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Lipide und Lipoproteide im Blutplasma

Biochemie — Pathophysiologie — Klinik

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Mit 73 Abbildungen und 80 Tabellen
XII, 399 Seiten (davon 22 in engl. Sprache) Gr.-8°. 1961
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„Die Kenntnisse auf dem Gebiet der Lipoide und Lipoproteine des Plasmas haben im Verlauf der letzten 20 Jahre — aufbauend auf den großen methodischen Fortschritten der Eiweiß- und Lipoidchemie — so sehr zugenommen, daß eine Monographie, die versucht, das Wissensgut in encyclopädischer Form zusammenzufassen, begrüßt werden muß. Der Herausgeber, der gleichzeitig Autor der meisten Kapitel ist, hat sich der mühevollen Arbeit unterzogen, zusammen mit 5 Coautoren den Inhalt von 2536 Arbeiten auf 293 Seiten zu referieren. Dementsprechend ist das Buch auch für den auf dem Lipoidgebiet bewanderten Leser eine Fundgrube.“ *Klinische Wochenschrift*

Untersuchung und Bestimmung der Lipoide im Blut

Herausgegeben von **Nepomuk Zöllner** und **Dietrich Eberhagen**
Bearbeitet von H. Betzing, D. H. Blankenhorn, E. Böhle, P. Böhm, H. Braunsteiner, D. Eberhagen, M. Eggstein, B. Frosch, K. Kirsch, G. Kremer, G. Richarz, S. Sailer, F. Sandhofer, J. Tiews, H. Wagener, H. Wiegandt, G. Wolfram, N. Zöllner

Mit 70 Abbildungen
XII, 408 Seiten Gr.-8° 1965
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„Das vorliegende Buch verdient besondere Beachtung ... eine hervorragende Z... ...rten und analytische ... der Lipoidchemie ... experimentierenden wi... Zeit erspart bleibe... bedient, und er wi... einen guten Überblick über die wichtigsten Eigenschaften der Lipoide erhalten.“ *Zeitschrift für Klinische Chemie*

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AM 28./30. APRIL 1965 IN MOSBACH/BADEN

LIPOIDE

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Cyclus wird in der letzten Abb. 11 vorgeschlagen und am Beispiel der Linolsäure, dem Prototyp einer Polyenfettsäure, explicit wiedergegeben.

Zum Schluß möchte ich besonderen Dank meinen Mitarbeitern, die an den hier zusammengefaßten Arbeiten maßgeblich beteiligt waren, aussprechen. Es sind dies HORST CAESAR, Dr. H.-G. SCHIEFER und Dr. R. DITZER.

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Glycolipids of Mammalian Red Blood Cells

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With 17 Figures

Historical

In 1951, a glycolipid called 'hematoside' was isolated in appreciable amount from equine erythrocyte stroma (YAMAKAWA and SUZUKI). It gave a beautiful purple color when heated with Bial's orcinol reagent, thus indicating the probable presence of neuraminic acid. Neuraminic acid was known, at that time, as a constituent of ganglioside and considered to be a polyhydroxyamino acid having the molecular formula of $C_{11}H_{21}O_9N$ or $C_{10}H_{19}O_8N$ (KLENK, 1941). In those days, ganglioside, which KLENK and co-workers had isolated from the brain of patient with TAY-SACHS disease (KLENK, 1935) and from bovine spleen (KLENK and RENNKAMP, 1942) was said to consist of stearic acid, sphingosine, 3 moles of hexose (glucose and galactose) and neuraminic acid (KLENK, 1942), though the presence of hexosamine in bovine brain was already reported by BLIX (1938). Afterward, galactosamine was isolated by BLIX, SVENNERHOLM and WERNER (1950) from a brain ganglioside preparation, but it seems they doubted the presence of neuraminic acid and regarded it as a degradation product of a crystalline disaccharide-like polyhydroxyamino acid, which BLIX had previously found in submaxillary mucin in 1936 and later named 'sialic acid' (BLIX, SVENNERHOLM and WERNER, 1952). This rather complicated situation was clearly understood when both neuraminic acid and galactosamine were found in ganglioside preparation (KLENK, 1951).

Hematoside contained no hexosamine but produced a substance like methoxy-neuraminic acid, named 'hemataminic acid' and was considered to be a complex glycolipid composed of fatty acid, sphingosine, 2 galactose and hemataminic acid. Hemataminic acid

was assumed to be identical with methoxy-neuraminic acid because of the similarity of color reaction, specific rotation and other properties (YAMAKAWA and SUZUKI, 1952) and this was later substantiated (KLENK and WOLTER, 1952). Since the molecular composition of hemataminic acid in view of C, H and N values by PREGL and DUMAS' method corresponded to $C_{10}H_{19}NO_8$, the methoxy-free parent compound, prehemataminic acid, was calculated as $C_9H_{17}NO_8$. But KLENK assumed $C_{11}H_{21}O_9N$ was pertinent because the nitrogen content by KJELDAHL's method could be explained by the C_{11} formula (KLENK, FAILLARD, WEYGAND and SCHÖNE, 1956). The structure proposed by us (YAMAKAWA and SUZUKI, 1952) was later proved to be erroneous (YAMAKAWA, 1956), but our original statement that the parent acid, now called neuraminic acid, is a 9 C compound has now been justified (GOTTSCHALK, 1960).

Just at the similar time as the discovery of hematoside, KLENK and LAUENSTEIN (1951) reported the presence of a glycolipid from human blood, which, however, gave no purple color with BIAL's orcinol reagent and yielded galactosamine after acid hydrolysis. In this way, it seemed there was an essential difference in the chemical constitution of human and equine erythrocyte glycolipids. It was thought that the discrepancy was most unusual and might be due to the probable change because the source material used by the German authors was aged, as mentioned in their paper, so the deterioration might occur during prolonged storage and neuraminic acid might have been converted into galactosamine by a supposed enzymic reaction. Therefore, we prepared dry stroma from a large amount of freshly obtained human blood and got a glycolipid by solvent extraction in a similar way as in the case of hematoside. The glycolipid was Bial-negative and Elson-Morgan positive after acid hydrolysis, just as Klenk's preparation. We named it 'globoside' for convenience (YAMAKAWA and SUZUKI, 1952). It was composed of fatty acid, sphingosine, 3 moles of hexose (glucose and galactose) and N-acetylgalactosamine. In this way we confirmed Klenk's finding, while our own results on hematoside were essentially confirmed by KLENK and WOLTER (1952). They found furthermore ceramide-dihexoside and also the presence of glucose in addition to galactose in these glycolipids. At the same time, KLENK and LAUENSTEIN (1952) reconfirmed their previous results about the presence of galactosamine instead of

neuraminic acid in human red cell glycolipid. Furthermore, they found glucosamine replaced galactosamine in bovine erythrocyte glycolipid. Later on, KLENK and LAUENSTEIN (1953) reexamined equine erythrocyte glycolipid fractions more thoroughly and found a small amount of glycolipid containing both neuraminic acid and hexosamine. Because of lack of material, they could not determine whether the substance was homogeneous or not. They suggested the amino group of neuraminic acid in hematoside was not substituted by a volatile acid such as acetic acid. It was later demonstrated that the sialic acid in hematoside was N-glycolyl-neuraminic acid (YAMAKAWA, 1956; KLENK and UHLENBRUCK, 1958), while in brain ganglioside it occurred as the acetylated form (SVENNERHOLM, 1955).

Thus it became evident that though both are from mammals, human erythrocyte glycolipid possesses galactosamine, whereas equine blood cells contain neuraminic acid in the glycolipid. On quantitative determination with chloroform-methanol extract of lyophilized blood stroma of various animals, the hexosamine and neuraminic acid contents were found to be very characteristic and two or three groups could be distinguished on the basis of their distribution. The erythrocyte glycolipids of human (irrespective of blood group), sheep, goat, ox, rabbit, pig and guinea-pig had hexosamine but no neuraminic acid, indicating that they were of a 'globoside type'. On the other hand, dog, horse and cat had in their stroma lipids neuraminic acid and very little hexosamine, if any, so were of a 'hematoside type'. Several samples of bovine stroma had both components, being a mixed or 'ganglioside type' (YAMAKAWA and SUZUKI, 1953; YAMAKAWA, IRIE and IWANAGA, 1960).

In the meantime, it was found that the hexosamine of pig erythrocyte glycolipid was exclusively galactosamine (MATSUMOTO, 1956) and the nature of hexosamine in several mammalian red cell glycolipid was found to be extremely specific with each species by Gardell's column chromatographic technique (YAMAKAWA, MATSUMOTO and SUZUKI, 1956). Mammalian red cell could be classified roughly into three or four groups with regard to the content of hexosamine and sialic acids of mucolipid (Tab. 1).

Of course, the classification is only an approximate and convenient one and the signs + or — cannot represent so strict distinction.

For example, as mentioned above, equine material contained a small proportion of hexosamine-containing mucolipid and there is a sialic acid-containing one as a minor component in human mucolipids shown later.

Table 1. *Nature of hexosamines and sialic acids in erythrocyte mucolipid of various mammals*

Animal	Glucosamine	Galactosamine	Sialic acid
Globosides			
Man	—	++	—
Pig	—	++	—
Guinea-pig	—	++	—
Rabbit	++	—	—
Ox	++	—	+ or —
Sheep	+	+	—
Goat	+	+	—
Hematosides			
Horse	—	—	++(NGNA)
Dog	—	—	++(NANA>NGNA)
Cat	—	—	++(NGNA)

Abbreviation: NGNA, N-glycolylneuraminic acid; NANA, N-acetylneuraminic acid.

As to the molecular weight of these glycolipid, YAMAKAWA, SUZUKI and HATTORI (1953) reported they behaved as if they were a high polymer in aqueous solution. They dissolved readily in water to form micelles and gave a high value of sedimentation coefficient and intrinsic viscosity.

Silicic Acid Column Chromatography of Erythrocyte Glycolipids

Recent advances in lipid chemistry have been brought about by the application of chromatography, especially using silicic acid. Silicic acid has been used for the purification of phospholipid (LEA, RHODES and STOLL, 1955; HANAHAN, DITTMER and WARASHINA, 1957) and brain glycolipids (WEISS, 1956). By this procedure, material which was considered as homogeneous was often separated into several fractions. Therefore, this technique was applied in the purification of the blood cell glycolipid (YAMAKAWA, OTA, ICHIKAWA and OZAKI, 1958; YAMAKAWA, IRIE and IWANAGA, 1960).

Dry stroma was extracted four times with methanol-ether (1:1) and so-called M.E.-glycolipid was obtained from this extract by the treatment with dry ether and pyridine. M.E.-glycolipid fraction consisted of a pyridine-soluble fraction of ether-insoluble white precipitate. The residual stroma after extraction with methanol-ether gave so-called C.M.-glycolipid upon continuous extraction with chloroform-methanol (Fig. 1). M.E.-glycolipid was thought to be rather loosely bound to stroma and most hexosamine-containing mucolipids (globoside-type) and ceramide hexosides were present

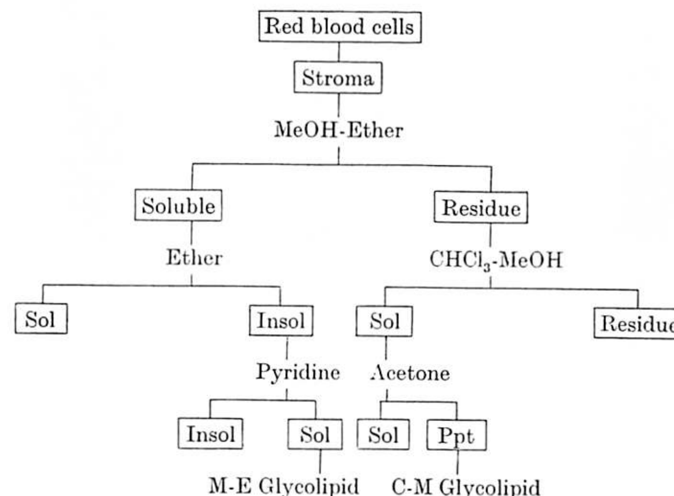


Fig. 1. Fractionation of lipids of erythrocyte stroma

in it. Sialic acid-rich mucolipids (hematoside-type) were released from the stroma by rather drastic treatment such as continuous extraction with chloroform-methanol.

By silicic acid chromatography using a mixture of chloroform and stepwise increasing concentration of methanol as eluent plotting the amount of hexose with each effluent, each glycolipid gave its characteristic chromatographic pattern, which was highly reproducible. Human M.E.- and C.M.-glycolipids were separated into two or three peaks (Fig. 2 and 3). The first peak (Fr. II) was ceramide dihexoside, *i.e.*, composed of N-acylsphingosine, glucose and galactose in equimolecular amount. Mucolipid, namely, glycosphingoside containing hexosamine and/or sialic acid, was eluted slower. The

second peak (Fig. 2, IV; Fig. 3, III) was main glycolipid (Globoside I) and consisted of N-acylsphingosine, 1 glucose, 2 galactose and N-acetylgalactosamine. The third peak represented by solid line

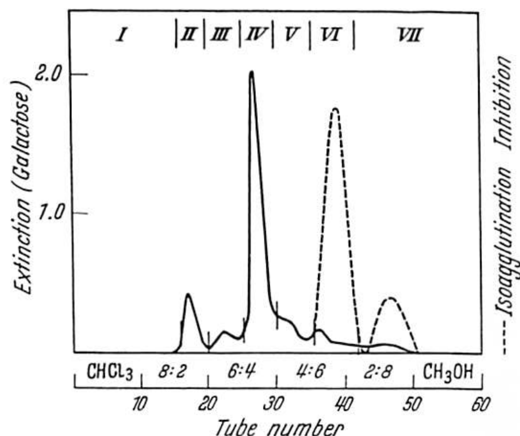


Fig. 2. Silicic acid chromatography of human erythrocyte M.E.-glycolipid. II, ceramide hexoside; IV, Globoside I

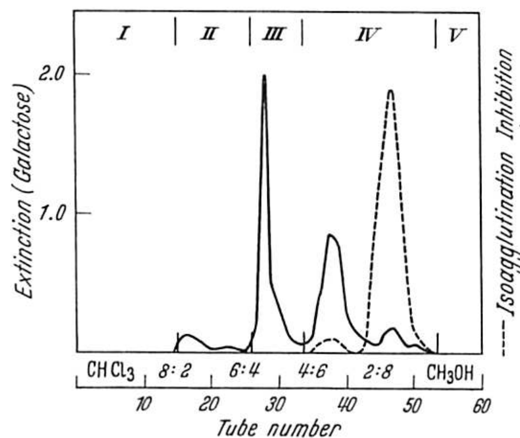
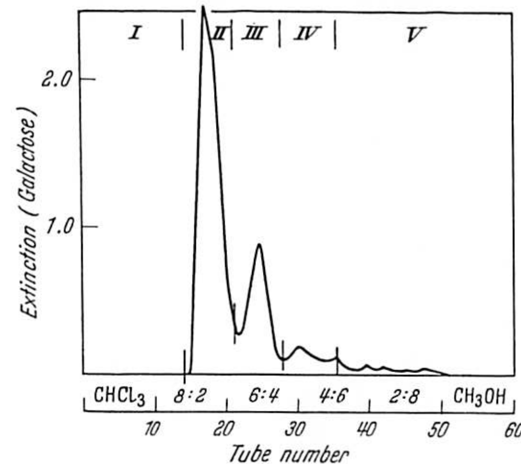


Fig. 3. Silicic acid chromatography of human erythrocyte C.M.-glycolipid. III, Globoside I; IV, Globoside II and III

in Fig. 3 (Fr. IV or Globoside II) contained fucose, glucosamine and a small amount of sialic acid in addition to glucose, galactose and galactosamine. The two peaks represented by dotted line in Figs. 2

and 3 corresponded to ABO group-active portion. In equine erythrocytes, M.E.-glycolipid contained almost nothing but ceramide hexosides (Fig. 4, II), and sialic acid-containing mucolipid (hem-



Fgg. 4. Silicic acid chromatography of equine erythrocyte M.E.-glycolipid. II, ceramid hexoside

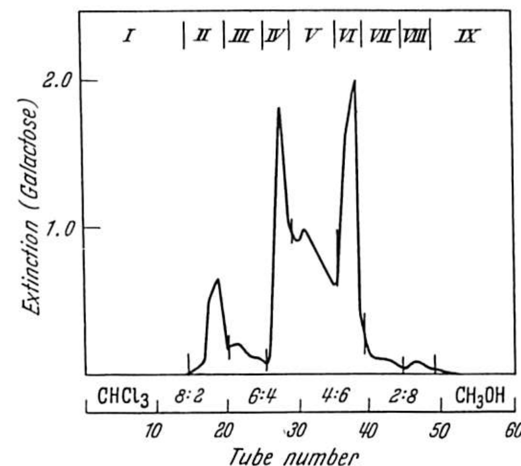


Fig. 5. Silicic acid chromatography of equine erythrocyte C.M.-glycolipid. II, ceramide hexoside; IV—VI, hematoside

atoside) was present only in C.M.-glycolipid. It was apparently heterogeneous (Fig. 5, IV to VI).

The two peaks were apparently the similar substances containing 2 hexoses and one sialic acid but no phosphorus. After the main hematoside was eluted, a small amount of hexosamine-containing mucolipid (Fig. 4, VII) appeared, which was probably the same material as previously reported by KLENK and LAUENSTEIN (1953). There was also a minor glycolipid in which the sialic acid content was very large (Fr. VIII).

In bovine preparations, most mucolipid was obtained by C.M.-extraction as in the case of equine material and the main mucolipid

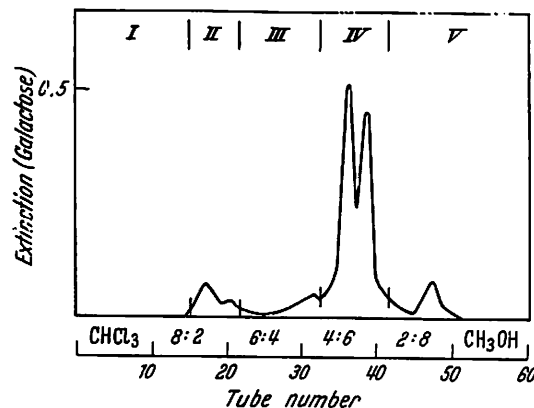


Fig. 6. Silicic acid chromatography of bovine erythrocyte C. M.-glycolipid. IV, mucolipid containing glucosamine, sometimes sialic acid in addition

contained glucosamine and in some cases sialic acid in addition (Fig. 6, IV).

M.E.-glycolipid of sheep erythrocytes gave, on chromatography, one main mucolipid (Fig. 7, IV) which contained both glucosamine and galactosamine but no sialic acid. This mucolipid had FORSSMAN haptenic activity, inhibiting sheep red cell hemolysis by immunhemolysin in the presence of complement.

Guinea-pig M.E.-glycolipid (Fig. 8, III) was similar to human globoside in several respects, namely, it contained only galactosamine and showed the similar infrared absorption. But, after purification by gradient chromatography its specific rotation in pyridine was —8.5°, whereas human globoside was dextro-rotatory, +19.5°.

The sugar moiety was composed of glucose, galactose and N-acetyl galactosamine in equimolar ratio and the glycolipid was considered probably the same as globoside occurred in the brain of TAY-SACHS disease in view of thin-layer chromatography and optical rotation. The latter substance showed $[\alpha]_D = -3.5°$ in pyridine and

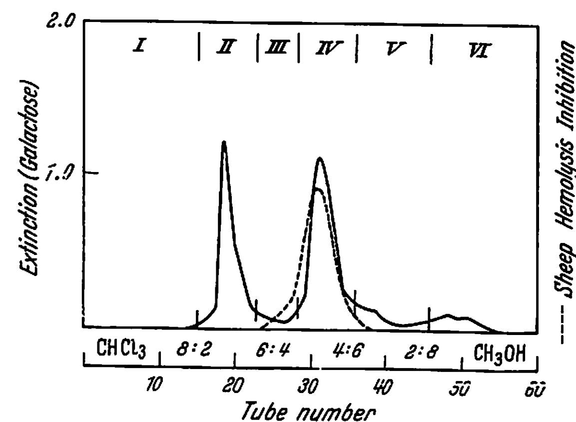


Fig. 7. Silicic acid chromatography of sheep erythrocyte M.E.-glycolipid. II, ceramide hexoside; IV, Forssman-active mucolipid

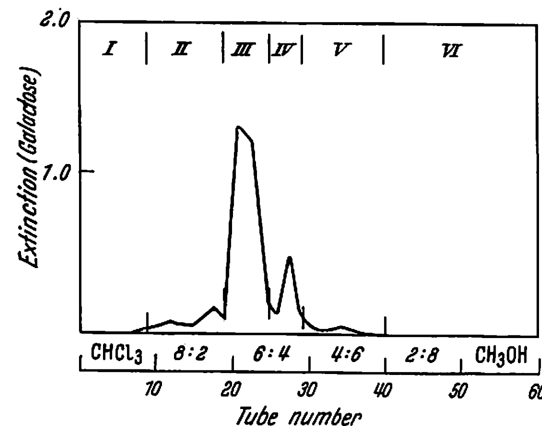


Fig. 8. Silicic acid chromatography of guinea-pig erythrocyte M.E.-glycolipid III ceramide hexoside and mucolipid

had the structure of N-acetylgalactosaminoyl (1→4) galactosyl (1→4) glucosyl-ceramide (MAKITA and YAMAKAWA, 1963). In Fig. 11, *B* represents several degraded products from partially hydrolyzed major ganglioside prepared from the brain of patient with

TAY-SACHS disease (*A*). Guinea-pig mucolipid (*C*) migrates similarly with one of the products, Tay-Sachs globoside. Tay-Sachs ganglioside used here is probably the same as Ganglioside B of KLENK and G_0 of KUHN.

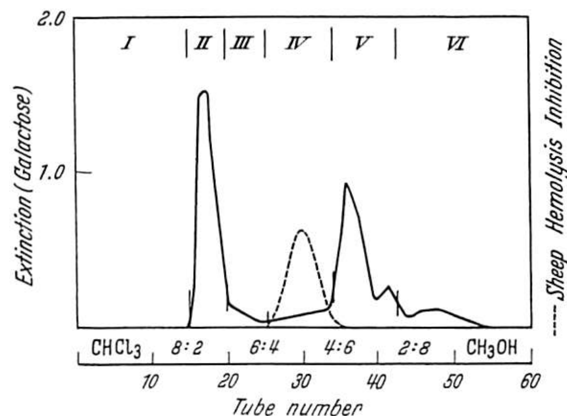


Fig. 9. Silicic acid chromatography of cat erythrocyte M.-E.glycolipid. II, ceramide hexoside; V, hematoside

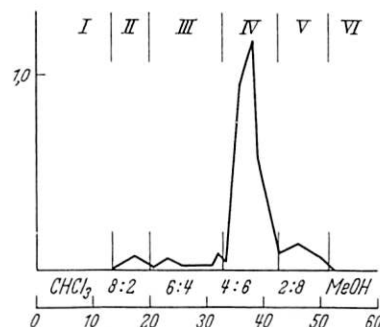


Fig. 10. Silicic acid chromatography of cat erythrocyte C.M.-glycolipid. IV, hematoside containing two moles of N-glycolyl neuraminic acids

The glycolipids of dog and cat were mainly contained in C.M.-glycolipid fraction and were hematoside-type (Fig. 9, V; Fig. 10, IV). The analysis of the recently purified preparation from cat erythrocytes, indicated that it had one more sialic acid than equine and dog materials (HANDA and HANDA, 1965).

In this way, it was established on column chromatographic

pattern that each animal species had its own characteristic mucolipid molecule, which might be connected with species-specificity revealed by, for instance, sero-specific hemagglutination, hemolysis or complement fixation test. In other words, at least among the blood cells examined, the mucolipid molecule differs from species to species.

Fig. 11. Thin-layer chromatogram of several glycolipids. A, ganglioside from Tay-Sachs' brain; B, after hydrolysis of A with dilute HCl; C, guinea-pig erythrocyte mucolipid; D, human erythrocyte Globoside I; E, Globoside II; F, Globoside III. Solvent; chloroform: methanol: water, 60:35:8; Wako Gel B O, sprayed with anthrone-sulfuric acid

Structural Studies of Erythrocyte Glycolipids

Since 1960 when KUHN and co-workers proposed the structure of bovine brain ganglioside by acetolysis technique, the study of glycolipid entered in the time of structural determination. Subsequently, KLENK and GIELEN (1960, 1961) proposed the structure of their ganglioside preparation. But the examination by newly developed thin-layer chromatography indicated the heterogeneity of these materials and the earlier results with such a mixed lipid seemed to be inconclusive. Actually, the brain ganglioside preparation was more and more purified and a number of fractions with

a different structure have been reported. The epoch-making advancement in the study of such a complex glycolipid was made by the improvement of permethylation technique devised by Kuhn, Trischmann and Löw (1955). The use of dimethylformamide as a solvent in the procedure was an excellent one and produced a complete material in good yield. Afterwards, dimethylsulfoxide and sodium hydride also contributed greatly in this line (Hakomori, 1964). Furthermore, big progress was established by the application of gas-liquid chromatography to the identification of methyl glycosides of fully and partially methylated monosaccharides (Yamakawa and Ueta, 1964; Kuhn and Egge, 1963). The isolation and examination of di- or oligo-saccharides produced by partial acid hydrolysis of glycolipid were also profitable in combination with the above procedures. The ratio of glucose to galactose in these materials was best determined by the recent gaschromatographic technique with trimethylsilyl derivative of the methanolysed product (Yamakawa and Ueta, 1964; Sweeley and Walker, 1964).

Ceramide Hexosides of Erythrocytes. Ceramide hexoside fractions (Fig. 2, II) were by no means homogeneous but heavily contaminated by cephalin-type glycerophospholipid. After its removal by passing through Florisil column or by mild alkaline hydrolysis, a pure glycolipid was obtained which consisted of fatty acid, sphingosine, glucose and galactose in equimolecular proportion. It was erroneously reported by us in previous report that this glycolipid was ceramide trigalactoside (Yamakawa, Irie and Iwanaga, 1960) but corrected later (Makita and Yamakawa, 1962), so it has the same composition as the glycolipid first isolated from bovine spleen (Klenk and Rennkamp, 1942). It was levorotatory, $[\alpha]_D = -9.7°$ in pyridine and homogeneous on thin-layer chromatography. Galactose was first split by mild acid hydrolysis and glucocerebroside was remained, which suggested the location of galactose at the non-reducing terminal. Peaks of methyl 2,3,4,6-tetra-*O*-methylgalactoside and anomers of methyl 2,3,6-tri-*O*-methylglucosides were evidently identified by gaschromatography after methanolysis of permethylated ceramide dihexoside, indicating the structure to be ceramide lactoside (Yamakawa, Kiso, Handa, Makita and Yokoyama, 1962). The material is identical with 'Cytolipin H' or Cytoside, which Rapport and co-workers isolated from human epidermoid cancer and bovine spleen and mentioned its configuration

from the immuno-chemical inhibition technique (Rapport, Graf and Yariv, 1961). The same material was obtained from spleen and kidney of various animals (Makita and Yamakawa, 1962, 1964; Makita, 1964) and also from blood serum (Svennerholm and Svennerholm, 1962). In the ceramide hexoside fraction of erythrocyte glycolipids of other animals, faint spots corresponding to ceramide mono- and tri-hexoside were frequently detected. But, ceramide lactoside was likely a predominating ceramide hexoside in erythrocytes. Organs such as spleen and kidney usually contain a considerable amount of ceramide mono- and tri-hexoside.

Human Erythrocyte Globoside I. Main glycolipid fractions of human erythrocytes (Fig. 2, IV; Fig. 3, III) were pooled and purified by rechromatography on silicic acid and on Florisil. The material homogeneous on thin-layer chromatography was rather easily obtained, which had the following analytical values: $C_{68}H_{126}N_2O_{23}$; C 60 to 61, H 9.5 to 10.0, N 2.23, Hexose (as galactose by anthrone-sulfuric acid) 50, Hexosamine (as HCl salt) 17.5, $[\alpha]_D = +19.5°$ in pyridine. It was composed of fatty acid, sphingosine, glucose, galactose and N-acetylgalactosamine in a ratio of 1:1:1:2:1.

The purified Globoside I was dissolved in water and was subjected to mild acid hydrolysis with 0.1 *N* HCl for 30 min., and then dialysed against water. The inner solution was again treated in a similar way and the procedure was repeated 7 times. The outer dialysable fluids were combined, passed through Dowex 50 (H^+) column to remove deacetylated material and the filtrate was concentrated to dryness. The neutral material was chromatographed on a charcoal column by eluting with water, then with dilute ethanol solution. Besides galactose, glucose and N-acetylgalactosamine, a crystalline disaccharide was obtained. This disaccharide melted at 191 to 192°, $[\alpha]_D = +68.6° \rightarrow +63.5°$ in water. $R_{Lactose}$ was 1.03 (ethylacetate-pyridine-water, 2:1:2, upper phase) and 1.25 (n-butanol-pyridine-water, 5:3:2). Infrared absorption band near 890 cm^{-1} indicated the probable presence of β-glycosidic linkage (Fig. 12). It was composed of galactose and N-acetylgalactosamine, the latter being located at the non-reducing terminal.

Its positive Morgan-Elson reaction suggested the presence of 1→3 or 1→6 glycosidic linkage (Kuhn, Gauhe and Baer, 1954). However, the results that it consumed two moles of periodate suggested the linkage to be 1→4. Periodate experiment was repeated further

and the newly obtained disaccharide consumed 1,5 to 1,8 moles of periodate and gave a distinct Morgan-Elson reaction (Fig. 13). Provided some over-oxidation might occur, it could reasonably be assumed that the disaccharide had 1→3 linkage instead of 1→6,

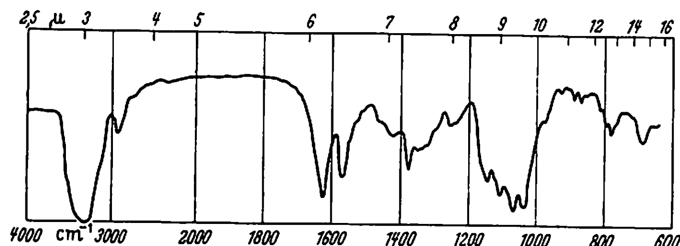


Fig. 12. Infrared spectrum of disaccharide liberated from Globoside I by mild acid hydrolysis, pressed in KBr

as concluded earlier (YAMAKAWA, YOKOYAMA and HANDA, 1963; YAMAKAWA, KAMIMURA and NISHIMURA, 1965).

More conclusive evidence for the structure of Globoside I was presented by permethylation and subsequent gaschromatography of the methanolysis products. In our previous report (1963), we erroneously concluded that main globoside (Globoside I) has the structure of N-acetylgalactosaminoyl (1→6) galactosyl (1→4) galactosyl (1→4) glucosyl ceramide. At present, I think it is not true. At that time, a false peak thought to be methyl 2,3,4-tri-*O*-methyl galactoside which occurred probably because of decomposition and adsorption on the gaschromatographic column led us to make a mistake and, what was worse, the uncertainty of periodate consumption of the disaccharide made some confusion. In our improved condition of gaschromatography, the separation of individual peaks

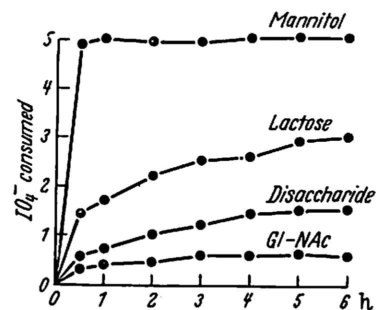


Fig. 13. Rate of periodate consumption during oxidation of mannitol, lactose, N-acetylglucosamine and disaccharide from Globoside I. The reaction, performed at 5 °C in the dark, consisted of 0.4 ml. of 0.01 M carbohydrate, 0.3 ml of 0.080 M sodium periodate, 3.0 ml of 0.1 M acetate, pH 5.0, and 1.3 ml of water. The periodate consumed was measured spectrophotometrically by a decrease in absorbancy at 305 mμ

of methylated sugars became clear and permethylated Globoside I gave evidently the peaks of 2,3,6-tri-*O*-methylglucoside, 2,3,6-tri-*O*-methylgalactoside and 2,4,6-tri-*O*-methyl galactoside (Fig. 14). Furthermore, ceramide mono-, di- and tri-hexosides obtained from the inner dialysis fluid of partial acid hydrolysis were permethylated, methanolysed and examined by gaschromatography. Considering all these results, the Globoside I of human erythrocytes has

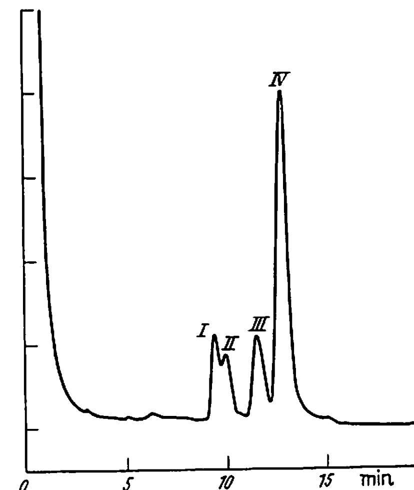


Fig. 14. Gaschromatogram of methanolysate of permethylated Globoside I. I, methyl 2,3,6-trimethyl-β-galactoside; II, methyl 2,3,6-trimethyl-β-glucolside; III, methyl 2,4,6-trimethyl-β-galactoside; IV, α-anomers of I, II and III. Separation was made with 3 mm × 2 m stainless steel column packed with 5% neopentylglycolsuccinate on Gas Chrom CLH at 175°, using Hitachi-Perkin Elmer model F-6 equipped with FID

a constitutional formula of N-acetylgalactosaminoyl (1→3) galactosyl (1→4) galactosyl (1→4) glucosyl ceramide (Fig. 15). Similar substance was obtained from human kidney (MAKITA and YAMAKAWA, 1964; RAPPORT, GRAF and SCHNEIDER, 1964) and the structure was elucidated (MAKITA, IWANAGA and YAMAKAWA, 1964). The fact that Globoside I cross-reacted with anti-human kidney serum in isofixation test (RAPPORT, private communication) would support the identity of these two substances.

Globoside II and Globoside III of Human Erythrocytes. The development of thin-layer chromatography revealed its excellency in resolving power rather than column chromatography and an apparently single peak on column was not always homogeneous by

thin-layer chromatography. In the course of purification of the third glycolipid peak (Fig. 3, IV) of human erythrocytes, a number of spots were detected by the thin-layer chromatography, even though

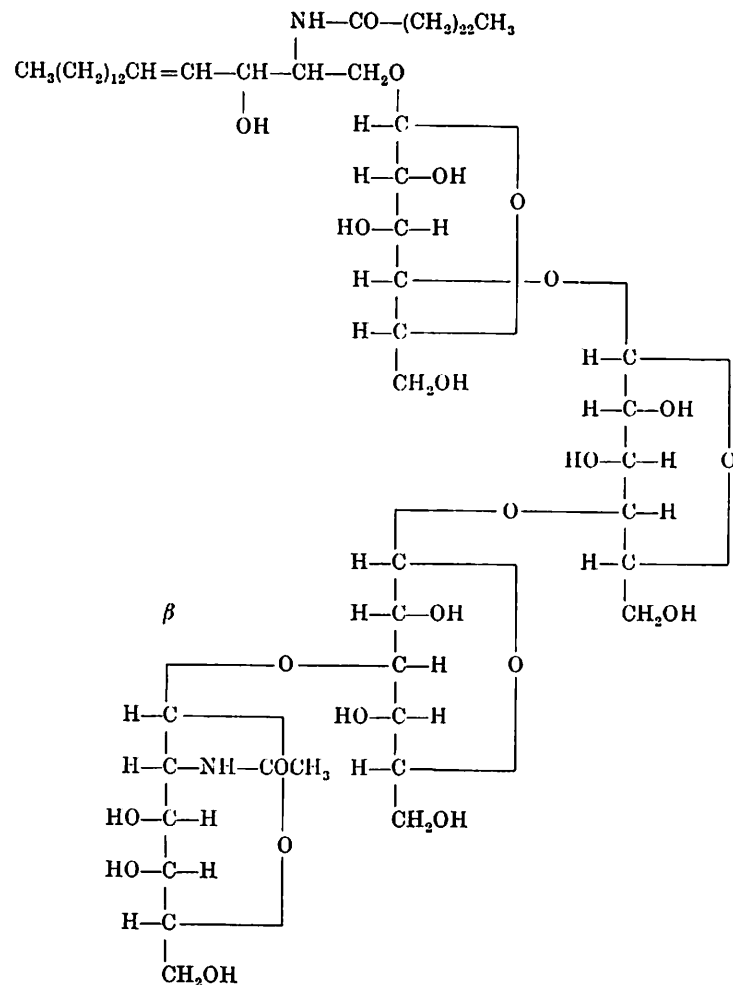


Fig. 15. Constitutional formula of Globoside I of human erythrocytes.

it had been purified into a single peak by column chromatography. The glycolipids eluted in this region was usually contaminated with sphingomyelin and its removal was difficult by column chromato-

graphy or alkaline hydrolysis. Therefore, the material was peracetylated with acetic anhydride in pyridine and the product was separated from acetylated sphingomyelin by gradient silicic acid chromatography. It was then hydrolysed with dilute alkali and examined on a thin-layer plate. Since it gave still several spots, preparative thin-layer chromatography was repeated and finally a homogeneous Globoside II was isolated in low yield. The slower

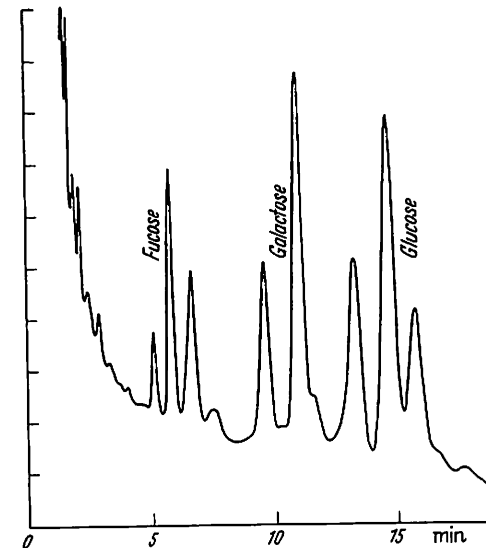


Fig. 16. Gaschromatography of methyl glycosides from human erythrocyte Globoside II as trimethylsilyl-derivatives. Separation was made with 2 m × 3 mm stainless steel column packed with 5% Ucon LB 550 X coated on Gas Chrom CLH, at 192°

migrating Globoside III was still inhomogeneous (Fig. 11). Both materials contained glucosamine, fucose and sialic acid in addition to glucose, galactose and galactosamine (Fig. 16) and inhibited the agglutination of erythrocytes by its corresponding iso-antibody.

The occurrence of fucose in glycolipid was reported by HORI, ITASAKA and HASHIMOTO in a shell-fish, Corbicula sandai (1964) and by KOSCIELAK (1963) and HANDA (1963) in group substance of human erythrocytes. Recently, HAKOMORI and JEANLOZ reported a glycosphingoside composed of glucose, galactose, fucose and glucosamine as the sugar moiety from human cancerous tissue (1964).

Hematosides. At first 'hematoside' was the name of equine erythrocyte mucolipid. Later on, when a variety of mucolipids were found in various species of mammalian erythrocytes and in several

Table 2. *Properties of human erythrocyte globoside II and III*

	Globoside II	Globoside III
R_f, relative to Globoside I (TLC of Kieselgel G, $CHCl_3$:MeOH:H_2O = 60:35:8)	0.81	0.65
Hexose (anthrone, as galactose)	38.5	36.7
Glucosamine......................	8.2	5.3
Galactosamine....................	7.4	2.2
Sialic acid........................	2.1	6.1
Fucose:Glucose:Galactose..........	ca. 1:2:3	ca. 1:2:4
Isoagglutination inhibition against 4 unit antibody	1.39 μg	4.4 μg

organs, I thought it would be better to use the term 'hematoside' to call the glycosphingoside having sialic acids in addition to neutral sugars but no hexosamines.

Now, too many complex glycolipids have been reported, especially from crude mixture of brain ganglioside fraction (KLENK and GIELEN, 1963; KUHN and WIEGANDT, 1963, 1964; SVENNERHOLM, 1964; JOHNSON and McCLUER, 1964) and the nomenclature is much confused, but the term 'globoside', 'hematoside' and 'ganglioside' are still convinient for the rough classification of these 'mucolipids'.

Equine hematoside has molecular composition of acylsphingosine, glucose, galactose and N-glycolylneuraminic acid in equimolar amount. The constitutional formula was reported as N-glycolylneuraminyl(2→3)galactosyl(1→4)glucosyl ceramide by KLENK and PADBERG (1962). The same result was reported later by HANDA and YAMAKAWA (1964). They also reported the formula of canine erythrocyte hematoside which was similar to equine material but the amino group of its sialic acid was substituted 73% by acetyl and 27% by glycolyl group, whereas KLENK and HEUER (1960) reported it was only N-acetylneuraminic acid. One more sialic acid was found to attach to the 8th hydroxyl of hematoside sialic acid in cat's material. They were exclusively N-glycolylneuraminic acids (HANDA and HANDA, 1965). The sialic acids were easily liberated by neuraminidase prepared from cholera vibrio culture filtrate.

Fatty Acid Composition of Red Cell Glycolipids

Glycolipids of erythrocytes were analyzed for their fatty acid composition by gas-liquid chromatography. The results are summarized in Table 3. It seems the aged material and the material purified by thin-layer chromatography gave relatively smaller amount of unsaturated acids. Therefore, some instances in this Table might have to be corrected with more fresh material.

Table 3. *Fatty acid composition of erythrocyte glycolipids*

	14:0	16:0	18:0	18:1	20:0	22:0	22:1	23:0	24:0	24:1	26:0
Human											
Ceramidelactoside	2	6	4	—	2	14	—	—	48	24	—
Globoside I	1	6	2	1	1	12	—	3	35	40	—
Bovine											
Mucolipid I	—	11	34	44	1	2	—	—	6	1	—
Mucolipid II	—	10	26	15	1	6	—	2	28	9	—
Sheep											
Mucolipid.......	—	5	13	5	1	11	—	3	39	24	—
Equine											
Mucolipid.......	—	2	12	—	—	8	3	—	64	2	8
Cat											
Mucolipid.......	—	2	2	—	1	9	1	3	33	50	—
Dog											
Mucolipid.......	—	6	45	2	—	6	—	—	15	27	—
Guinea-pig											
Mucolipid.......	—	—	—	—	—	26	—	5	69	—	—
Human											
Group-mucolipid											
MeOH-insoluble*	—	—	—	—	—	8.1	—	2.4	89.5	—	—
MeOH-soluble* ..	—	19.7	10.7	2.3	3.8	22.9	1.1	2.3	26.8	7.8	—
Equine											
Mucolipid**.....	—	—	—	—	—	14.2	—	3.0	76.4	6.0	—
Dog											
Mucolipid***....	—	0.8	1.4	—	0.8	11.5	—	3.1	34.2	47.6	—

* KOSCIELAK (1963)
** KLENK and PADBERG (1962)
*** KLENK and HEUER (1960)

The Serological Significance of Erythrocyte Glycolipids

The elucidation of the structure of mucolipid led us to assume the sugar moiety of these material might be correlated to the serological specificity. Actually, YAMAKAWA and IIDA (1953) found human erythrocyte glycolipid (formerly designated as 'globoside')

could inhibit the hemagglutination of erythrocytes of a given blood group by its corresponding isoantibody. The glycolipid indicated the greatest inhibitory activity as compared with other fractions, such as phospholipid and polysaccharide fraction and the activity was group-specific (YAMAKAWA, MATSUMOTO and SUZUKI, IIDA, 1956). Meanwhile, a glycolipid from sheep erythrocytes inhibited the hemolysis of sheep cells by heterophile antibody (PAPIRMEISTER and MALLETTE, 1955).

In early days, attempts were made to extract organ-specific or blood group-specific materials with organic solvents and the results suggested that the effective components were lipoidal in nature. Later, this concept became out-dated and a role of polysaccharide in antigenicity was indicated. Only the classical examples of FORSSMAN and WASSERMANN antigens, which were first obtained as alcoholic extract of guinea-pig kidney and bovine heart were considered to be of lipoidal nature. As for the ABO blood group, the earlier view that isoagglutinogen was lipid was replaced by the carbohydrate theory and the investigation by using mucoid material from secretions such as gastric mucin, pseudomucinous ovarian cyst fluid, saliva, meconium, *etc.* have made a splendid advance of elucidating structures involved in antigenic specificity. Our results that the active substance of erythrocyte is a mucolipid was criticized by KABAT (1956) who doubted the possible contamination of group mucoid. However, RADIN (1957) fractionated the glycolipid of human erythrocytes by cellulose chromatography and a number of anthrone-positive peaks were found but the group activities were separated from the main glycolipid peak. In the case of AB-red cells, partial separation of the A and B activities occurred. However, probably the poor yield of active material discouraged him from further work. Similar studies were carried out by HAKOMORI and JEANLOZ (1961). KLENK (1960) mentioned in his review of 'hemagglutination' that he had recognized group-specific activity in his glycolipid. Furthermore there appeared a number of results supporting the lipoidal character of erythrocyte antigen (HAMASATO, 1950; NOWOTNY and BACKHAUSZ, 1957; KOSCIELAK and ZAKRZEWSKI, 1960). After purification by silicic acid chromatography (YAMAKAWA, OTA, ICHIKAWA and OZAKI, 1958; YAMAKAWA, IRIE and IWANAGA, 1960), active material was separated from main glycolipid peak (Globoside I) and further divided into two separate fractions; the glucosamine/galactosamine ratio of anterior fraction was 1 and that of the posterior fraction was 1.5 (Figs. 2 and 3, dotted line). By the same procedure, group-active mucoid from ovarian cyst fluid could not be eluted by the organic solvents but passed straight through by water, suggesting the active material of erythrocyte surface was not a mucoid. Furthermore, it was precipitated by potent anti-A rabbit serum. The material recovered from the precipitate was found to exert a characteristic mucolipid spectrum in the infra-red determination (YAMAKAWA and IRIE, 1960). KOSCIELAK (1963) and HANDA (1963) carried out more detailed studies on the erythrocyte group-mucolipid and presented the chemical and immunochemical properties of the final products. The group materials from erythrocytes differ from mucoid material in that they are mucolipids composed of ceramide, glucose, galactose, N-acetylglucosamine, N-acetylgalactosamine, fucose and sialic acid, whereas the latter contains amino acids in addition but is devoid of ceramide, glucose and sialic acid. The analytical values of purified group mucolipids differed from preparation to preparation but the results by KOSCIELAK and by HANDA are fairly similar except the content of sialic acid (Tab. 4).

Table 4. *Analytical data of group materials from erythrocytes*

	Sugar	Hexosamine	Sialic acid	Fucose
KOSCIELAK				
A-substance				
Methanol-soluble	43.0*	15.8	10.4	1.2
Methanol-insoluble	41.4*	12.1	10.9	2.3
HANDA				
A-substance				
from E-M	38.2	14.5	4.7	2.8
from C-M	23.2	9.2	2.5	2.9
B-substance				
from E-M	39.4	12.9	0.8	0.6
YAMAKAWA-NISHIMURA				
Globoside II	38.5	15.6	2.1	3.1
(mostly from A-cell)				

* reducing value

From the relatively high value of glucosamine/galactosamine of Koscielak's material, 3.0, it can reasonably be assumed that his preparation is closely similar to Globoside III (Ref. Tab. 2). It is

of some interest that the contents of sialic acid and fucose are less in B-material than A-material (HANDA, 1963). Among the materials in Tab. 4, it appears only Globoside II is homogeneous on thin-layer chromatography. These materials were of the same order of activity as substances from secretions on precipitin assays or in hemagglutination inhibition. KOSCIELAK noticed an interesting phenomenon that the low-active methanol-soluble glycolipid became much more active after addition of inactive 'carrier' lipid. Since these glycolipids form a micellar solution in water the potency

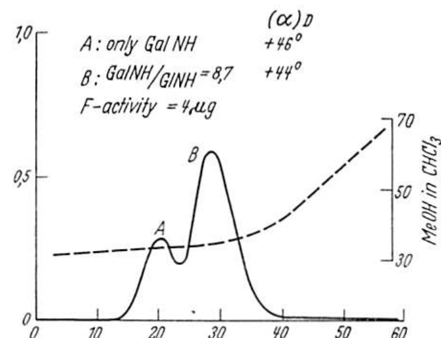


Fig. 17. Further separation of Forssman-active sheep erythrocyte mucolipid by gradient elution chromatography on silicic acid with chloroform-methanol. Fr. IV of Fig. 7 was separated into peaks A and B. A contained no glucosamine. Both materials were F-active

could not often be adequately measured by simple serological assay without addition of a proper auxially lipid. The A-group mucolipid gave a single line in agar gel with a rabbit antiserum against human A red cells, but the line showed only partial fusion with the band formed by ovarian cyst A substance or by hog gastric mucin (HANDA). Furthermore, the activity of erythrocytes, mucoid and mucolipid could be reduced by the decomposing enzyme (FUJISAWA, FURUKAWA and ISEKI, 1963). These findings indicate that the determinant sugar moieties of both type of group substances are very similar in structure.

Forssman Activity. It is of interest that the FORSSMAN haptenic activity of sheep and cat erythrocytes was easily split off by methanol-ether extraction of stroma and the C.M.-glycolipid had almost no activity, while ABO-blood group activity of human erythrocytes was present in both M.E.- and C.M.-extracts. The F-activity was eluted in the same position by silicic acid chromatography in every case of erythrocytes and organs such as guinea-pig kidney, equine spleen and kidney (Figs. 7, IV; 9, IV).

The F-active sheep erythrocyte mucolipid (Fig. 7, IV) contained both glucosamine and galactosamine. By the gradient elution with chloroform-methanol on silicic acid column chromatography, the glycolipid peak was divided into two (Fig. 17). The former glycolipid was free from glucosamine and in the latter the ratio of glucosamine to galactosamine was 1:8.

The F-activity of them were about the same. Both materials contained glucose and galactose in a ratio of 1:2 and resembled to human Globoside I in this respect. However, the $[\alpha]_D$ of sheep material was $+45°$ and human mucolipid was $+19°$.

Recently, F-active mucolipid was purified from equine kidney which had also $[\alpha]_D = +48°$ in pyridine (MAKITA, unpublished). Probably the non-reducing terminal sugar structure responsible for F-activity is probably α N-acetylgalactosaminoyl(1→3)galactoside (CHEESE and MORGAN, 1961), which might be a reason why F-active mucolipid has larger dextrorotation than Globoside I.

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