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THE CHEMISTRY OF LIPID OF POSTHEMOLYTIC RESIDUE OR STROMA OF ERYTHROCYTES

IX. SILICIC ACID CHROMATOGRAPHY OF MAMMALIAN STROMA GLYCOLIPIDS

By TAMIO YAMAKAWA, REIKO IRIE AND MICHIKO IWANAGA

(*From the Department of Chemistry, the Institute for Infectious Diseases, the University of Tokyo, Tokyo*)

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In 1951, Yamakawa and Suzuki isolated a ganglioside-like lipid, named hematoside, from equine red blood cells stroma (*1*). It was later confirmed by Klenk and Wolter (*2*) and further reexamined by Klenk and Lauenstein (*3*). Hematoside is now assumed to consist of lignoceric acid, sphingosine, two moles of hexose (mainly galactose besides a small amount of glucose) and N-glycollyl neuraminic acid (*4*). Klenk and Lauenstein were the first, however, to obtain a glycolipid from human erythrocytes (*5*). Further investigations by Yamakawa and Suzuki (*6*) as well as by Klenk and Lauenstein (*7*) have shown that the main glycolipid (named globoside) has an approximate structure of lignoceryl (or nervonyl) sphingosine acetyl-galactosamine tri- or tetra-hexoside. Yamakawa and Iida (*8*) found that globoside carries the capacity to inhibit the agglutination of erythrocytes of a given blood group by its corresponding isoagglutinin. From studies on the bovine erythrocytes by Klenk and Lauenstein (*7*), the hexosamine constituent is glucosamine, while Matsumoto (*9*) isolated from hog red cells a glycolipid, whose hexosamine is exclusively galactosamine. As has been emphasized by Yamakawa and co-workers (*10*), the glucosamine/galactosamine ratio of the lipid fraction of erythrocytes is quite characteristic of animal species.

On the other hand, Papirmeister and Mallette (*11*), while purifying the Forssman hapten from sheep red cells, noted that the essential substance for the activity was a glycolipid similar to globoside. Meanwhile, Kabat (*12*) doubted the group activity exhibited by globoside might be ascribed to a possible contamination of group mucoid. In contradiction to this, Yamakawa *et al.* (*13*) pointed out that the activity of globoside was much more stable toward dilute acid than mucoid group substance obtained from gastric mucin. More recently, Radin (*14*) attempted to fractionate globoside by cellulose column chromatography and reported that the ABO-activity was separated from main glycolipid peak. Shortly afterward, Yamakawa *et al.* (*15*) reported a similar finding using silicic acid column

and indicated the active portion was further divided into two fractions.

The present study is primarily concerned with the application of silicic acid chromatography to these and other several mammalian erythrocytes glycolipids. As the results, closely related glycolipids have been separated on the column by means of fractional elution with chloroform and methanol and the patterns obtained are remarkably characteristic and highly reproducible.

EXPERIMENTAL

Preparation of the Column—Columns of different sizes were prepared from 2 parts of silicic acid (Mallinckrodt, AR., suitable for chromatographic analysis, without activation) and one part of thoroughly washed dry Hyflo Super-Cel (Johns Mannville). In a typical experiment, 60 g. (40 g.+20 g.) of mixed powder was suspended in chloroform to a rather thin slurry which was then poured into a chromatographic tube of 2.2 cm. diameter and allowed to settle.

Preparation of the Sample—Erythrocytes of various species of mammals were hemolyzed with 10 volumes of 0.3 per cent acetic acid solution, stroma precipitated was spun down, washed and freeze-dried. Extraction of this dry stroma with methanol-ether (1 : 1) mixture was repeated four times, and from this extract was obtained so-called M.E.-glycolipid by treatment with anhydrous ether and pyridine. M.E.-glycolipid was contained in the pyridine-soluble portion of ether-insoluble white matter separated from the extract. Methanol-ether extracted residual stroma gave so-called C.M.-glycolipid upon continuous extraction with chloroform-methanol (1 : 1); the dark brown extract thus obtained was concentrated *in vacuo*, dissolved in a small volume of chloroform and precipitated with acetone. The yield of total glycolipid amounted to about 2 per cent of dry stroma, and was variable depending on the procedure and animal species.

Addition of the Sample to the Column—About 1.2 g. of the glycolipid sample dissolved in minimal amount of chloroform was applied on top of the column and allowed to drain in slowly. The inner walls of the tube were then washed with a small amount of chloroform.

Elution of the Material—The elution of the material from the column could be accomplished by the use of chloroform-methanol mixture of stepwise increasing concentration of the latter. In a typical experiment with a column composed of 40 g. of silicic acid and 20 g. of Hyflo Super-Cel, the elution was carried out by successive application of each 200 ml. portion of chloroform, then 20, 40, 60 and 80 per cent methanol-containing chloroform and finally pure methanol.

Analysis of the Fractions—In all case, a quantitative determination of carbohydrate was performed on each fraction. A suitable aliquot of fraction was pipetted into a graduated test tube and after the solvent was evaporated to dryness by immersing in boiling water-bath, the residue was dissolved in 0.5 ml. of water. Five ml. of anthrone reagent (100 mg. of anthrone and 2 mg. of thiourea dissolved in 200 ml. of 66 per cent sulfuric acid) was added and after thorough mixing, the mixture was heated at 100° for 15 min. Optical density was measured in a Coleman 6A electro-photometer using D-galactose as standard. In some cases, sialic acid was determined as described previously (*16*). To 1 ml. of aqueous solution was added 1 ml. of Bial's oricinol reagent and heated at 100° for 22 minutes. As reference standard, N-acetyl neuraminic acid from human erythrocytes (*4*) was used in this determination.

In case of human stroma, ABO-group activity was assayed with each fraction by

isoagglutination inhibition technique and in case of sheep and cat blood cells, Forssman activity determination was carried out by sheep cell hemolysis inhibition.

Isolation and Analysis of Purified Glycolipid—Fractions corresponding to glycolipid peak on chromatogram were pooled. After the solvent was evaporated *in vacuo* nearly to dryness at 40°, the residue was redissolved in minimal amount of chloroform, precipitated by acetone, dried in a desiccator, weighed and analyzed.

Phosphorus (*17*), hexose, reducing value (as galactose) (*18*), hexosamine (as glucosamine HCl) (*19*) and sialic acid (*16*) were measured with this material. Glucosamine/galactosamine ratio was determined according to Gardell (*20*). For optical rotation measurement, Hitachi spectrophotometer (EPU-2A) was used.

RESULTS AND DISCUSSION

In general, M.E.-glycolipid contains rather more ceramide-oligohexoside, which is usually more readily soluble in chloroform. On the other hand, to C.M.-glycolipid belong such glycolipids as are hardly soluble in pure chloroform unless a small amount of methanol is added. It can be said that M.E.-glycolipids are rather faster eluted from silicic acid column than C.M.-glycolipids.

Moreover, evidences have been accumulated, that phospholipid, especially lecithin and sphingomyelin were completely extracted with methanol-ether. However, an additional amount of cephalin-type phospholipid was contained in C.M.-glycolipid portion, and on chromatography, the slower eluted mucolipid fractions were often observed to be contaminated by this phospholipid. The removal of this non-choline-containing phospholipid could be accomplished by repeated chromatography on silicic acid, but not by Florisil.

Note on Nomenclature—The name, ceramide-oligohexoside*, is given to the class of glycolipids containing neither hexosamine nor sialic acid. The authors use the term, mucolipid, for such glycosphingoside as possessing hexosamine and/or sialic acid.

Fractionation of Human Erythrocytes Glycolipids (*Globoside*)—Crude globoside (*6*) was divided into several fractions (Fig. 1.). Fr. II were considered to contain ceramide-trihexosides. Fr. IV was highest in yield and composed of ceramide, three hexose and acetyl-galactosamine. Blood group activity appeared thereafter in Fr. VI and VII. In these more slowly eluted lipids, glucosamine and sialic acid were also present besides galactosamine. The detailed immunochemical study of these group-active fractions will be reported shortly after elsewhere.

Fr. II isolated by silicic acid chromatography of mixed lipids revealed the presence of a significant amount of phosphorus. That this phosphorus was indeed a contaminant and not a constituent of glycolipid was illustrated by the fact that it could be removed by rechromatography on silicic acid and on Florisil column according to the procedure of Radin (*21*). Fr. II of M.E.- as well as C.M.-glycolipid were collected and rechromatographed on silicic acid column by eluting with solvent mixtures of chloroform-methanol 9:1, 8.5:1.5, and 8:2. The low-phosphorus (P 0.38) lipid (550 mg.) thus obtained was dissolved in a small amount of chloroform-methanol (2:1), passed through a column of Florisil (25 g.) and eluted with 300 ml. of this solvent mixture. Thus, an apparently pure phosphorus-free glycolipid was obtained.

* Rapport, M. M., and Alonzo, N. (*Federation Proc.*, **18**, 307 (1959)) have proposed the name 'cytoside' for tumor-specific glycolipid of similar structure. But it seems not yet to be determined whether cytoside is really ceramide-oligohexoside itself.

Analysis: C 63.4, H 10.2, N 1.36, Hexose 48.4, Reducing value, 49.2, Hexosamine, 0.35, $[\alpha]_{589}$ −9.7° (10 per cent in pyridine).

On the basis of quantitative determination, it appears the material is most probably lignoceryl sphingosine trigalactoside.

Calculated for $C_{60}H_{113}NO_{18}$ (1135)
C 63.4 H 10.0 N 1.23 Hexose 47.6

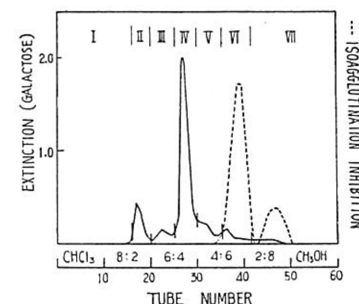

Fig. 1. Silicic acid chromatography of human erythrocytes M.E.-glycolipid.

TABLE I

Composition of a Typical Case of Human Erythrocytes M.E.-Glycolipid (Corresponding to Fig. 1.)

	Original Mixture	Fraction No.						
		I	II	III	IV	V	VI	VII
Weight, mg	1200	14	**200**	48	**449**	55	30	152
Hexose, %	28.9	3.30	**24.8**	38.2	**45.3**	39.3	25.6	2.43
Hexosamine, %	—	0	**0**	1.00	**13.3**	10.7	11.2	2.20
Phosphorus, %	1.52	—	**2.20**	—	—	—	—	—

One g. of Fr. IV (Fig. 1) or Fr. III (Fig. 2) was loaded on a column composed of 50 g. of Florisil. Each 310 ml. of solvent mixtures of chloro-

form-methanol, 2 : 1 and 1 : 1, was sufficient to allow separation of essentially phosphorus-free mucolipid. 538 mg. of this mucolipids was further purified on silicic acid column (60 g. of mixed powder) by eluting with 600 ml. of chloroform-methanol (7 : 3). An apparently single glycolipid peak was obtained.

Analysis:

Found: C 59.23-60.95, H 9.49-10.23, N 2.23, Hexose 49.5-50.0, Hexosamine 17.4-17.5, Reducing val. 62.4-67.5

Sialic acid was not detectable. $[\alpha]_{589} = +19.5°$ (10% in pyridine)

Calculated for lignoceryl sphingosine acetylgalactosamine trihexoside $C_{69}H_{126}N_2O_{23}$: C 60.96, H 9.48, N 2.09, Hexose 53.8, Hexosamine 16.1

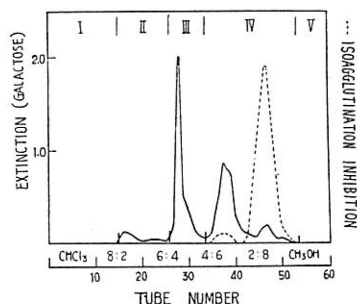

Fig. 2. Silicic acid chromatography of human erythrocytes C.M.-glycolipid.

Table II

Composition of a Typical Case of Human Erythrocytes C.M.-Glycolipid (corresponding to Fig. 2.)

	Original Mixture	Fraction No. I	II	III	IV	V
Weight, mg	980	—	188	**125**	179	73
Hexose, %	14.6	—	5.12	**35.2**	23.7	3.41
Hexosamine, %	—	—	0.47	**11.6**	10.0	1.43
Phosphorus, %	3.00	—	3.26	**1.07**	1.96	—

By Gardell's procedure, the existence of only galactosamine as amino sugar was established. The results of the analysis corresponds to lignoceryl sphingosine acetylgalactosamine trihexoside previously assumed for globoside (*6*). The value of optical rotation formerly reported, +10.3°, was too low, possibly since the material was mixed with levo-rotatory ceramide oligohexoside. On hydrolysis, galactose and glucose were detected by paper chromatography. This purified main glycolipid gave clear aqueous solution, and when heated it turned turbid and became clear again by followed cooling. Contrary to the previous finding (*22*), this aqueous solution was not viscous and gave apparently single boundary and sedimentation coefficient comparable with those of brain ganglioside (*22*), strandin (*23*, *24*) and brain mucolipid (*25*) in the ultracentrifugal field (Fig. 3, 4).

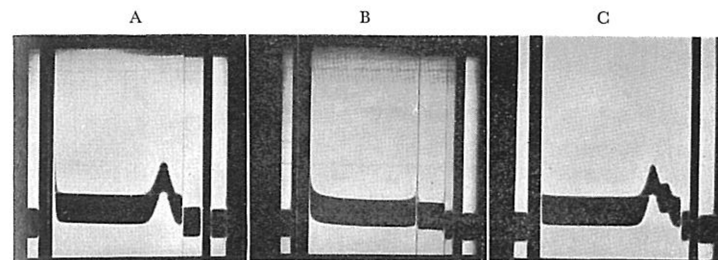

Fig. 3. Sedimentation patterns of purified mucolipids.
A. Globoside (purified Fr. IV., Fig. 1), 0.4% in 0.85% NaCl 35,600 r.p.m., $s_{20,w} = 10.9$ S
B. Hematoside, the latter peak (Fr. VI, Fig. 5), 0.4% in water 35,600 r.p.m., $s_{20,w} = 24.4$ S
C. Hematoside, the former peak (Fr. IV, Fig. 5), 0.4% in water 35,610 r.p.m.

Blood group active portions were concentrated in Frs. VI and VII in case of M.E.-, and in Fr. IV in case of C.M.-glycolipid. In both cases, rechromatography on silicic acid divided them into two distinctly separated group-active fractions and the analytical values of these materials, although not yet pure, are as follows.

Active portion *I* Hexose 33.2, hexosamine 13.2, sialic acid 5.3
,, ,, *II* ,, 40.7, ,, 14.5, ,, ,, 2.1
Glucosamine/galactosamine ratio of active portion *I* is 1.0 and *II* is 1.5.

Fractionation of Equine Erythrocytes Glycolipids (Hematoside)—In this case, the yield of M.E.-glycolipid was found to be extremely small and the mucolipids (hematosides) were present abundantly in C.M.-glycolipid. This tendency was already observed as reported previously (*1*). Data from a typical experiment are presented in Figs. 4 and 5 together with Tables III and IV.

The relatively high content of phosphorus in the fast running peak (Fr. II) made it seem desirable to remove phospholipid further by usual Florisil technique. However, in this case, the trial was accomplished only with difficulty. Moreover, the clean-cut separation of hematosides fractions (Frs. IV, V and VI of Fig. 5) was not successful by rechromatography of each fraction, but the individual existency of Frs. IV and VI were repeat-

edly indicated. Because of the similarity of composition, a postulation that Fr. VI might be a salt form of Fr. IV is indeed possible, though several attempts with or without acidification as well as electrodialysis prior to

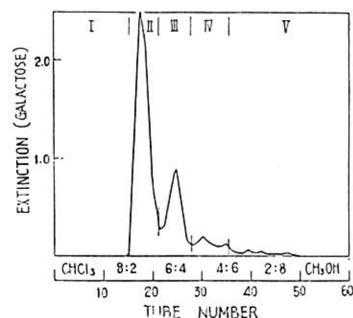

Fig. 4. Silicic acid chromatography of equine erythrocytes M.E.-glycolipid.

TABLE III

Composition of a Typical Case of Equine Erythrocytes M.E.-Glycolipid (corresponding to Fig. 4.)

	Original Mixture	Fraction No.				
		I	II	III	IV	V
Weight, mg	600	—	356	19	65	8
Hexose, %	6.26	—	8.72	18.5	2.74	2.91
Hexosamine, %	—	—	0.13	0.40	0.31	0.26
Sialic acid, %	—	—	—	10.3	0.73	0
Phosphorus, %	3.09	—	—	1.56	2.98	—

chromatography did not yet give concrete evidence for this assumption. The phosphorus-free specimen of Frs. IV and VI showed similar optical activity, $[\alpha]_{589} = -14.5°$ (1.86 per cent in pyridine) and $-17.2°$ (1.16 per cent), respectively, but aqueous solution of the latter gave similar extraordinary boundary (Fig. 3, B) in the ultracentrifugal field as reported already with blood cells mucolipids (*22*), whereas the sedimentation pattern of purified Fr. IV represented apparently two different components (Fig. 3, C).

In several experiments, Frs. VII as well as VIII contained considerable amount of sialic acid and hexosamine, but in another instance only sialic acid was present. The presence of such a hexosamine-containing glycolipid in equine stroma was already described by Klenk and Lauenstein (*3*).

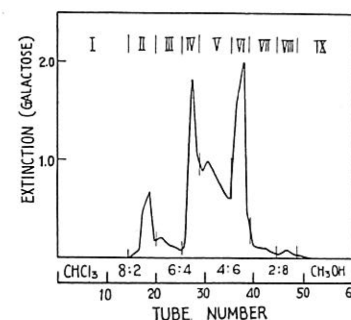

FIG. 5. Silicic acid chromatography of equine erythrocytes C.M.-glycolipid.

TABLE IV

Composition of a Typical Case of Equine Erythrocytes C.M.-Glycolipid (corresponding to Fig. 5.)

	Original Mixture	Fraction No.								
		I	II	III	**IV**	V	**VI**	VII	VIII	IX
Weight, mg	900	—	275	18	**131**	185	**200**	22	16	6
Hexose, %	16.0	—	5.25	1.67	**28.0**	34.5	**35.9**	27.5	15.4	—
Hexosamine, %	1.10	—	0.44	—	**0.71**	0.44	**0.71**	4.51	3.22	—
Sialic acid, %	20.7	—	1.25	0.68	**19.8**	26.7	**29.3**	22.5	58.7	—
Phosphorus, %	1.34	—	3.36	—	**0.47**	0	**0.08**	0.96	—	—

Fractionation of Bovine Erythrocytes Glycolipids—As illustrated in Figs. 6 and 7, the M.E.-glycolipid contains nearly whole amount of ceramide-oligohexoside and only a small portion of glucosamine-containing mucolipid, while in the C.M.-glycolipid, the former is almost negligible and the latter larger in amount. In this fraction (Fr. IV) the existence of sialic acid was also indicated, however in another experiment, this mucolipid possessed hexosamine but no sialic acid. This strange finding was also already described by Klenk and Lauenstein (*7*).

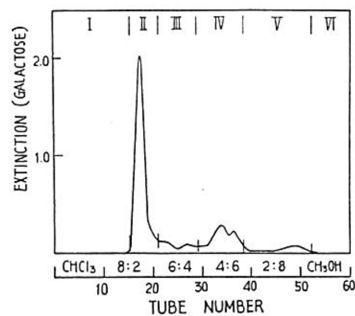

Fig. 6. Silicic acid chromatography of bovine erythrocytes M.E.-glycolipid.

Table V

Composition of a Typical Case of Bovine Erythrocytes M.E.-Glycolipid (corresponding to Fig. 6.)

	Original Mixture	Fraction No.					
		I	II	III	**IV**	V	VI
Weight, mg	530	—	136	30	**16**	187	18
Hexose, %	6.71	—	14.2	10.5	**34.3**	1.86	2.76
Hexosamine, %	—	—	0.14	1.66	**7.97**	0.48	—
Sialic acid %	—	—	—	—	**2.73**	0	—
Phosphorus, %	3.30	—	2.92	—	**0.96**	3.77	—

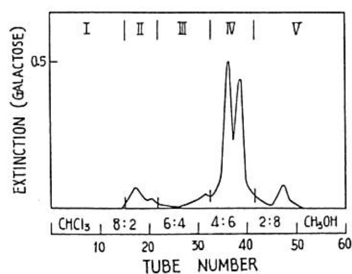

Fig. 7. Silicic acid chromatography of bovine erythrocytes C.M.-glycolipid.

Table VI

Composition of a Typical Case of Bovine Erythrocytes C.M.-Glycolipid (corresponding to Fig. 7.)

	Original Mixture	Fraction No.				
		I	II	III	**IV**[a)]	V
Weight, mg	850	—	533	84	**61**	9
Hexose, %	4.55	—	0.82	2.02	**25.8**	1.51
Hexosamine, %	—	—	0.02	0.32	**6.59**	4.15
Sialic acid, %	—	—	—	0	**10.8**	—
Phosphorus, %	3.44	—	3.87	—	**1.56**	—

a) In another experiment: Hexose 24.6, Hexosamine 8.70, Sialic acid 0.76.

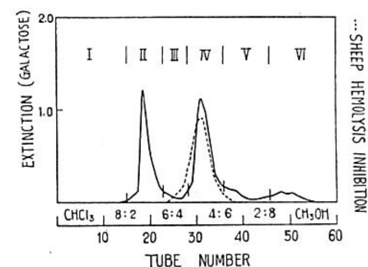

Fig. 8. Silicic acid chromatography of sheep erythrocytes M.E.-glycolipid.

Table VII

Composition of a Typical Case of Sheep Erythrocytes M.E.-Glicolipid (corresponding to Fig. 8.)

	Original Mixture	Fraction No.					
		I	II	III	**IV**	V	VI
Weight, mg	504	—	180	10	**54**	46	100
Hexose, %	9.24	—	7.36	11.2	**23.3**	6.05	0.97
Hexosamine, %	—	—	0.21	—	**14.4**	2.64	—
Sialic acid %	—	—	0	0	**0**	0	—
Phosphorus, %	2.80	—	2.64	2.48	**0.36**	2.66	3.18

Fractionation of Sheep Erythrocytes Glycolipids—Sheep red cells are well known carrier of Forssman antigen. Forssman activity existed in M.E.-glycolipid and on silicic acid column, the active fraction coincided in position with the main mucolipid, which contained galactosamine and glucosamine at a ratio of 4 : 1 but no sialic acid. In C.M.-glycolipid there was no Forssman activity, indicating the active material is more or less loosely bound with stroma.

The main mucolipid (Fr. IV in Fig. 8) inhibited sheep cell hemolysis in amount as little as 1 μg. and after purification, showed the following composition.

Hexose 36.9, hexosamine 17.4, reducing value 56.6, N 2.37, P 0.15

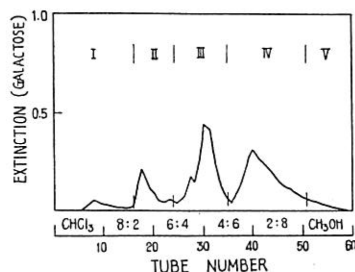

Fig. 9. Silicic acid chromatography of sheep erythrocytes C.M.-glycolipid.

Table VIII

Composition of Sheep Erythrocytes C.M.-Glycolipid
(corresponding to Fig. 9.)

	Original Mixture	Fraction No.				
		I	II	III	IV	V
Weight, mg	600	—	243	48	51	1
Hexose, %	4.00	—	1.72	6.30	5.64	—
Hexosamine, %	—	—	0.32	2.46	3.69	—
Sialic acid, %	—	—	0	0	0	—
Phosphorus, %	3.28	—	3.07	1.75	0.43	—

The structural relationship between these blood cells mucolipids and the Forssman hapten obtained from equine spleen and kidney (*26*) will be discussed elsewhere*.

* Yamakawa, T., and Makita, A., unpublished.

Fractionation of Cat Erythrocytes Glycolipid—It is said that cat red cells have also Forssman antigen. Similarly as in the case of sheep cells, the activity was found only in M.E.-glycolipid, but in far less degree. In Figs. 10 and 11, the later peak composed the main mucolipid, which, resemblance to hematoside, contained much sialic acid and a negligible amount of hexosamine. Forssman active portion was eluted before this main mucolipid, corresponding to the active portion in case of sheep red cells (Fig. 8). The data on the composition of these fractions are tabulated in Tables IX and X.

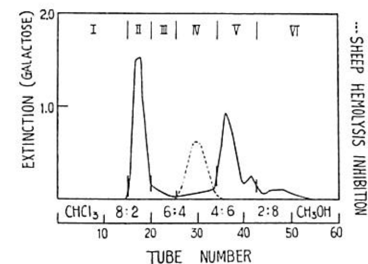

Fig. 10. Silicic acid chromatography of cat erythrocytes M.E.-glycolipid.

Table IX

Composition of a Typical Case of Cat Erythrocytes M.E.-Glycolipid
(corresponding to Fig. 10.)

	Original Mixture	Fraction No.					
		I	II	III	IV	**V**	VI
Weight, mg	300	—	88	39	19	**37**	120
Hexose, %	9.05	—	10.5	1.45	10.1	**23.6**	2.67
Hexosamine, %	—	—	0.23	0.36	2.16	**2.04**	0.43
Sialic acid, %	2.79	—	0.88	0.50	3.33	**28.3**	2.26
Phosphorus, %	3.08	—	2.04	—	—	**1.44**	3.69

After rechromatography on silicic acid and recrystallization from methanol, the main mucolipid (Fr. IV of Fig. 11) gave following analytical value.

C 55.58, H 8.07, N 2.62, Hexose 23.4–24.4, Hexosamine 0.11, P 0.17
Sialic acid 53.0, $[\alpha]_{589} = -20.1°$ (3.78 per cent in pyridine)

From these data, it might be assumed that this mucolipid possibly

consist of ceramide, three moles of hexose and three or four moles of sialic acids.

Calcd. for ceramide trihexoside plus 3 sialic acids, $C_{93}H_{164}N_4O_{42}$,
C 55.6 H 8.17 N 2.79 Hexose 26.9 Sialic acid 46.1
Calcd. for ceramide trihexoside plus 4 sialic acids, $C_{104}H_{181}N_5O_{50}$,
C 54.5 H 7.86 N 3.05 Hexose 23.5 Sialic acid 53.5

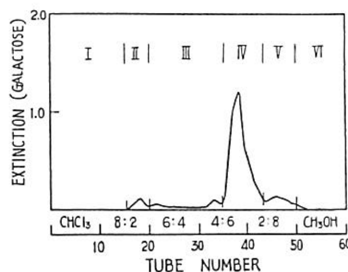

Fig. 11. Silicic acid chromatography of cat erythrocytes C.M.-glycolipid.

Table X

Composition of a Typical Case of Cat Erythrocytes C.M.-Glycolipid (corresponding to Fig. 11.)

	Original Mixture	Fraction No.					
		I	II	III	**IV**	V	VI
Weight, mg	450	—	27	58	**275**	44	26
Hexose, %	23.2	—	7.99	8.69	**27.8**	13.9	8.53
Hexosamine, %	—	—	—	0.75	**0.76**	2.08	1.45
Sialic acid, %	38.5	—	2.93	6.03	**37.2**	47.0	18.0
Phosphorus, %	1.32	—	1.62	1.42	**0.42**	1.50	—

Fractionation of Guinea-Pig Erythrocytes Glycolipids—In the M.E.-glycolipid (Fig. 12), main mucolipid was eluted so fast that it could be hardly separable from ceramide-oligohexoside. Rechromatography of this fraction (Fr. III) with Florisil column and infra-red spectrum indicated the presence of ceramide-oligohexoside but a complete separation in a single peak was not accomplished. Analysis showed it as a mucolipid containing only galactosamine as hexosamine component and no sialic acid. In this respect, it resembles to main globoside of human erythrocytes. However, the direction of optical rotation was found quite opposite.

Analysis for purified mucolipid from Fr. III,

Hexose 47.1, hexosamine 15.7, reducing val., 45.5,
N 2.08, P 0.08, $[\alpha]_{589} = -14.6°$ (2.67 per cent in pyridine)

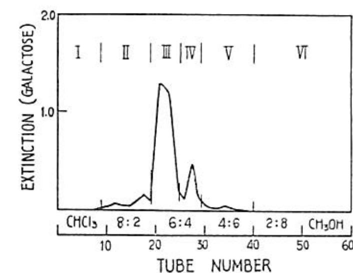

Fig. 12. Silicic acid chromatography of guinea-pig erythrocytes M.E.-glycolipid.

Table XI

Composition of Guinea-Pig Erythrocytes M.E.-Glycolipid

	Original Mixture	Fraction No.					
		I	II	**III**	**IV**	V	VI
Weight, mg	800	—	170	**402**	**77**	30	2
Hexose, %	25.3	—	5.00	**36.9**	**23.4**	8.95	—
Hexosamine, %	—	—	0.42	**8.10**	**6.97**	1.89	—
Sialic acid, %	—	—	0	**0**	**0**	0	—
Phosphorus, %	1.12	—	2.14	**0.24**	**0.13**	—	—

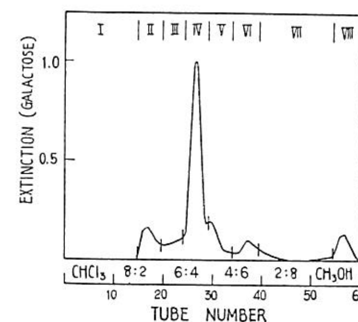

Fig. 13. Silicic acid chromatography of guinea-pig erythrocytes C.M.-glycolipid.

C.M.-glycolipid of guinea-pig erythrocytes consisted mainly of mucolipid (Fr. IV, Fig. 13), which appeared to be the same as described above for M.E.-glycolipid, but the amount of the material was so small that no detailed examination could be carried out.

TABLE XII

Composition of Guinea-Pig Erythrocytes C.M.-Glycolipid

	Original Mixture	Fraction No.							
		I	II	III	**IV**	V	VI	VII	VIII
Weight, mg	160	—	26	3	**19**	3	4	1	17
Hexose, %	8.53	—	6.55	95.9	**25.8**	1.92	9.16	—	4.58
Hexosamine, %	—	—	—	5.73	**10.9**	—	—	—	—
Sialic acid, %	—	—	—	0	**0**	—	—	—	—

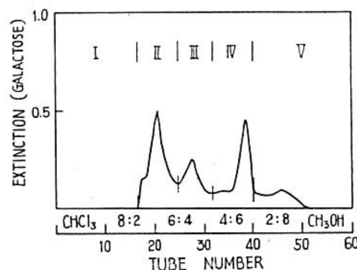

FIG. 14. Silicic acid chromatography of rabbit erythrocytes M.E.-glycolipid.

TABLE XIII

Composition of Rabbit Erythrocytes M.E.-Glycolipid (corresponding to Fig. 14.)

	Original Mixture	Fraction No.				
		I	II	III	IV	V
Weight, mg	150	—	43	13	8	37
Hexose, %	7.47	—	8.91	18.0	24.5	1.72
Hexosamine, %	—	—	0.23	1.2	5.0	0.51
Sialic acid, %	—	—	0	0	0	0

Fractionation of Rabbit Erythrocytes Glycolipids—Since the yield of stroma

from rabbit erythrocytes was low, freeze-dried erythrocytes were used as the starting material. In this case, too, analysis was carried out only with partially purified specimen because of the small amounts of glycolipid obtained. However, the chromatographic pattern was remarkably peculiar and several glycolipids peaks were demonstrated. Because the starting material was erythrocyte itself, the fractions were heavily contaminated with considerable amounts of non-lipid, probably inorganic materials.

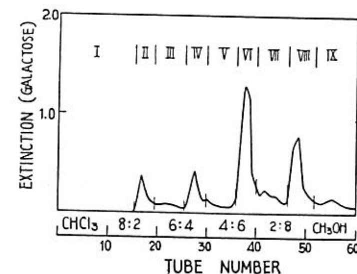

FIG. 15. Silicic acid chromatography of rabbit erythrocytes C.M.-glycolipid.

TABLE XIV

Composition of Rabbit Erythrocytes C.M.-Glycolipid (corresponding to Fig. 15.)

	Original Mixture	Fraction No.								
		I	II	III	IV	V	**VI**	VII	**VIII**	IX
Weight, mg	730	—	14	12	10	27	**65**	100	**127**	56
Hexose, %	2.41	—	—	—	14.7	1.38	**12.9**	2.15	**3.42**	2.84
Hexosamine, %	—	—	—	—	—	—	**2.42**	—	**1.70**	—

After partial purification by removal of contaminant inorganic materials, the main mucolipids from C.M.-glycolipid gave the following value.

Fr. VI Hexose 40.8, hexosamine 9.9, sialic acid 0.
Fr. VIII ,, 16.0, ,, 4.9, ,, ,, 0.

Analysis indicated that the mucolipids of rabbit erythrocytes contained hexosamine but no sialic acid.

Infra-Red Determination—During the chromatographic procedure, the process of purification was checked by infra-red spectrum. Purified glycolipid specimen gave no absorption band at 1750 cm^{-1}. assigned to ester C=0 stretching frequency. As illustrated in Fig. 16, the absorption patterns in region from 1650 cm^{-1}. to 1000 cm^{-1}. were characteristic of each glycolipid. The samples of cerebroside (I) and cerebroside sulfuric ester (II) were

prepared from hog brain and purified by means of silicic acid chromatography. The pattern of (I) is quite in accordance with the diagram reported by Chatagnon (*27*). Strong absorption found for (II) at near 1245 cm^{-1}. is considered to be that of sulfuric acid (*28*). In general, cerebroside or ceramide-oligohexoside shows sharp absorption band at 1475 cm^{-1}. as compared with mucolipids. Mucolipids from various erythrocytes gave essentially similar pictures; apparently no significant differences are observed whether the mucolipid contains hexosamine or sialic acid.

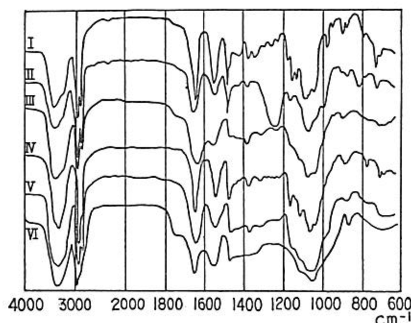

FIG. 16. Infra-red spectra of various glycolipids. (Hitachi EP-I 2, double-beam automatic recording spectophotometer, pressed in KBr) (I) Hog brain cerebroside, (II) Hog brain CSE, (III) Beef brain ganglioside, (IV) Ceramide oligohexoside of human erythrocytes, (V) Main mucolipid of human erythrocytes, (VI) Main mucolipid of cat erythrocytes.

SUMMARY

1. Silicic acid column chromatography was applied to the purification of crude glycolipids obtained from human, equine, bovine, sheep, cat, guinea-pig blood cells stroma as well as rabbit erythrocytes.

2. Glycolipids were separated to methanol-ether soluble and chloroform-methanol soluble materials and each of these was further divided by chroma- into ceramide-oligohexoside and mucolipid.

3. Chromatographic pattern and nature of mucolipids were remarkably peculiar to each animal secies. Generally speaking, muco lipids of human, sheep, guinea-pig and rabbit contain hexosamines, whereas those of equine and cat sialic acids instead.

4. Blood group active lipid as well as Forssman hapten were purified by this procedure from human, sheep and cat erythrocytes.

5. Infra-red spectra of these and other glycolipids are presented.

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