den Thrombozyten-Sphingolipoiden vor; die Summe der gesättigten und ungesättigten C_{16}- und C_{20}-Homologen liegt um 4% der Gesamtbasen. Sphinganin wurde in größeren Mengen in einer der drei verschiedenen Ceramidklassen und der Hauptgangliosidfraktion angetroffen. 4D-Hydroxysphinganin wurde in einer der Ceramidfraktionen gefunden *und Hydroxyfettsäuren in großen Mengen in* einer weiteren Ceramidfraktion und in kleineren Mengen in den Glycosylceramiden und der Sulfatidfraktion.

The lipids of blood platelets are thought to be involved in blood coagulation. It has been suggested that a phospholipid acts as a coagulation factor[1–8]. Therefore considerable interest has been directed to the analysis of the lipid composition of whole platelets and subcellular fractions particularly of human origin. The total lipid content of platelets amounts to 15–20% of the dry weight of these particles[4,5]. Phospholipids represent about 80% of the total lipids of platelets[1,6,9–11], out of which more than 15% have been identified as plasmalogens predominantly of the "cephalin-type"[3,12,13]. Fatty acid analyses indicated that stearic acid is the main saturated and arachidonic acid the main unsaturated fatty acid component[3,12,14].

Cholesterol amounted up to 20% of the lipid mixture[9,15,16]. So far the occurrence and the structure of glycosphingolipids in platelets have not been examined with great care. Only Marcus *et al.*[17] have reported the occurrence of five different gangliosides, the main component of which has been tentatively identified as hematoside. Glycosylceramides and sulfatides could not be detected by these authors[11]. The present work concerns the analyses of the sphingolipids and glycosphingolipids in pig platelets.

Results and Discussion

The glycosphingolipid fraction was isolated from the total lipid extract, previously distributed according to Folch[18] and the ester lipids were removed by alkaline hydrolysis. Thin-layer chromatographic separation of this fraction yielded four different glycosphingolipids. They were identified as monoglycosylceramides, diglycosylceramides, sulfatides and triglycosylceramides by comparison of their properties in co-chromatography with well characterized standards prepared from bovine spleen and human brain. The purity of these fractions is indicated in Fig. 1.

The absence of other and more complicated neutral glycolipids such as tetraglycosylceramides (globosides) indicates the high purity of the pooled platelets which appeared essentialy free of erythrocytes. Pig erythrocytes contain globosides and

[1] Hecht, E. R. (1955) in Lipids of Blood Clotting, p. 113, Charles Thomas, Springfield, Ill.

[2] Fischer, M. & Deutsch, E. (1969) *Hämatologie und Bluttransfusion (Sonderbände „Blut") 6*, 89–103.

[3] Eggstein, M. & Gross, R. (1968) in Stoffwechsel u. Membranpermeabilität v. Erythrocyten u. Thrombocyten (Deutsch, E., Gerlach, E. & Moser, K., eds.) pp. 222–224, G. Thieme Verlag, Stuttgart.

[4] Deutsch, E. & Stockinger L. (1963) *Wien. Z. Inn. Med.* **44**, 61–84.

[5] Marcus, A. J., Ullman, H. L. & Wolfman, M. (1960) *J. Lipid Res.* **1**, 179–187.

[6] Troup, S. B., Reed, C. F., Marinetti, G. V. & Swisher, S. N. (1961) in Blood Platelets, p. 265, Little, Brown & Co., Boston.

[7] Karach, M. & Stefanini M. (1966) *J. Lab. Clin. Med.* **67**, 229–245.

[8] Okany, A. & Palagy, C. (1966) *Can. J. Biochem.* **44**, 1235.

[9] Marcus, A. J. & Zucker, M. B. (1965) in The Physiology of Blood Platelets p. 2, Grune & Stratton Inc., New York.

[10] Pries, C., Aumont, A. & Böttcher, C. J. F. (1966) *Biochim. Biophys. Acta* **125**, 277–287.

[11] Marcus, A. J., Ullman, H. L. & Safier, L. B. (1969) *J. Lipid Res.* **10**, 108–114.

[12] Marcus, A. J., Ullman, H. L., Safier, L. B. & Ballard, H. S. (1962) *J. Clin. Invest.* **41**, 2198–2212.

[13] Gross, R. (1961) in Blood Platelets p. 407–421, Little, Brown & Co., Boston.

[14] Marcus, A. J. (1966) in *Advan. Lipid Res.* **4**, p. 1–32.

[15] Barkman, P., Silver, M. J. & O'Keefe, L. M. (1961) in Blood Platelets p. 303–318, Little, Brown & Co., Boston.

[16] Smith, E. P. (1967 *Cardiovasc. Res.* **1**, 111.

[17] Marcus, A. J., Ullman, H. L., Safier, L. B. & Ballard, H. S. (1970) *Fed. Proc.* **29**, 315.

[18] Folch, J., Lees, M. & Sloane Stanley, G. H. (1957) *J. Biol. Chem.* **226**, 497–509.

cerebrosides as main components, whereas pig platelets afford triglycosylceramides as the predominant component of the neutral glycolipid fraction, followed by diglycosylceramides and sulfatides in about equal amounts, whereas cerebrosides represent by far the smallest fraction.

Fig. 1. Thin-layer chromatogram of purified glycolipids isolated from pig platelets.

1) Cerebrosides (monoglycosylceramides); 2) diglycosylceramides; 3) sulfatides; 4) triglycosylceramides. Solvent system: chloroform/methanol/water 65:25:4, silica gel *H*.

Ceramides

A rather large fraction consisting of three less polar lipids free of sugar and phosphate and very similar in their chromatographic behaviour were found to be associated with the neutral glycolipid fraction, using the separation technique described under Experimental. Thin-layer chromatography (solvent system: chloroform/methanol 15:1) separated this fraction into three groups of ceramide species, which were designated ceramide A, B and C. The total ceramide fraction amounted to 45% of the total glycolipid fraction. The result of the separation by preparative thin-layer chromatography is documented in Fig. 2. The most abundant ceramides were those of group A followed by B and C. The different chromatographic behaviour is due to the individual fatty acid and long chain normal fatty acids with 20 to 26 C-atoms and about 60% 4*t*-sphingenine and 40% sphinganine.

The ceramides B contained a completely different fatty acid pattern compared with the ceramides A and C and with sphingomyelins. Unsaturated fatty acids such as oleic and linoleic acids and arachidonic acid were typical for this fraction, and the main long chain bases were 4*t*-sphingenine (46%) and sphinganine and 4*D*-hydroxysphinganine in approximately equal amounts (22 and 24%).

The ceramide C fraction is the slowest moving band. The higher polarity is due to the presence of hydroxy fatty acids as acyl moieties which are absent from ceramides A and B. About 42% of the

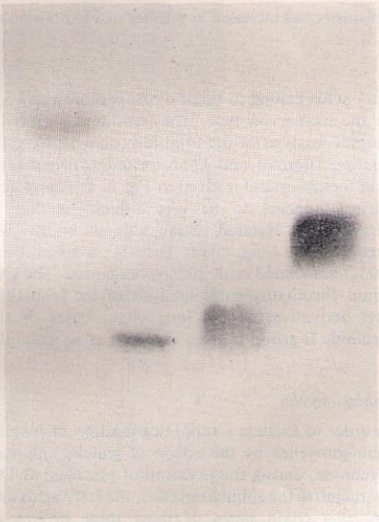

Fig. 2. Thin-layer chromatographic analysis of the components of the ceramide fraction separated by preparative thin-layer chromatography.

1) Cholesterol; 2) ceramide C; 3) ceramide B; 4) ceramide A. Solvent system: chloroform/methanol 15:1.

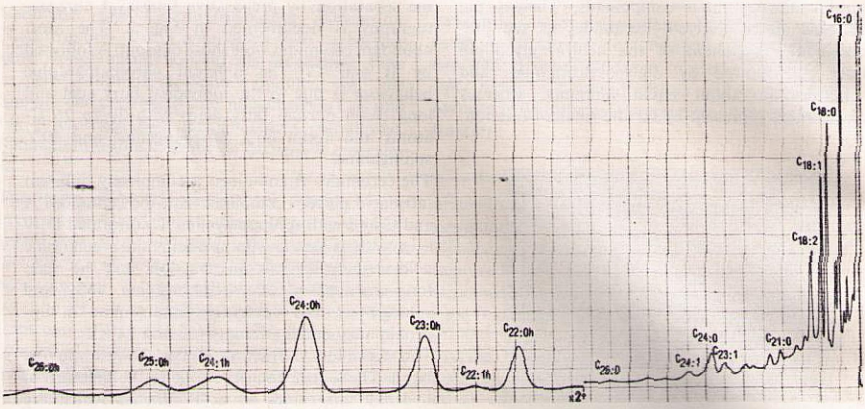

Fig. 3. Gas-liquid chromatographic analysis of total mixture of fatty acid methyl esters of ceramides C isolated from pig platelets.
Sensitivity was increased by a factor of 2 at position x2.

fatty acids belong to those of the cerebron and 5% to the oxynervon type. The gas-liquid chromatographic analysis of the total fatty acid methyl ester mixture (normal and *O*-acetylated hydroxy fatty acid methyl esters) is given in Fig. 3. The fatty acid pattern appears to be very similar to that of cerebrosides isolated from human brain. The predominant long chain base is 4*t*-sphingenine (80%) associated with 20% sphinganine. The gas-liquid chromatographic analysis of the trimethylsilyl derivatives of the long chain bases of the ceramide B group is given in Fig. 4 as an example.

Sphingomyelin

In order to exclude a rapid degradation of platelet sphingomyelins by the action of platelet sphingomyelinase, during the isolation of platelets, and to characterize the sphingomyelins, the fatty acids and the long chain bases of these three groups of ceramides were compared with those of the sphingomyelin fraction. For this purpose sphingomyelin was hydrolyzed with phospholipase C[19]. The resulting ceramide fraction, isolated by preparative thin-layer chromatography was hydrolyzed according to Gaver and Sweeley[20]. The initial enzymatic hydrolysis of the phosphodiester followed by the acid hydrolysis proves to be essential for a quantitative hydrolysis of sphingomyelin as demonstrated earlier[21]. The prolongation of the acid hydrolysis leads to an increased production of 5-*O*-methylsphingenine. The summary of these analyses and the comparison with the ceramide fractions is given in Table 1 and 2. These results underline the remarkable difference of the ceramides and the ceramide portion of sphingomyelins. Half of the fatty acid residues in sphingomyelin is represented by palmitic acid (42%) and stearic acid (9%), while nervonic ($C_{24:1}$) and tetracosadienoic acid ($C_{24:2}$) comprise 23%. 4*t*-Sphingenine accounts for 86% and sphinganine for 10% of the long chain bases present in sphingomyelin. This component analysis of the sphingomyelin fraction points to the distinct difference between the three ceramides and sphingomyelin and it excludes an enzymatic hydrolysis during the isolation procedure.

[19] Kleiman, J. H. & Lands, W. E. M. (1969) *Biochim. Biophys. Acta* **187**, 477–485.

[20] Gaver, R. C. & Sweeley, C. C. (1965) *J. Amer. Oil Chem. Soc.* **42**, 294–298.

[21] Stoffel, W. & Assmann, G. (1972) *this J.* **353**, 65–74.

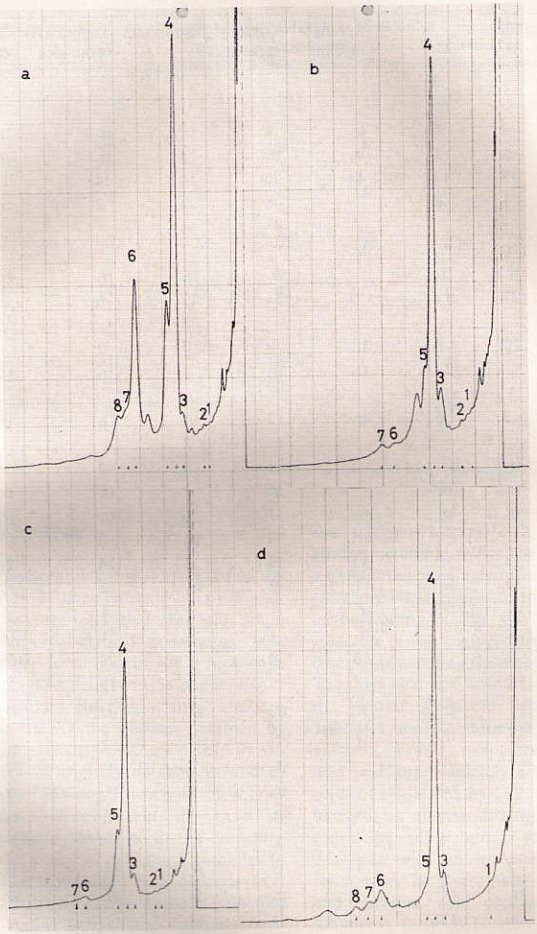

Fig. 4. Gas-liquid chromatogram of the trimethylsilyl derivatives of the long chain bases isolated from sphingo- and glycosphingolipids from pig blood platelets.

Bases from a) ceramides B; b) glycosylceramides; c) triglycosylceramides; d) main ganglioside; 1) C_{16}-4*t*-sphingenine; 2) C_{16}-sphinganine; 3) 5-*O*-methylsphingenine; 4) 4*t*-sphingenine; 5) sphinganine; 6) 4*D*-hydroxysphinganine; 7) C_{20}-4*t*-sphingenine; 8) C_{20}-sphinganine. Conditions used are described under Experimental.

Table 1. Percentage composition of normal and α-hydroxy fatty acids in the sphingolipids and glycosphingolipids from pig platelets.

Compound	Ceramides A	Ceramides B	Ceramides C	Sphingomyelins	Glycosylceramides	Sulfatides	Diglycosylceramides	Triglycosylceramides	Gangliosides
14:0			2						
14:1			tr.						
15:0	tr.*	2	2	tr.	1	tr.	tr.	tr.	tr.
15:1	tr.	tr.	1	tr.	tr.	1	tr.	tr.	tr.
16:0	20	13	14	42	20	14	14	12	18
16:1	tr.	1	2		6	2	2	tr.	2
12:0h**						4			
17:0	tr.	tr.	tr.	tr.					2
16:2		tr.	tr.		tr.	tr.	tr.	tr.	tr.
18:0	24	27	11	9	14	12	6	6	12
13:0h						2			
18:1	3	18	9	tr.	9	7	2	4	4
19:0	tr.			tr.	tr.		tr.	tr.	
14:0h						tr.			
18:2	tr.	12	6	tr.	3	tr.	tr.	tr.	3
15:0h						2			
20:0	4	tr.	tr.	2	1	4	3	4	7
18:3	tr.	tr.	tr.		tr.		tr.	tr.	tr.
21:0	tr.	tr.	tr.	tr.	1	6	tr.	tr.	5
16:0h				tr.	4	tr.	2	5	tr.
22:0	10	3	2	12	6	8	24	26	20
20:4	2	14	tr.	1	2	tr.	2	2	3
23:0	2	tr.	tr.	tr.	1	4	1	1	2
23:1	tr.	1	1	tr.	1	tr.	1	tr.	2
24:0	22	2	3	10	10	18	26	25	10
24:1	8	2	tr.	16	8	3	13	11	7
25:0	1		tr.		tr.	tr.	tr.	tr.	tr.
24:2	tr.		tr.	7	tr.	3	4	3	2
25:1		1			tr.	1	tr.	tr.	1
26:0	4	tr.	tr.		tr.	2	tr.	tr.	tr.
26:1	tr.				1	3	tr.	tr.	tr.
27:0					tr.	tr.			tr.
22:0h		2	7	1	2	tr.	tr.	tr.	tr.
22:1h		tr.	tr.		tr.	tr.	tr.	tr.	
23:0h		tr.	11		3	tr.	tr.	tr.	tr.
23:1h			tr.		1		tr.		
24:0h		tr.	17		4	2	tr.	1	
24:1h		tr.	5		2		tr.	tr.	
25:0h		tr.	4		tr.	2	tr.	tr.	
26:0h		tr.	3		tr.	tr.			

* Traces means < 1%. ** h = α-hydroxy fatty acid.

Table 2. Percentage composition of long chain bases (LCB) as TMS derivatives in the sphingolipids and glycosphingolipids from pig platelets.

Compound	Ceramides A	B	C	Sphingomyelins	Glycosylceramides	Sulfatides	Diglycosylceramides	Triglycosylceramides	Gangliosides
5-*O*-Methyl-LCB	3	4	6	8	10	7	12	9	11
C_{16}-4*t*-Sphingenine	tr.	tr.	tr.	tr.	tr.	tr.		tr.	tr.
C_{16}-Sphinganine	tr.	tr.	tr.	tr.	tr.	tr.		tr.	tr.
C_{18}-4*t*-Sphingenine	59	46	75	78	72	83	81	70	67
C_{18}-Sphinganine	38	22	19	10	17	7	7	21	8
C_{18}-4*D*-Hydroxy-sphinganine		24	tr.		tr.	1	tr.	tr.	7
C_{20}-4*t*-Sphingenine		2	tr.	2	1	2	tr.	tr.	3
C_{20}-Sphinganine		2		2					4

Neutral glycosphingolipids

The three glycosylceramide fractions purified by preparative thin-layer chromatography were analyzed for their hexose, fatty acid and long chain base moieties. Microanalytical procedures were elaborated in order to deal with these problems, which are described in detail in this paper. Fig. 5 presents the gas-liquid chromatographic analyses of the alditol acetates of the hexoses obtained by hydrolysis of a) monoglycosylceramides, b) diglycosylceramides, c) triglycosylceramides and d) main gangliosides. The sugar analysis of monoglycosylceramides indicated that the ratio of glucosyl- and galactosylceramide in the "cerebroside-fraction" was 1 mol of galactosylceramides to 2.5 mol of glucosylceramides. The diglycosylceramide fraction afforded equal amounts of galactose and glucose on acid hydrolysis and the triglycosylceramide 2 mol of galactose and 1 mol of glucose. When these two fractions were partially hydrolyzed, only glucosylceramide could be observed in both cases in thin-layer chromatography[22]. Therefore the diglycosylceramide is a lactosylceramide and the triglycosylceramide a galactosyl-galactosyl-glucosylceramide. When the latter was treated with α-galactosidase, isolated from ficin[23], the terminal galactose was hydrolyzed to a large extent yielding lactosylceramide, Fig. 6. Therefore the terminal galactose in this triglycosylceramide must be linked by an α-galactosidic linkage to the central galactose. The fatty acid and long chain base composition of the neutral glycolipids are summarized in the Tables 1 and 2. Monoglycosylceramide carries palmitic, stearic, oleic, cerebronic and nervonic acid and approximately 10% of α-hydroxy fatty acids of chain lengths C_{23} and C_{24}. It is interesting to note, as summarized in Table 1, that the concentration of palmitic, stearic and oleic acid decreases with an increasing number of hexose residues in the glycolipids, whereas the cerebronic and nervonic acid concentration increases. No α-hydroxy fatty acids were detected in the di- and triglycosylceramide fractions. Studies on the structure of the glycolipids of pig erythrocytes led to a similar observation[24]. Only long chain bases

[22] Kean, E. L. (1966) *J. Lipid Res.* 7, 449–452.

[23] Hakomori, S. & Siddiqui, B. (1971) *J. Biol. Chem.* **246**, 2271–2277.

[24] Coles, E &. Foote, J. L. (1970) *J. Lipid Res.* **11**, 433–438.

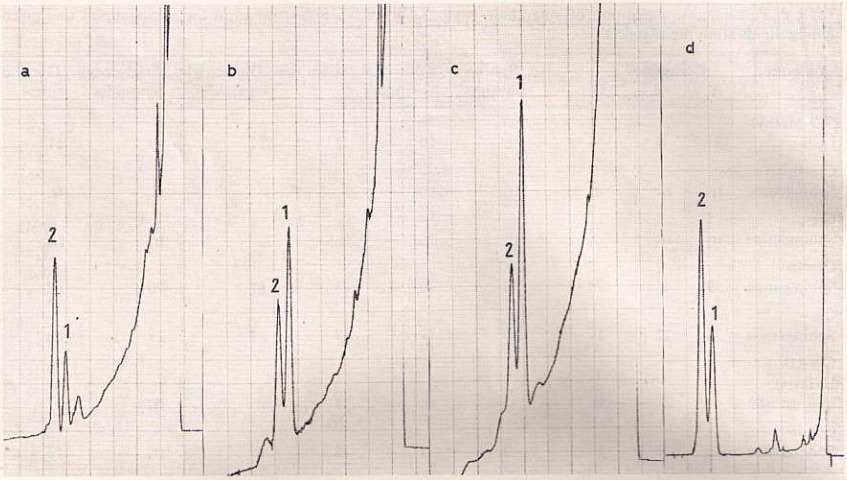

Fig. 5. Gas-liquid chromatogram of alditol hexaacetates of sugars released from pig platelet glycosphingolipids from
a) monoglycosylceramides; b) diglycosylceramides; c) triglycosylceramides; d) main ganglioside fraction; 1) galactitol hexaacetate; 2) glucitol hexaacetate. For conditions see Experimental.

with 18 carbon atoms were detected in the neutral glycolipids; more than 90% 4*t*-sphingenine was detected in mono- and diglycosylceramides; about 80% 4*t*-sphingenine with 20% sphinganine was detected in triglycosylceramides.

Acidic glycosphingolipids

The *sulfatides* were characterized by co-chromatography with brain sulfatides. The carbohydrate group was identified as pure galactose. The sulfatides were gradually converted into galactosylceramides by treatment with ammonia as previously reported by Svennerholm and Thorin[25]. Acid hydrolysis according to Mårtensson[26] also yielded galactosylceramide. Sulfatides exhibited a component analysis rather similar to the cerebroside fraction except for the diminished content of long chain hydroxy fatty acids and of sphinganine.
The *ganglioside fraction* offers a rather complex picture when separated into its components by two

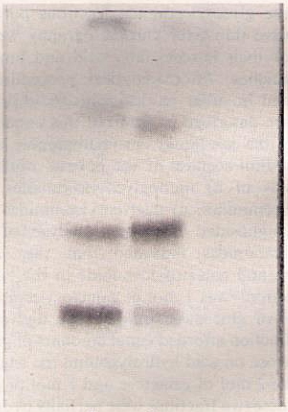

Fig. 6. Thin-layer chromatographic analysis of a triglycosylceramide fraction from pig platelets after
1) α-galactosidase treatment; 2) partial acid hydrolysis. Solvent system: chloroform/methanol/water 65:25:4, silica gel H.

[25] Svennerholm, L. & Thorin, H. (1962) *J. Lipid Res.* **3**, 483–485.
[26] Mårtensson, E. (1966) *Biochim. Biophys. Acta* **116**, 521–531.

consecutive runs in thin-layer chromatography, Fig. 7. Among the eight different gangliosides one predominates. It co-chromatographed in different solvent systems with a standard bis(*N*-glycoloyl-neuraminyl)-lactosylceramide isolated from cat erythrocytes. The main ganglioside band (Fig. 7) consisted of a double band. Also the diglycosylceramide obtained after the enzymatic release of the neuraminic acid by neuraminidase or repeated mild acid hydrolysis (10mM acetic acid, by neuraminidase or repeated mild acid hydrolysis (10mM acetic acid, 80°C, 90 min) separated into two bands in thin-layer chromatography, Fig. 8. The neuraminic acid derivative recovered from the hydrolysis procedures consisted of *N*-glycoloylneuraminic acid exclusively as proven by gas-liquid chromatography as trimethylsilyl derivative, Fig. 9. The resulting diglycosylceramide fraction was further analyzed. No α-hydroxy n-fatty acids were present in the ceramide portion. Only unsubstituted n-fatty acids, predominantly $C_{16:0}$, $C_{18:0}$, $C_{22:0}$, $C_{24:0}$ and $C_{24:1}$, were found, (Table 1); and only 7% of 4*D*-hydroxysphinganine in addition to 78% 4*t*-sphingenine and 8% sphinganine. The reason for the different chromatographic behaviour within this group was therefore of interest. The carbohydrate analysis yielded a molar ratio of galactose/glucose of 1:1.86 (Fig. 5d). The monoglycosylceramide obtained during partial hydrolysis studies proved to be only glucosylceramide. Therefore one band consisted of lactosylceramide and the other of a diglucosylceramide which has never been described before.

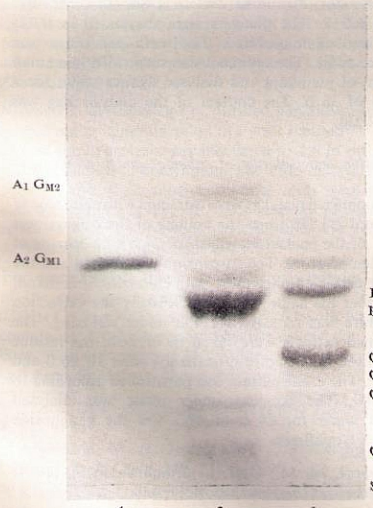

Fig. 7. Thin-layer chromatogram of gangliosides.
1) Ganglioside $A_2(G_{M1})$, $A_1(G_{M2})$ tests; 2) ganglioside mixture of pig platelets; 3) ganglioside mixture of human brain $A_2(G_{M1})$, $B_1(G_{D1a})$, B_3, $C_1(G_{D1b})$, C_2, $C_3(G_{T1})$, C_4. S = start.

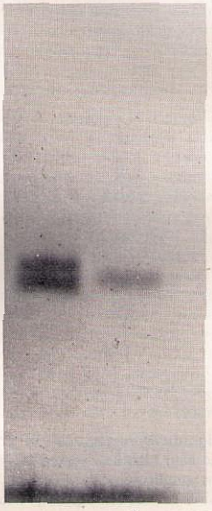

Fig. 8. Thin-layer chromatography of the degradation product of the main ganglioside component of pig platelets after complete release of neuraminic acid by neuraminidase.
1) Test: lactosylceramide; 2) neuraminic acid-free product. Solvent system: chloroform/methanol/water 65:25:4, silica gel H. Two consecutive runs in the same solvent system.

Further research on the components of this ganglioside mixture from pig platelets is required. It should be mentioned that this ganglioside bis-(*N*-glycoloylneuraminosyl)-lactosylceramide might prove to be involved in the serotonin uptake into platelets, since other observations refer to its

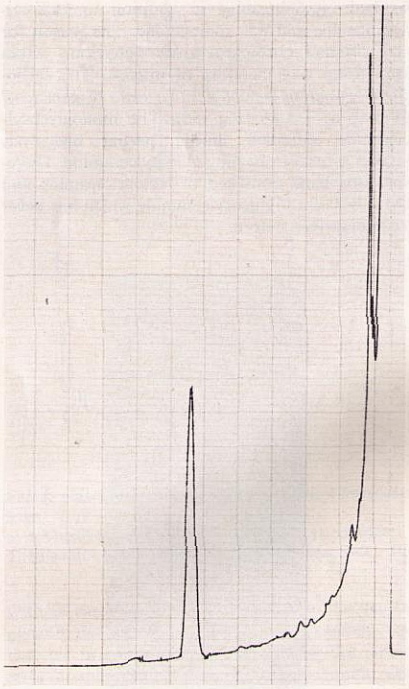

Fig. 9. Gas-liquid chromatography of TMS derivative of neuraminic acid residue released by neuraminidase from the main ganglioside of pig platelet. The retention time corresponds to that of N-glycolyl neuraminic acid.

receptor function for serotonin[27–30]. Also they may be involved in immunochemical reactions, since the hapten function of mono- and polyglycosylceramides has been demonstrated[31–36].

[27] Wooley, D. W. & Gommi, B. W. (1965) *Proc. Nat. Acad. Sci. USA* **53**, 959–963.

[28] Wooley, D. W. & Gommi, B. W. (1964) *Nature (London)* **202**, 1074–1075.

[29] Gielen, W. (1966) *Z. Naturforsch.* **21**b, 1007–1008.

[30] Gielen, W. (1968) *Z. Naturforsch.* **23**b, 117–118.

[31] Rapport, M. M., Graf, L., Skipski, V. P. & Alonzo, N. F. (1959) *Cancer* **12**, 438.

[32] Rapport, M. M. (1961) *J. Lipid Res.* **2**, 25–36.

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Experimental

Preparation of platelets

Pig blood was collected with gentle swirling in siliconized (Silikonöl WS 60, Wacker-Chemie, München, West Germany) vessels or plastic containers and clotting was prohibited by 3.8% sodium citrate (8.5 of blood to 1.5 of sodium citrate solution by vol.). The blood was left at 4°C for 3–4 h during which a platelet enriched supernatant (appr. 35–45% of the total blood volume) was obtained. This procedure is not suitable for beef blood, since only a very slow sedimentation can be observed. The supernatant was sucked off and immediately centrifuged for 13 min at $330 \times g$ in a Stock centrifuge. The precipitate was discarded and the platelet-enriched supernatant again collected by drainage and centrifuged under the conditions described before. After three centrifugations a platelet-rich plasma was obtained, which contained 4–5 erythrocytes per 10^5 platelets. Cell counting was carried out with a Coulter counter, model A, Fa. Coulter Electronics Ltd., St. Alban, Great Britain. The platelets were sedimented finally at $20000 \times g$ for 20 min at 2°C in a Sorvall centrifuge, model RC 2. The platelets were suspended in 0.9% NaCl and again pelleted. The wash procedure was repeated twice. The sediment was suspended in a small amount of methanol and dialysed against water for a period of 36 h. The content of the dialysis bag was lyophilized.

Extraction and isolation of sphingo- and glycosphingolipids

The lyophilized platelets were extracted with chloroform/methanol 2:1 (10 times the volume of the original wet weight of the packed platelets) for 24 h with stirring at room temperature. The suspension was filtered and the residue extracted once more with chloroform/methanol 1:2 under the same conditions. Finally the extraction procedure was repeated for 4 h under refluxing. The combined extracts were taken to dryness, the residue redissolved in chloroform/methanol/water 10:20:3 and filtered. The crude extract was partitioned according to Folch[18]. The aqueous upper phase was dialysed against water for 24 h at 4°C and the ganglioside solution lyophilized.

[33] Rapport, M. M., Graf, L. & Schneider, H. (1964) *Arch. Biochem. Biophys.* **105**, 431–438.

[34] Rapport, M. M., Schneider, H. & Graf, L. (1967) *Biochim. Biophys. Acta* **137**, 409–411.

[35] Naiki, M. & Taketomi, T. (1969) *Jap. J. Exp. Med.* **39**, 549–571.

[36] Kuhn, R. & Wiegandt, H. (1964) *Z. Naturforsch.* **19**b, 256–257.

The lower phase, containing neutral lipids, glycosphingolipids and phospholipids, was separated by silicic acid chromatography according to Vance and Sweeley[37]. Ester lipids, including plasmalogens, were hydrolyzed by the $HgCl_2$-saponification of Abramson *et al.*[38]. Sphingomyelin was obtained in this manner free of lysoplasmalogens from the phospholipid fraction.

Thin-layer chromatography of the different lipid classes was carried out in the following solvent systems for analytical as well as preparative purposes:

gangliosides: chloroform/methanol/2.5N NH_4OH 60:35:8;

glycosphingolipids and *sphingomyelin:* chloroform/methanol/water 65:25:4 or chloroform/methanol/water 100:42:6;

ceramides: chloroform/methanol 15:1;

diglycosylceramides and *sulfatides:* n-propanol/12.5N NH_4OH 80:20;

glucosyl- and *galactosylceramides* were separated on borate impregnated plates (1% $Na_2B_4O_7 \times 10H_2O$, pH 9.2) in the solvent system: chloroform/methanol/water/ 15N NH_4OH 280:7:6:1[22].
Silica gel H coated plates were used in all instances. All lipids were visualized by spraying with Bial's reagent[39], other sugar-containing lipids with α-naphthol/ sulfuric acid spray[40], phosphorus-containing lipids with Zinzadze's reagent[41].

Neuraminic acid analyses of purified gangliosides were carried out by a modification of Puro's method[42]. The time of the hydrolysis was shortened to 1 h in order to avoid an increased deacylation. The TMS derivatives of *N*-acetyl and *N*-glycoloylneuraminic acids were chromatographed on a 1% SE 30 column at 230°C. The retention time of *N*-glycoloylneuraminic acid relative to *N*-acetylneuraminic acid was 1.95.

For *neuraminidase* treatment of the gangliosides 1 mg of the pure main ganglioside was dissolved in 1 ml 0.001M $CaCl_2$ solution adjusted to pH 6.0 with Na_2CO_3 containing 2 mg neuraminidase (Behringwerke, Marburg, West Germany) at 37°C. The enzyme preparation had been freed from the stabilizing sugar by dialysis prior to the incubation. The enzymatic hydrolysis was completed within 1 h. No orcin positive lipid material could be detected in the upper or lower phase of the total extracts.
0.5 mg of triglycosylceramide was treated with 2 mg α-*galactosidase* for 48 h at 37°C in a total volume of 1.0 ml of 0.05M citrate buffer, pH 4.5; 100000 U of penicillin were added in order to prevent bacterial contamination. The reaction was stopped by extracting with chloroform/methanol 2:1. The lower phase was analyzed by thin-layer chromatography.
For sequence studies the *partial hydrolysis* of the ceramide polyhexosides and main ganglioside was carried out in sealed tubes with 0.1N HCl at 100°C for 1 h. The hydrolysate was extracted with chloroform/methanol 1:1 and the degradation products identified by thin-layer chromatography.

Quantitative analysis of carbohydrates

The ratios of the hexose residues of the individual glycosphingolipids were determined as their alditol acetates [43–47]. Standards of the alditol acetates of fucose, mannose, galactose, glucose and mannosamine were prepared from their respective aldohexose[48–50]. Glucosaminol and galactosaminol were acetylated with pyridine/acetic anhydride 1:1 at 80°C and the hexaacetate was recrystallized from methanol, ethyl ether and pentane[51,52]. It should be mentioned that glucosaminol hexaacetates are readily water-soluble. 100–200 μg of the respective glycosphingolipid fraction was transferred into small tapered tubes. The vacuum-dried sample was heated with 0.5 ml 1N HCl in these tubes sealed with Teflon lined screw caps for 12 h at 100°C. The fatty acids were extracted 4 times with 0.5 ml hexane and twice with 0.5 ml chloroform. The

[37] Vance, D. E. & Sweeley, C. C. (1967) *J. Lipid Res.* **8**, 621–630.

[38] Abramson, M. B., Norton, W. T. & Katzman, R. (1965) *J. Biol. Chem.* **240**, 2389–2395.

[39] Klenk, E. & Gielen, W. (1961) *this J.* **326**, 144–157.

[40] Jacin, H. & Mishkin, A. R. (1965) *J. Chromatogr.* **18**, 170.

[41] Dittmer, J. C. & Lester, R. L. (1964) *J. Lipid Res.* **5**, 126–127.

[42] Puro, K. (1969) *Biochim. Biophys. Acta* **187**, 401–413.

[43] Sawardeker, J. S., Slonecker, J. H. & Jeanes, A. (1965) *Anal. Chem.* **37**, 1602–1604.

[44] Crowell, E. P. & Burnett, B. B. (1967) *Anal. Chem.* **39**, 121–124.

[45] Lehnhardt, W. F. & Winzler, R. J. (1968) *J. Chromatogr.* **34**, 471–479.

[46] Kim, J. H., Shome, B., Liao, T. H. & Pierce, J. G. (1967) *Anal. Chem.* **20**, 258–274.

[47] Weinstein, D. B., Marsh, J. B., Glick, M. C. & Warren, L. (1970) *J. Biol. Chem.* **245**, 3928–3937.

[48] Abdel-Akher, M., Hamilton, J. K. & Smith, F. (1951) *J. Amer. Chem. Soc.* **73**, 4691–4692.

[49] Beilsteins Handbuch d. Org. Chemie (1968) 4. Aufl., 3. Erg.-W., Vol. II, pp. 341–346, Springer-Verlag, Heidelberg.

[50] Roth, W., Pigman, W. & Danishefsky, I. (1964) *Tetrahedron* **20**, 1675–1676.

[51] Crimmin, W. R. C. (1957) *J. Chem. Soc.* 2838.

[52] Levene, P. A. & Christman, C. C. (1937) *J. Biol. Chem.* **120**, 575–590.

fatty acids were analyzed as described below. The aqueous phase was concentrated to dryness and excess HCl removed in a desiccator over KOH-pellets under vacuum. The residue was dissolved in 0,5 m*l* water and portions of a stock solution of $NaBH_4$ (1 mg/m*l*) added (appr. 400–500 μg). After 2 h excess $NaBH_4$ was decomposed by the addition of 1–3 drops of glacial acetic acid and the solution concentrated to dryness and the residue acetylated with 100 μ*l* pyridine/acetic anhydride 1:1 at 80°C for 12 h. 50 μ*l* water were then added and the solvents removed under vacuum. The residual alditol acetates were dissolved in three 0.2 m*l* portions of chloroform. These extracts were transferred into a small tapered vial and concentrated to dryness in a stream of nitrogen. The residue was dissolved in 10 μ*l* of chloroform and a portion taken for the gas-liquid chromatography. The gas-chromatographic analysis of the alditol acetates was carried out on 3% ECNSS-M on Gaschrom Q 100–200 mesh (Applied Science Laboratories Inc.) isothermally at 190°C, column length 120 cm. The hexaacetates of galactitol eluted after 8 min, of glucitol after 9 min, glucosaminol after 44.5 min and galactosaminol after 53 min, see Fig. 5. A flame ionisation detector was used for quantitation.

Analysis of long chain bases and fatty acids

Sphingolipids and glycosphingolipids were hydrolyzed in 1N aqueous methanolic HCl at 75°C for 18–22 h[53]. The *long chain bases* were isolated according to Carter and Hirschberg[54] and transferred into small vials, followed by 10–20 μ*l* of *N*-methyl(trimethylsilyl)trifluoroacetamide as silylation reagent. The tubes were sealed with Teflon caps and warmed in a 60°C water bath for 20 min. The trimethylsilyl derivatives of the long chain bases were chromatographed on 1% SE 30 on Chromosorb G, 80–100 mesh at 230°C, column length 200 cm.

The long chain bases were also identified after periodate oxidation[55] and gas-liquid chromatographic analysis of their long chain aldehydes on 2.5% EGS on Kieselguhr (0.15–0.20 mm) at 140°C. The partially methylated *fatty acids* from this hydrolysis were combined with those obtained from the hydrolysis procedures for the analysis of the carbohydrate residues and esterified with BF_3/methanol[56]. Prior to the methylation 10% ether was added to improve the solubility. The methylation was carried out for 10 min at 100°C. The solvents were evaporated in a stream of nitrogen. 10 μ*l* of acetylation reagent (pyridine/acetic anhydride 1:1) were added, the mixture allowed to stand overnight at room temperature. Fatty acid methyl esters were extracted with chloroform after the addition of 100–200 μ*l* water. The analysis was carried out on 2.5% EGS on Kieselguhr (0.15–0.20 mm) at 190°C. Normal and acetylated hydroxy fatty acid methyl esters separated well in one run.

[53] Gaver, R. C. & Sweeley, C. C. (1965) *J. Amer. Oil Chem. Soc.* **42**, 294–298.

[54] Carter, H. E. & Hirschberg, C. B. (1968) *Biochemistry* **7**, 2296–2300.

[55] Sweeley, C. C. & Moscatelli, E. A. (1959) *J. Lipid Res.* **1**, 40–47.

[56] Morrison, W. R. & Smith, L. M. (1964) *J. Lipid Res.* **5**, 600–608.