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Glycosphingolipids in Lysosomal Membranes[1]

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Summary: Glycosphingolipids, isolated from the membrane fraction of rat liver lysosomes filled with Triton WR 1339, were analyzed qualitatively and quantitatively. Glucosyl-, lactosyl- and galactosyl-galactosyl-glucosylceramide are the predominant components of the neutral glycosphingolipid fraction. *N*-Acetylneuraminyl-lactosylceramide (hematoside) is the main ganglioside.

The sugar composition was determined by gas liquid chromatography and the sugar sequence was established by partial hydrolysis studies. Palmitic, stearic and lignoceric acid are the most prevalent fatty acids. C_{18}-Sphingenine is the main long chain base. Lactosyl and trihexosylceramide also contain substantial amounts of C_{20}-sphingenine and C_{18}-4*D*-hydroxysphinganine.

In the view of the possible participation of the plasma membrane in the production of the secondary lysosomal membrane, the glycosphingolipids of these two membrane fractions were compared by thin-layer chromatography. Trihexosylceramide was found to be absent from the neutral glycolipids of the plasma membrane. The ganglioside composition of the two membranes is completely different. Since the composition of primary lysosomal membranes is not known, the glycosphingolipids in secondary lysosomal membranes may be derived from the plasma membrane by Triton-induced pinocytosis; alternatively, this class of lipids may be a genuine lysosomal constituent.

Glykosphingolipide in Lysosomenmembranen

Zusammenfassung: Aus der Membranfraktion von Triton-WR-1339 gefüllten Rattenleberlysosomen (Tritosomen) wurden Glykosphingolipide isoliert und qualitativ und quantitativ untersucht. Glucosyl-, Lactosyl- und Galaktosyl-galaktosyl-glucosylceramid bilden die Hauptkomponenten der neutralen Glykosphingolipide und unter den Gangliosiden wurde *N*-Acetylneuraminyl-lactosylceramid (Haematosid) als Hauptbestandteil analysiert.

Die Zuckerzusammensetzung wurde gaschromatographisch ermittelt und die Zuckersequenz durch partielle Hydrolyse bestimmt. Die Ganglioside enthalten *N*-Acetylneuraminsäure als einziges Neuraminsäurederivat.

Palmitin-, Stearin- und Lignozerinsäure sind die hauptsächlichen Fettsäuren der neutralen Glykolipide und des Haematosids. C_{18}-Sphingenin (C_{18}-Sphingosin) ist die überwiegende Sphingosinbase in allen untersuchten Glykosphingolipiden. In Dihexosyl- und Trihexosylceramid wurden außerdem in größeren Mengen C_{20}-Sphingenin und C_{18}-4*D*-Hydroxysphinganin gefunden.

Ausgehend von der Frage nach einer Beteiligung der Plasmamembran bei der Entstehung sekundärer Lysosomenmembranen wurden die Glykosphingolipide der Plasmamembran und der Lysosomenmembran dünnschichtchromatographisch verglichen. Nur das Trihexosylceramid fehlte unter den neutralen Glykolipiden in der Plasmamembran. Die Gangliosidmuster sind in beiden Membranfraktionen völlig verschieden. Da die Zusammensetzung primärer Lysosomenmembranen nicht be-

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Enzymes:

5'-Nucleotidase, 5'-ribonucleotide phosphohydrolase (EC 3.1.3.5)
Acyl-CoA- transferase (EC 2.8.3.?) not yet listed.

kannt ist, kann die Herkunft von Glykosphingolipiden entweder durch den Einbau von Plasmamembran bei dem durch Triton WR 1339 induzierten Pinocytoseprozeß erklärt werden oder Glykolipide müssen als echt lysosomale Bausteine betrachtet werden.

The glycosphingolipids originally detected in brain have been isolated from several extraneural tissues (for references see[2]). Due to the great variability of the composition of the long chain bases, fatty acids and sugars, this lipid class exhibits the highest tissue specificity of all known lipids.

Only a few data exist on the glycolipid composition of liver. Svennerholm[3] reported the occurrence of neutral glycosphingolipids in human liver and detailed data have been reported recently by Kviterovitch *et al.*[4]. These authors found dihexosylceramide as the predominant neutral glycosphingolipid and they also identified monohexosyl- and trihexosylceramide. Hematoside was the predominant ganglioside. Only a few investigations have been carried out on rat liver. Siddiqui and Hakomori[5] *compared normal rat livers with hepatoma* glycolipids, and they described glucosylceramide as the main component with traces of lactosyl- and tetrahexosylceramide. The main gangliosides were hematoside and monosialoganglioside (GM_1).

Among subcellular organelles the plasma membrane has gained considerable interest. This is due to the fact that one of the best studied events in surface membranes of cells undergoing viral transformation to malignancy are changes in the glycosphingolipid pattern[6,7] concomitant with changes of enzymes of the glycolipid-synthesizing pathway[8]. The phenomenon of loss of contact inhibition[9], the altered surface charge[10] and the changed agglutinability[11] may be related in part to glycolipid structures and may underline their importance to the surface architecture of the cell.

Up to now it has generally been accepted that the glycolipids are localized exclusively in the surface membrane of animal cells[2]. Since our previous studies[12,13] dealt with the question of a membrane flow of the plasma membrane into the secondary lysosome during the probably pinocytotic uptake of the detergent Triton WR 1339 by rat liver lysosomes, an analysis of the glycolipids in the membrane of this organelle should provide further insight into the problem of the relationship between these two membranes.

The data of previous studies on this subject[12,13], including the composition of neutral lipids, phospholipids, proteins and some plasma-membrane-specific enzymes in secondary lysosomal membranes, gave no clear answer. Nearly all features in the lipid sector known to be characteristic in plasma membranes could also be detected in the membrane fraction of Triton WR 1339 filled lysosomes (tritosomes). On the other hand neither the protein profile in polyacrylamide gel electrophoresis nor the comparison of plasma-membrane-specific enzymes in the two membranes supported a pinocytosis-induced plasma membrane transfer to the lysosomal membrane in this system.

In addition the presence of ubiquinone[14] as well as cytochromes b_{563}[15,16] and P_{420}[16] in the membrane fraction of tritosomes lends strong support to the idea that autophagy participates in the formation of tritosomes and that these substances are derived from autophagized subcellular organelles.

These considerations and the above-mentioned surface-specific localization of glycosphingolipids

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[3] Svennerholm, E. & Svennerholm, L. (1963) *Nature (London)* **198**, 688–689.

[4] Kviterovitch, P. O., Jr., Sloan, H. R. & Fredrickson, D. S. (1970) *J. Lipid Res.* **11**, 322–330.

[5] Siddiqui, B. & Hakomori, S. (1970) *Cancer Res.* **30**, 2930–2936.

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[11] Burger, M. M. (1969) *Proc. Nat. Acad. Sci. U.S.A.* **62**, 994–1001.

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[15] Hanes, D. M. & Tappel, A. L. (1971) *Biochim. Biophys. Acta* **245**, 42–53.

[16] Henning, R., Brauser, B. & Plattner, H., in preparation.

prompted us to analyze this lipid class thoroughly in the tritosomal membrane fraction and to compare their pattern with that of the plasma membrane.

Results

Tritosomes were prepared by a slight modification[12] of the method of Wattiaux[17] and were essentially free from contamination by other cell fractions as checked by marker enzymes[12] and by electron microscopy[14]. The pellet obtained by centrifugation at 105000 × *g* (60 min) of an osmotically ruptured tritosome fraction was designated the tritosomal membrane fraction. The membraneous character of this fraction is established by electron microscopy[14] as well as by the analysis of its chemical composition.

Fig. 1 shows the neutral glycosphingolipids of the tritosomal and the plasma membrane. Mono- and diglycosylceramide predominate in both membranes, whereas trihexosylceramide is absent in the plasma membrane. Triglycosylceramide could not be detected in five different fractions, each pooled from 15–20 rats. Two more anthrone- and α-naphthol-positive substances can be seen. These bands have been observed constantly in the tritosomal fraction, whereas the upper spot has never been found in the plasma membrane fraction. The complexity of these structures and the microgram quantities available did not permit further analysis.

It is obvious from Fig. 2 that the ganglioside pat-

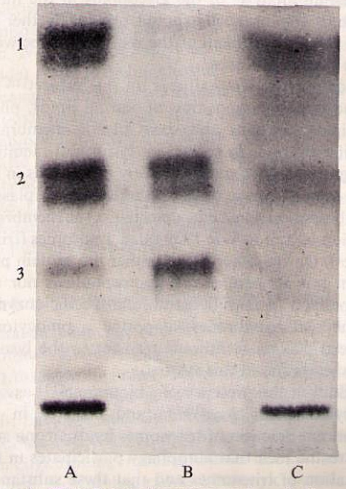

Fig. 1. Thin-layer chromatography of the neutral glycosphingolipid fraction of the tritosomal and the plasma membrane fraction.

Solvent system: chloroform/methanol/water 65:25:4. Staining by α-naphthol/sulfuric acid.

A) Tritosomal membrane;
B) test mixtures;
C) plasma membrane.

1 = Monoglycosylceramide;
2 = diglycosylceramide;
3 = triglycosylceramide.

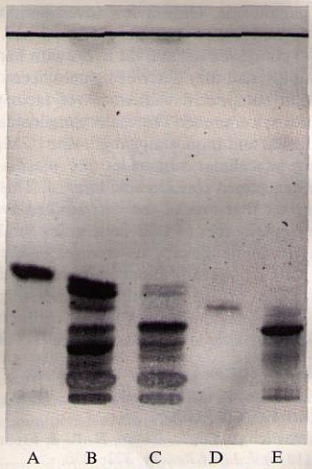

Fig. 2. Thin-layer chromatography of gangliosides of the tritosomal and the plasma membrane fractions.

Solvent system: chloroform/methanol/2.5N ammonia. Staining by Bial's orcinol reagent.

A) Hematoside (dog erythrocytes, *N*-acetylneuraminyl-lactosylceramide);
B) ganglioside fraction of tritosomal membranes;
C) ganglioside fraction of plasma membranes;
D) hematoside (*N*-glycolylneuraminyl-lactosylceramide, horse erythrocytes);
E) GM_1-ganglioside [galactosyl-*N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)-lactosylceramide, human brain].

[17] Wattiaux, R., Wibo, M. & Baudhuin, P. (1963) in A Ciba Foundation Symposion on Lysosomes (Reuck, A. V. S. de & Cameron, M. P., eds.) p. 176, Little Brown and Comp., Boston.

Table 1. Sugar composition, quantitative analysis of the identified glycosphingolipids isolated from the tritosomal membrane fraction, and the results of sequence determination.

	Concentration[a] [μg/mg prot.]	Molar sugar ratio[c]	Sugar sequence[d]
Monohexosylceramide	11.8	Glc	cer-Glc
Dihexosylceramide	9.4	Glc/Gal 1:1	cer-Glc-Gal
Trihexosylceramide	3.9	Glc/Gal 1:2	cer-Glc-Gal-Gal
Hematoside	8.4[b]	Glc/Gal 1:1	cer-Glc-Gal

[a] Based on anthrone determinations according to Razin[19].
[b] Calculated determinations of sialic acid by the thiobarbituric acid procedure[20].
[c] Ratios obtained by the results of gas liquid chromatography (see Fig. 3).
[d] Determined by partial hydrolysis.

terns of the two membrane fractions are completely different. In the tritosomal membrane fraction hematoside is the predominant ganglioside, whereas in the plasma membrane, GM_1-ganglioside is the main component. Hematoside is found only in small amounts in the latter fraction. The neutral glycosphingolipids shown in Fig. 1 were isolated from 3.5 mg protein of plasma membrane and from 1.5 mg protein of tritosomal membrane. The spot intensities indicate that the concentrations of glycosphingolipids are by far the higher in the tritosomal membrane; the same is true for the gangliosides, as revealed by the intensity of Bial-positive bands in thin-layer chromatography.

Component analysis of glycosphingolipids

The individual glycosphingolipids were identified by thin-layer chromatography. Their concentrations and the structures are summarized in Table 1. The concentrations of the glycosphingolipids decrease as the number of their constituent carbohydrate residues increases. The content of hematoside is not corrected and it might be higher, since several authors[18] have described an incomplete release of sialic acid by the hydrolysis procedures commonly used.

According to Table 1 the total glycolipid content accounts for about 12% (33,5 μg/mg protein) of the total tritosomal membrane lipids (270 μg/mg protein).

The total lipid-bound sialic acid was found to be 6.9 μg/mg of protein; hematoside-bound sialic acid was 2.1 μg/mg of protein.

a) Sugars

The molar ratio of sugars in the isolated glycosphingolipids was determined by gas-liquid chromatography of the alditol acetates of monosaccharides obtained after hydrolysis of the glycosphingolipids. Fig. 3 represents the results of gas liquid chromatography of the sugar moieties of the isolated glycosphingolipids.

The molar sugar ratios obtained by this method are summarized in the legend of Fig. 3. Amino sugars could not be detected in the identified glycolipids. The ganglioside fraction separated from hematoside contained galactosamine as the only amino sugar.

The carbohydrate sequence of the neutral glycosphingolipids and of hematoside was determined by partial hydrolysis. The resulting glycosylceramide mixtures were separated by thin-layer chromatography on borate impregnated plates[21]. The only monohexosylceramide derived from each identified glycolipid by partial hydrolysis was glucosylceramide. From this result and the molar sugar ratios obtained by gas liquid chromatography, the carbohydrate sequences given in Table 1 were derived.

The solvent system used for the isolation of trihexosylceramide does not separate this lipid from dihexosylceramide sulfate. The possibility of the presence of dihexosylceramide sulfate could be excluded by treatment of this compound under mild acid hydrolysis conditions (0.05N HCl, 4 h, 20°C).

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

[19] Razin, N. S., Lavin, F. B. & Brown, J. R. (1955) *J. Biol. Chem.* **245**, 789–796.

[20] Warren, L. (1959) *J. Biol. Chem.* **234**, 1971–1975.

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

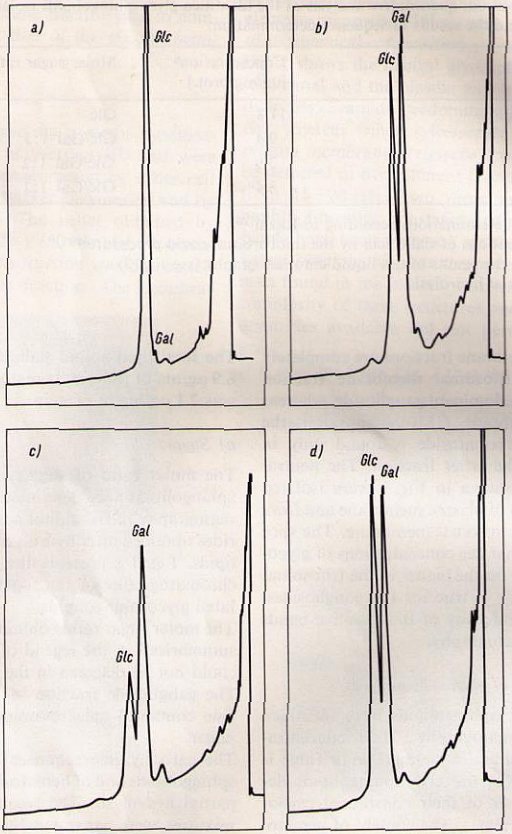

Fig. 3. Gas-liquid chromatograms of the alditol acetates of isolated glycosphingolipids.
a) Monoglycosylceramide: glucose, traces of galactose; c) triglycosylceramide: galactose/glucose, 1.9:1;
b) diglycosylceramide: galactose/glucose, 1.1:1; d) hematoside: galactose/glucose, 0.88:1.

According to Mårtensson[22] this treatment completely removes the $SO_3^{\ominus}$ group, whereas the glycosidic bond is resistant against this procedure. No dihexosylceramide could be detected by thin-layer chromatography after this treatment.

[22] Mårtensson, E. N. (1966) *Biochim. Biophys. Acta* **116**, 521–531.

b) Fatty acids

Table 2 summarizes the fatty acid composition of the glycosphingolipids obtained by gas-liquid chromatographic analysis of their methyl esters. The esterification was achieved by borontrifluoride/methanol. This method was carefully checked for the occurrence of artifacts from unsaturated fatty

acids as described by several authors[23,24], which could be avoided by the use of freshly prepared reagents.

Table 2. Fatty acid composition of the glycosphingolipids of the tritosomal membrane fraction (values given in % of the total).

Chain length: number of double bonds	Monohexosylceramide	Dihexosylceramide	Trihexosylceramide	Hematoside
14:0	0.7	1.5	2.7	0.9
14:1	–	–	–	0.4
15:0	0.5	1.0	2.3	0.3
16:0	14.3	16.4	16.7	7.5
16:1	3.4	3.5	0.5	1.6
17:0	0.7	1.1	2.5	1.0
18:0	6.9	8.2	10.8	15.5
18:1	7.7	5.7	7.2	3.4
18:2	2.3	1.0	1.2	0.7
18:3	–	0.5	–	–
19:0	0.4	–	0.9	0.7
20:0	1.7	3.2	2.4	2.7
20:4	1.5	2.1	4.7	2.5
21:0	0.8	1.2	1.2	0.5
22:0	10.5	9.2	3.4	8.7
22:1	–	1.7	1.3	0.9
23:0	9.0	7.5	4.3	7.0
23:1	2.0	1.1	0.8	2.1
24:0	23.0	19.1	11.8	29.3
24:1	3.4	6.5	3.7	9.4
26:0	7.5	9.0	19.2	3.5
26:1	–	–	–	1.4
unidentified	3.7	0.5	2,4	–

Monohexosyl- and dihexosylceramide appear as double spots in most solvent systems used (Fig. 1). Hydroxyfatty acids could not be detected by gas-liquid chromatography of the acetylated methyl esters or by thin-layer chromatography. Since the fatty acid methyl esters run as double spots in thin-layer chromatography (solvent system: 1,2-dichloroethane), the faster and the slower running spot were analyzed separately by gas liquid chromatography. The faster running spot contained mainly long chain fatty acids ($C_{22}-C_{24}$), whereas the slower running band consisted mainly of palmitic and stearic acid.

[23] Fulk, W. K. & Shore, M. S. (1970) *J. Lipid Res.* **11**, 276–277.

[24] Klopfenstein, W. E. (1971) *J. Lipid Res.* **12**, 773–776.

c) Long chain bases

The amount of glycosphingolipids available from subcellular material was too small for a direct analysis of the sphingosine bases. As extensively reviewed by Karlsson[25] the different types of bases are subjected to various acid- and alkali-induced chemical changes by the hydrolysis procedures. None of the reported procedures was found to give complete hydrolysis without producing substantial amounts of byproducts. The most widely used method of Gaver and Sweeley[26], which produces only small amounts of byproducts, cleaves quantitatively only the amide bond, whereas the glycoside bond is hydrolyzed incompletely. This agrees well with results reported from this laboratory[27] indicating an incomplete hydrolysis of sphingomyelin by this procedure. Prolonged hydrolysis results in allylic rearrangements leading to increasing amounts of *O*-methylsphingosine as revealed by gas-liquid chromatography of the trimethylsilyl derivatives of the long chain bases.

Aqueous 1N hydrochloric acid cleaves the glycosidic bond quantitatively but not the amide bond. Again the yield in long chain bases was not quantitative. Therefore we took advantage of the periodate oxidation of the long chain bases[28] after acid hydrolysis of the glycosphingolipids, since this procedure cleaves free bases and those substituted at the primary hydroxyl group as well. Although the analysis of the aldehydes prepared by the periodate oxidation gives only indirect evidence of the original base composition, this procedure has the advantage of the nearly quantitative production of derivatives from the original bases and it gives better resolution on gas-liquid chromatography than the widely used trimethylsilylation of long chain bases. The results of the gas-liquid chromatographic analysis of the aldehydes derived from the long chain bases are summarized in Table 3.

The aldehydes were identified by comparison with standards. The resulting patterns of identified aldehydes of the isolated neutral glycosphingolipids are very similar. They all contain C_{18}-sphingenine as the principal component, appreciable amounts of C_{20}-sphingenine shown by the presence of the

[25] Karlsson, K. A. (1970) *Chem. Phys. Lipids* **5**, 6–43.

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

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

Table 3. Results of gas-liquid chromatographic analysis of the aldehydes derived from the long chain bases by periodate oxidation.
CMH: monohexosylceramide; CDH: dihexosylceramide; CTH: trihexosylceramide.
−18:0 OH = C_{18}-4*D*-hydroxysphinganine (C_{18}-phytosphingosine).

Aldehyde	Parent base	CMH	CDH	CTH	Hematoside
14:0	16:0	2.4	4.1	5.2	2.1
14:1	16:1	–	< 0.5	–	–
15:0	18:0 OH	5.1	17.7	13.3	2.1
16:0	18:0	5.6	7.9	6.9	3.9
16:1	18:1	77.6	50.5	57.3	85.3
18:0	20:0	–	–	–	< 0.5
18:1	20:1	9.3	19.8	17.3	6.5

$C_{18:1}$ aldehyde, and of 4*D*-hydroxysphinganine (C_{18}-phytosphingosine) as indicated by the appearence of a $C_{15:0}$ aldehyde. Theoretically $C_{15:0}$ aldehyde may also be derived from C_{17}-sphinganine, but this compound has been found only in minute amounts as a component of the glycosphingolipids of other mammalian tissues[25]. Hematoside shows a very uniform pattern containing 85% sphingenine.

d) Sialic acids

The sialic acids of gangliosides have been analyzed by gas liquid chromatography of the trimethylsilyl derivatives of the methyl esters of the sialic acid methyl ketoside. Furthermore, the identity of the hematoside that contains *N*-acetylneuraminic acid was established by thin-layer chromatography as shown in Fig. 2. This solvent system gives a good separation of the hematoside containing neuramic acid and the hematoside containing *N*-glycolylneuraminic acid. *N*-Acetylneuraminic acid was the only sialic acid detected. *N*-Glycolylneuraminic acid could not be found in hematoside or in the other gangliosides.

Discussion

In rat liver plasma membranes, Benedetti *et al.*[29] reported that 95% of the sialic acid was protein bound, whereas Ray *et al.*[30] estimated that 5 to 6% of the total lipids of the surface membrane of the liver cell is glycolipid. Dod and Gray[31] described the presence of mono-, di- and trihexosylceramide and hematoside in rat liver plasma membranes. They calculated a glycolipid content of 7% of total lipids.

Regarding the intracellular occurrence of glycosphingolipids only indirect evidence has been reported. Weinstein *et al.*[18] identified lactosylceramide and monosialosyl ganglioside in a glycolipid preparation of total L-cells. However, they could not identify these compounds in surface membrane fractions prepared by various methods. The concentration of glycosphingolipids in tritosomal membranes (12% of total lipid) appears to be high as compared with data reported from plasma membranes.

The neutral glycolipid pattern is similar to that reported in total liver and other extraneural organs, in which mono- and diglycosylceramide are the predominant fractions. The sugar composition found in tritosomal glycolipids corresponds to data available from other extraneural tissues. Glucose is the characteristic carbohydrate moiety of extraneural monohexosylceramide. The tritosomal membranes contain lactosylceramide, which is also the most widely occurring dihexosylceramide (spleen[32], erythrocytes[33], pig lung[34], pig thrombocytes[35],

[29] Benedetti, E. L. & Emmelot, P. (1968) in The Membranes (Dalton, A. J. & Haguenau, F., eds.) p. 99, Academic Press, New York and London.
[30] Ray, T. K., Skipski, V. P., Barclay, M., Essner, E. & Archibald, F. M. (1969) *J. Biol. Chem.* **244**, 5528–5536.
[31] Dod, B. J. & Gray, G. M. (1968) *Biochim. Biophys. Acta* **150**, 397–404.
[32] Klenk, E. & Rennkamp, F. (1942) *this J.* **273**, 253–268.
[33] Klenk, E. & Lauenstein, K. (1953) *this J.* **295**, 164–173.
[34] Adams, E. P. & Gray, G. M. (1967) *Chem. Phys. Lipids* **1**, 368–375.
[35] Heckers, H. & Stoffel, W. (1972) *this J.* **353**, 407–418.

exception: Cer-Gal-Gal in kidney[36]). The carbohydrate composition and sequence in trihexosylceramide is the same as that originally described by Klenk and Lauenstein[33]. The presence of trihexosylceramide-containing amino sugars, as described in the brain of Tay-Sachs patients and in minor amounts in normal brains[37], could be excluded since no amino sugar was found in gas-liquid chromatography. It should be noticed that no sulfatides could be detected either in plasma or in tritosomal membranes. This is in contrast to observations of Dod and Gray[31], who reported the presence of sulfatides in plasma membranes of rat liver.

The most widely occurring extraneural ganglioside is hematoside[38–41]. Few data are available on the more complex gangliosides with the exception of bovine and human tissues[40]. In the tritosomal membrane fraction we were able to establish the structure of the main ganglioside as *N*-acetylneuraminyl-lactosylceramide. The correct carbohydrate sequence was confirmed by partial hydrolysis, which yielded glucosylceramide as a fragment, and by the molar ratio of 1:1 for galactose/glucose, as determined by gas liquid chromatography.

The fatty acids of the tritosomal membrane glycosphingolipids show a typical composition when compared with other tissues. Brain, kidney and spleen glycosphingolipids[40] contain a high concentration of saturated long chain fatty acids, mainly C_{24}-acids. Polyunsaturated fatty acids are only present in very low concentrations or are essentially absent. The overall fatty acid composition of tritosomal membrane glycolipids corresponds well to this general feature. Another characteristic detail is the absence of 2-hydroxyfatty acids in tritosomal glycolipids. The lack of hydroxyfatty acids has been reported in glycolipids of liver, spleen and leucocytes, but they have been found in brain and in several other tissues[39,40]. Hematosides of horse erythrocytes[42], dog intestine[43] and liver[40] generally contain more than 60% of long chain fatty acids. This agrees well with the hematoside isolated from tritosomal membranes.

Comparing the fatty acid patterns of the isolated glycolipids, it is evident that there is a close relationship between mono- and dihexosylceramide, whereas trihexosylceramide has a lower content of long chain fatty acids, mainly in lignoceric acid. This suggests a common precursor pool for mono- and dihexosylceramide, whereas the third glycosyl transferring step appears to show a slight preference for other fatty acid patterns. This also holds true for hematoside. The fatty acid composition of glycolipids may be controlled in several steps of the biosynthetic pathway; but experimental evidence is available only at the level of acyl-CoA-transferase, in the synthesis of ceramide with the aid of brain microsomes[44]. On the other hand, the multiglycosyltransferase system proposed by Roseman[45] would not allow the formation of glycolipid subclasses with different fatty acid compositions. The determination of the fatty acid specificity might also be possible by a fatty acid exchange mechanism as proposed by Lands[46] for the phospholipids. The determining step could be found in both the hydrolyzing and the reacylating step. In the case of sphingomyelin, however, such a mechanism has been excluded by Stoffel[27].

The heterogeneity of the long chain base composition again argues against a common precursor pool. The occurrence of 4*D*-hydroxysphinganine (phytosphingosine) has been reported in several organs, but mainly in kidney[25]. C_{20}-Sphingenine has been found recently in brain[47].

In summary, the results of the glycolipid component analysis of the tritosomal membrane fraction are comparable to that found in other extraneural organs or surface membranes. No characteristic features can be concluded for the tritosomal membrane since the glycolipid composition of other intracellular membranes to our knowledge has not been investigated.

The main purpose of this analysis was to investigate whether a pinocytosis-induced membrane flow of

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the plasma membrane to the secondary lysosomal membrane takes place during the uptake of Triton WR 1339. The concentration of glycolipids in the plasma membrane apparently is much lower than in the tritosomal membrane as roughly estimated from band intensities in thin-layer chromatography. This is in line with higher concentrations of sugars and sialic acid in the total tritosomal membrane fraction[12]. Furthermore, the presence of terminal and subterminal carbohydrate residues, which are most probably not bound to glycolipid, can be clearly demonstrated by agglutination of tritosomes by several carbohydrate specific heterophile agglutinins from plants and snails[48].

In a first approach only the tritosomal glycolipids have been investigated rigorously and compared qualitatively with those of the plasma membrane. The neutral glycolipids in the two membranes are similar, with the exception of trihexosylceramide, which could not be detected in the plasma membrane. The ganglioside patterns, however, are completely different (Fig. 2). There are at least two explanations of the differences in the detailed composition of this lipid class. Firstly, according to electron micrographs after carbohydrate staining[49,50], glycolipids are very likely present in primary lysosomes, although this is not definitively proven. The plasma membrane lipids integrated into the lysosome might be altered by the lysosomal enzymes. Furthermore the heterogeneity of the plasma membrane, composed of the sinusoidal site, the bile canaliculus and the intercellular region must be taken into consideration. It can be demonstrated by subfractionation of the plasma membrane[51]. A possible enrichment of the sinusoidal part has been reported by House and associates[52] using hormone sensitive receptors as marker substances. The two-cell system of the liver also contributes to the apparent heterogeneity of the plasma membrane, since the parenchymal cells account for 90–95% of the liver cell weight, but only 60–65% of the cell population, and the Kupffer cells contribute 5–10% of the cell weight and approximately 35% of the cell population[53]. These data restrict conclusions on relationships of the tritosomal membrane and the plasma membrane fraction in rat liver which are based only on the chemical analysis of the two membrane fractions.

The second aspect which already has been discussed in previous papers[12,13] is the possibility of a modification or transformation of the plasma membrane after its interiorization. Attempts to test this idea have been reported at the histochemical level in capillary endothelial cells[54] and macrophage cells[55]. Recent experiments of Werb and Cohn[56] explain our previous findings of an absence of plasma-membrane-specific enzymes in the tritosomal membrane. These authors showed the continuous disappearance of 5′-nucleotidase in phagolysosomes of macrophages which had been isolated after phagocytosis of polystyrene latex particles. The enzyme had a phagolysosomal half-life of 2 h starting with the addition of latex to macrophage cultures. These experiments unambiguously show the change or the loss of plasma-membrane-specific features after the incorporation into the lysosomal membrane.

In view of these aspects, the origin of glycosphingolipids in the tritosomal membrane fraction may be either plasma membrane derived or originate from subcellular membranes which have been taken up by autophagocytosis, or this lipid class has to be regarded as a genuine lysosomal constituent. None of these proposals is more probable than the others on the basis of experimental evidence now available. Glick *et al.*[57] reported a high content of sialic acid and hexosamines in a lysosome-rich fraction of tissue culture cells (L-cells), indicating the presence of glycolipids and/or glycoproteins. In cell fractionation studies the activity of acid phosphatase paralleled the content of sialic acid. Recent results in our laboratory suggest an important role of sialic acid in providing the lysosomal membrane with a strong anionic charge. Only the isolation and

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the analysis of true primary lysosomes would allow a decision on the origin of glycosphingolipids in lysosomes.

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Experimental

Glycosphingolipids used as test substances were kindly provided by *Professor Dr. E. Klenk* and *Dr. G. Tschöpe* from this institute. The following test substances have been used: cerebroside (Cer-Gal, ox brain), glucosylceramide (ox spleen), lactosylceramide (ox spleen), trihexosylceramide (Cer-Glc-Gal-Gal, ox spleen), globoside (Cer-Glc-Gal-Gal-GalNAc, human erythrocytes), tetrahexosylceramide (asialoganglioside, Cer-Glc-Gal-GalNAc-Gal, ox brain), sulfatide (Cer-Gal-SO_3H, ox brain), hematoside (Cer-Glc-Gal-NeuNAc, dog erythrocytes), GM_1-ganglioside (Cer-Glc-Gal-NeuNAc-GalNAc-Gal, human brain). *N*-acetylneuraminic acid (NeuNAc) and *N*-glycolylneuraminic acid were kind gifts of *Prof. Dr. W. Gielen* (University of Cologne) and *Dr. H. Schoop* (University of Bochum). Triton WR 1339 and boron trifluoride in methanol (14%) were purchased from Serva (Heidelberg, Germany). All other chemicals were of analytical grade.

Preparation of subcellular fractions

The homogenization procedures, the isolation of plasma membranes, of Triton WR 1339 filled lysosomes and their enzymatic and electron microscopic criteria of purity have been described previously[12–14]. The tritosomal membrane fraction was prepared by centrifugation at $105000 \times g$ for 1 h after disrupting the tritosomes either by brief sonication or by osmotic shock with distilled water. It has been shown previously[12] that both methods lead to the same results with respect to the lipid composition. The $105000 \times g$ sediment was designated the tritosomal membrane fraction.

Extraction and isolation of glycolipids

The lyophilized membrane fractions were extracted by a slightly modified Folch[58] procedure. Briefly, about 20 mg of membrane protein was extracted twice with 4 m*l* of chloroform/methanol 2:1 (10 h and 6 h at 22°C). The residue was reextracted with chloroform/methanol 1:2 for 15 min under reflux. The combined filtrates were evaporated under N_2. The lipids were redissolved in 5 m*l* of chloroform/methanol 2:1, washed with 1 m*l* of 0.1M KCl, then with Folch's theoretical upper phase containing KCl and three times with Folch's theoretical upper phase without KCl in order to afford an optimal extraction of hematoside from the lower phase. The combined upper phases were dialyzed for 72 h at 4°C against distilled water and stored at −20°C.

Isolation of neutral glycosphingolipids

The lower phase was evaporated under N_2, redissolved in chloroform and subjected to column chromatography with silicic acid (column diameter 0.5 cm, 0.6 g silicic acid, Merck). The neutral lipids were eluted with 25 m*l* of chloroform, the neutral glycolipids with 25 m*l* of acetone and 15 m*l* of acetone/methanol 9:1[59]. By this procedure a contamination with phospholipids could generally be avoided. If contaminating phospholipids were present they were hydrolyzed by mild alkaline hydrolysis[60] with subsequent column chromatographic separation. Mono-, di- and trihexosylceramides were isolated by preparative thin-layer chromatography (silica gel H, Merck, solvent system chloroform/methanol/water 65:25:4). The glycolipids were localized by a brief exposure to iodine vapour. After evaporation of iodine the marked spots were scraped off and eluted with chloroform/methanol 1:1 (5×5 m*l*). For identification and detection of the glycolipids the plates were sprayed with α-naphthol/sulfuric acid[61] and heated for 3–5 min at 110°C.

Isolation of hematoside

The dry residue of the lyophilized upper phases was extracted three times with chloroform/methanol/water 10:20:3. Hematoside was isolated by preparative thin-layer chromatography (silica gel H, solvent system: chloroform/methanol/ammonia 2.5N, 60:35:8). Hematoside-containing spots were detected with Bial's orcinol spray[62].

Quantitative estimations of the glycosphingolipids were carried out by the anthrone method according to Razin[19]. The procedure was standardized with galactosylceramide and standard mixtures of glucose and galactose. Glucose was found to produce 1.52 times higher extinctions than galactose. Extinctions obtained with pure cerebroside corresponds well to the expected galactose content. From the molar ratios obtained by gas liquid chromatography it was possible to calculate the quantity of the isolated glycolipid. The hematoside content in membrane extracts was determined by the thiobarbituric assay[20] from hematoside-containing spots in thin-layer chromatograms.

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Carbohydrate analysis

The hydrolysis procedure was performed as described by Weinstein *et al.*[29]. 50–100 μg of the isolated glycolipid was hydrolyzed with 0.5 ml of 1N HCl in Teflon-lined screw cap tubes at 100°C under a nitrogen atmosphere for 12 h. After cooling, the samples were transferred into new tubes with several rinses of water. The fatty acids were removed with three extractions with n-hexane (3 ml) and additional lipid material with 2×2 ml of chloroform. The aqueous phase was evaporated and excess of HCl was removed under vacuum in a desiccator over KOH. The sugars were reduced in 0.5 ml of a 0.02% solution of sodium borohydride[63]. After 1 h, an additional 100 μg of $NaBH_4$ was added. After 2 h excess $NaBH_4$ was destroyed with 0.25 ml of acetic acid. The samples were evaporated several times after adding methanol. After vigorous drying, acetylation of the alditols was performed according to Crowell and Burnett[64] in 0.1 ml of a mixture of acetic anhydride/pyridine 1:1 at 75°C. Both reagents were distilled just before use. After 12 h, 0.5 ml of water was added, the samples were taken to dryness, the alditol acetates dissolved in 5 μl of chloroform and analyzed by gas-liquid chromatography. Galactose, glucose, galactosamine and glucosamine were separated on 3% ECNSS-M on a Chromosorb Q column (1.2 m, 2 mm diameter) in one run isothermally at 190°C[65]. Alditol acetates and hexosaminitol acetates were synthesized and crystallized in this laboratory[35] and used as standards. Fatty acid methyl esters were prepared by treating the glycolipids with boron trifluoride in methanol (14%) containing 20% benzene at 100°C in Teflon lined screw cap tubes[66]. After 90 min the fatty acid methyl esters were isolated by extraction with 4×2 volumes of n-hexane after the addition of half a volume of water. Gas-liquid chromatographic analysis was carried out on a 2.5% diethylene glycol succinate polyester on a Chromosorb W column at 185°C with a Perkin Elmer gas chromatograph, model F 20, equipped with a flame ionization detector. Unsaturated fatty acids were analyzed before and after catalytic hydrogenation (PtO_2).

Sialic acid was analyzed by gas-liquid chromatography of the trimethylsilyl derivative of the methyl ketoside methyl ester of neuraminic acid. The ganglioside sample (about 50 μg) was hydrolyzed as described by Puro[67] (0.1N methanolic HCl, 80°C, 2 h). Excess lipid material was removed by partitioning between chloroform and water. The aqueous phase was evaporated and the methyl ketoside methyl esters were silylated with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide according to Donike[68].

Sphingosine bases prepared from the isolated glycolipids by the hydrolysis procedure described by Gaver and Sweeley[26] were subjected to periodate oxidation according to Sweeley and Moscatelli[28]. The aldehydes were analyzed by gas-liquid chromatography on 1% SE 30 on a Chromosorb W column at 170°C.

The determination of the sugar sequence in the carbohydrate backbone of the glycolipids was achieved by partial hydrolysis in 0.1N HCl (100°C, 2 h). The reaction products were extracted three times with chloroform/methanol 2:1. The sugar moiety of the resulting monohexosyl ceramide was analyzed by thin-layer chromatography on 2.5% borate-impregnated silica gel G plates (solvent system: chloroform/methanol/water 24:7:1)[21].

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