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THE SEPARATION OF NEUTRAL BLOOD-SERUM GLYCOLIPIDS BY THIN-LAYER CHROMATOGRAPHY

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SUMMARY

A method is described for the quantitative isolation of neutral glycolipids of blood serum on thin-layer plates of silica gel. Four different glycolipids have been isolated and characterized: ceramide-monohexoside, -dihexoside, -trihexoside and -trihexoside-*N*-acetylgalactosamine. The ceramide-monohexoside is a glucocerebroside. All the hexosides, except the amino sugar containing one, contained both normal and hydroxy fatty acids. The glucocerebroside and the ceramide-dihexoside constitute more than 4/5 of total neutral serum glycolipids.

INTRODUCTION

The concentration of glycolipids in blood plasma and their physiological significance have been subject to only a few investigations. Some years ago we elaborated a method for the quantitative estimation of lipid hexose in blood plasma[1]. The method has been applied for the study of plasma glycolipids in Gaucher's disease[2], late infantile metachromatic leucodystrophy and Krabbe's diffuse sclerosis[3,4]. In six splenectomized patients with chronic Gaucher's disease the plasma level of cerebrosides was increased in all[2]. The glycolipids which accumulate in the spleen of patients with Gaucher's disease have been identified as glucocerebrosides[5]. Therefore, it was of interest to investigate whether the increased plasma glycolipids of the patients were glucocerebrosides or whether they were galactocerebrosides—the normal constituent of brain. It soon turned out that there were several different glycolipids in plasma both from normal persons and from patients with Gaucher's disease. Before the glycolipid profile was studied in normal and pathological conditions we assumed it necessary to establish the chemical structure of the different glycolipids. A large-scale preparation of the glycolipids from normal human serum was therefore started.

In a recent report[6] we described the isolation of total serum glycolipids and their separation into a neutral and an acid fraction. By chromatography on paper and silica gel plates it could be shown that the neutral fraction consisted of four main components. These have now been isolated by thin-layer chromatography and their carbohydrate moieties have been determined.

MATERIALS AND METHODS

Crude neutral glycolipids

The lipids from 17 l of normal human blood serum were extracted with 4 vol. of ethanol. After hydrolysis of the evaporated lipid extract with mild alkali, two crude glycolipid fractions were obtained by column chromatography on silicic acid and alumina. Each of them was separated into a neutral and an acid fraction by chromatography on DEAE-cellulose[7]. The yield of glycolipids, determined as lipid hexose,

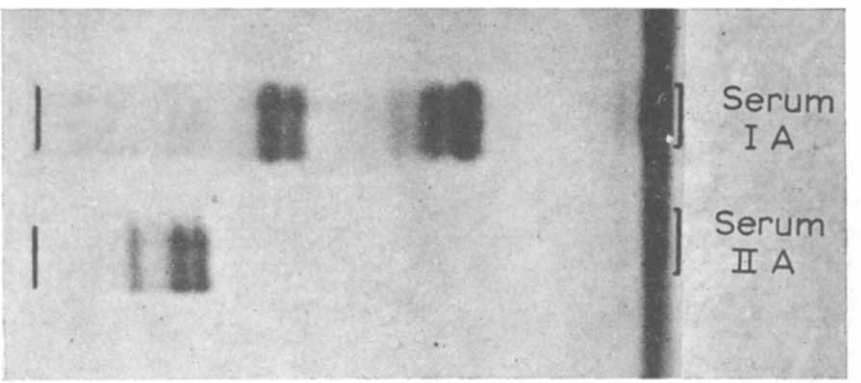

Fig. 1. Thin-layer chromatogram of crude neutral serum glycolipid Fractions IA and IIA. Solvent: chloroform–methanol–water (65:25:4, v/v). Spray reagent: perchloric acid–molybdate[8].

was about 60%. The two neutral fractions IA = 340 mg and IIA = 16 mg have been the starting material for the present investigation. Fraction IA contained 18.0% of hexose (determined as galactose) and 0.32% of hexosamine. The corresponding figures for Fraction IIA were 21.4% and 3.1%. In Fig. 1 a thin-layer chromatogram of the two fractions is shown.

Preparation of thin-layer plates of silicic acid

For five plates 30 g Kieselgel G according to STAHL (E. Merck, A.G. Darmstadt, Germany) and 65 ml distilled water were shaken vigorously for 1 min in a flask fitted with an outlet tube for attachment to a water pump. The gel suspension was deaerated at reduced pressure for 1 min and then the thin layers (0.25 mm) of silicic acid on 20 × 20 cm glass plates were prepared using a commercial apparatus (DESAGA Nr. 600, C. Desaga GmbH, Heidelberg, Germany). The plates were dried for 30 min at 120° and let to cool at room temperature for at least 15 min before the lipid extract was applied.

Preparative chromatography on thin-layer plates

Three-fourths of Fraction IA (255 mg) was dissolved in 5 ml chloroform–methanol (2:1, v/v) and on 25 plates 200 μl of the extract was put as a narrow band at the starting line with a Carlsberg micropipette. The plates were developed with chloroform–methanol–water (65:25:4, v/v)[8] for 60 min at 21°. The plates were let to dry at room temperature and the separated glycolipids were visualized by spraying the plates with distilled water. By this means the lipid bands (A–D) were seen as white streaks against a transparent surrounding. While still moist the bands were removed

with a right angled spatula of rust-free steel. As there were only very small amounts of band D, it happened sometimes that it was not possible to separate between C and D. They were then scraped off together.

The same bands from all the 25 plates were sampled in a beaker. The beakers were placed in a vacuum-desiccator above P_2O_5 for 24 h to remove most of the water from the samples. The lipids were then extracted from the silica gel with 50 ml chloroform–methanol (2:1, v/v). The extract was filtered on a sintered glass filter. The silica gel was re-extracted twice with the same solvent, this time under warming to boiling point. To the combined extracts 25 ml of distilled water were added and the solvents mixed by vigorous shaking. After 24 h the lower phase was drained off and the upper phase was re-extracted once with 25 ml of chloroform. The pooled two lower phases were evaporated to a small volume, dried with ethanol and finally precipitated with acetone.

Three-fourths of Fraction IIA and the combined bands C and D from Fraction IA were separated on two thin-layer plates. The chromatograms were run for 90 min, instead of for 60 min, in which manner a much better separation of the slow fractions was obtained. The separated bands C and D were isolated as described above.

Analytical methods

Hexose was determined by orcinol-[9] or anthrone-[10] methods after hydrolysis according to RADIN *et al.*[11]. Glucose was assayed in the following manner. The glycolipids were hydrolysed with chloroform–ethanol–HCl[11], the organic solvent was removed and after addition of water the fatty acids and sphingosine were extracted into light petroleum (b.p. 40–50°). The aqueous phase was hydrolysed with 1 N HCl for one additional hour. After neutralisation, glucose was determined with glucose oxidase[12]. Galactose did not react with this preparation.

Semiquantitative estimation of hexose was done by paper chromatography and electrophoresis. About 2 mg glycolipid was hydrolysed with 1 ml 2 N HCl for 2 h. The hydrolysate was poured through a column with 1 g strong anion exchange resin (Dowex-1 X8) in acetate form. The column was eluted with 10 ml distilled water and the eluate evaporated to dryness. The residue was dissolved in 200 μl distilled water and 10 and 20 μl of the extract was analysed. The paper chromatograms (Whatman No. 1 papers) were developed with *n*-butanol–pyridine–water (6:4:3, v/v), ascending technique, overnight and then sprayed with $AgNO_3$–NaOH (ref. 13). The high-voltage electrophoresis was also run on Whatman No. 1 papers in a borate buffer[14] and after drying sprayed with anilin phthalate. Hexosamine was determined by a modified Elson–Morgan procedure[15] after hydrolysis for 16 h in 2 ml 2 N HCl at 100°. Paper chromatographic identification of the hexosamines was performed according to STOFFYN AND JEANLOZ[16]. Total nitrogen was analysed with Nessler's reagent[17]. Phosphorus was determined as earlier described[18].

Analytical thin-layer chromatograms were run with chloroform–methanol–water (65:25:4, v/v)[8] and the glycolipids were visualized by spraying with perchloric acid–molybdate[8]. Optical rotations were taken in pyridine in a Hilger Standard polarimeter. Infrared spectra were obtained on a Perkin–Elmer spectrophotometer Model 21 equipped with NaCl prism. The fatty acids of the isolated glycolipids were transesterified by 7 % methanolic HCl in closed tubes at 100° for 4 h. The fatty-acid methyl esters were extracted with light petroleum (b.p. 40–50°), and after rinsing

the petroleum phase with water, it was dried with Na_2SO_4. The fatty acid methyl esters were separated into normal and hydroxy fatty acids on thin-layer plates of silica gel G by CH_2Cl_2. The methyl esters were isolated in the same manner as described for the glycolipids.

RESULTS

From 255 mg of Fraction IA 172.8 mg or 68% of total glycolipids were recovered (Table I). The recovery of hexose was, however, 95.3% which means that there has been a partial purification of the glycolipids at the thin-layer chromatography. At the chromatographic separation of Fraction IIA and rechromatography of component C plus D the recovery of hexose was not better than about 80%. The recovery of hexosamine was as low as 50%.

TABLE I

SEPARATION OF SERUM GLYCOLIPIDS ON THIN-LAYER PLATES

255 mg of Fraction IA (total hexose, as galactose, = 45.1 mg) were separated on 25 plates and isolated as described in the text. The samples have not been dried to constant weight before weighing and determination of hexose.

Material	*Recovered amount (mg)*	*Hexose as galactose*	
		(%)	*(mg)*
Component A	61.2	15.3	9.65
Component B	82.8	28.1	24.67
Component C	20.1	32.8	6.59
Component D	3.7	31	1.15
Component C + D	5.0	18	0.90
[Total recovery]			
in mg	172.8		42.96
in %	67.7		95.3

All the three components A–C showed at the thin-layer chromatography double spots or bands (Fig. 2). By isolating the two bands separately it could be shown by gas–liquid chromatography that the faster moving spot contained normal fatty acids while the slower moving band contained α-monohydroxy fatty acids.

The fractions were still admixtured with silica gel and small amounts of nonlipid nitrogenous material which stayed at the starting line at chromatography. The admixture of the glycolipid fractions with silicic acid can be reduced by cold precipitation of the glycolipids dissolved in hot CH_3OH. As the isolated fractions were to be used for the determination of their fatty acid composition, no purification by precipitation was done because we have earlier found this procedure to give a relative increase of saturated and hydroxy fatty acids. Therefore, we have used as criterion for purity that no other lipid should be indicated at chromatography in three different solvent mixtures.

Component A

At paper chromatography and electrophoresis only one major spot was detected with the same R_F value and M_G value as glucose. A very weak spot could be seen

in ultraviolet light at the place of galactose. Experiments with known amounts of galactose and glucose showed that the amount of galactose was less than 1/20 of that of glucose.

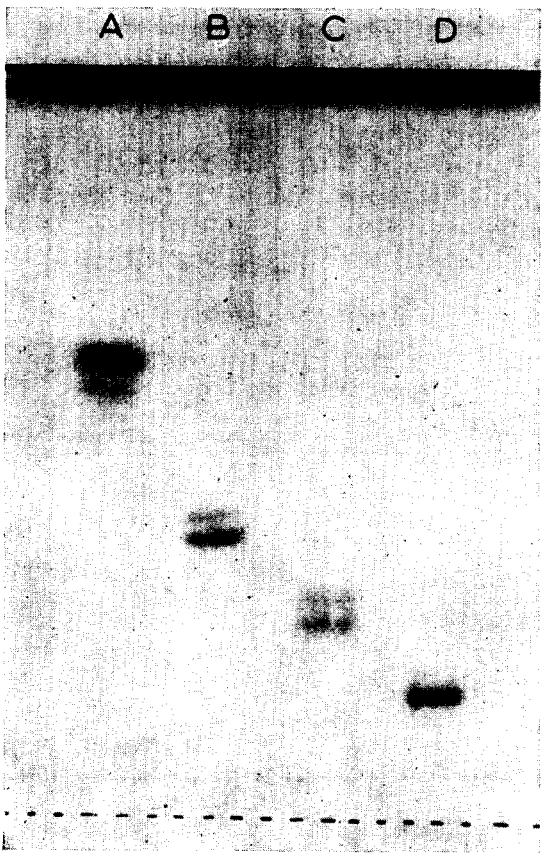

Fig. 2. Thin-layer chromatogram of isolated serum glycolipids. A, ceramide-monohexoside; **B, ceramide-dihexoside**; C, ceramide-trihexoside; D, amino glycolipid. Solvent and spray reagent as in Fig. 1.

When the hexose content was analysed with glucose as standard, a hexose-value of 19.7 % in the orcinol- and 20.3 % in the anthrone-method was found. The concentration of glucose analysed with the glucose oxidase method was 18.9 %. Nitrogen was 1.78 %. The molar ratio nitrogen to hexose was 1.1 : 1. From 27.8 mg of component A 6.4 mg of normal and 1.7 mg of α-hydroxy fatty acid methyl esters were isolated. The fatty acid composition was analysed by gas chromatography by Dr. S. STENHAGEN[23].

$$[\alpha]_{589}^{20} = -6.4^\circ \text{ (}c\text{, 2.2 \% in pyridine)}.$$

The infrared spectrum of component A (Fig. 3) was similar to that described for Gaucher cerebrosides[24]. There was no absorption band at 1750 cm^{-1}, assigned to ester-C=O stretching frequency.

The results indicate that component A of human blood serum is a glucocere-

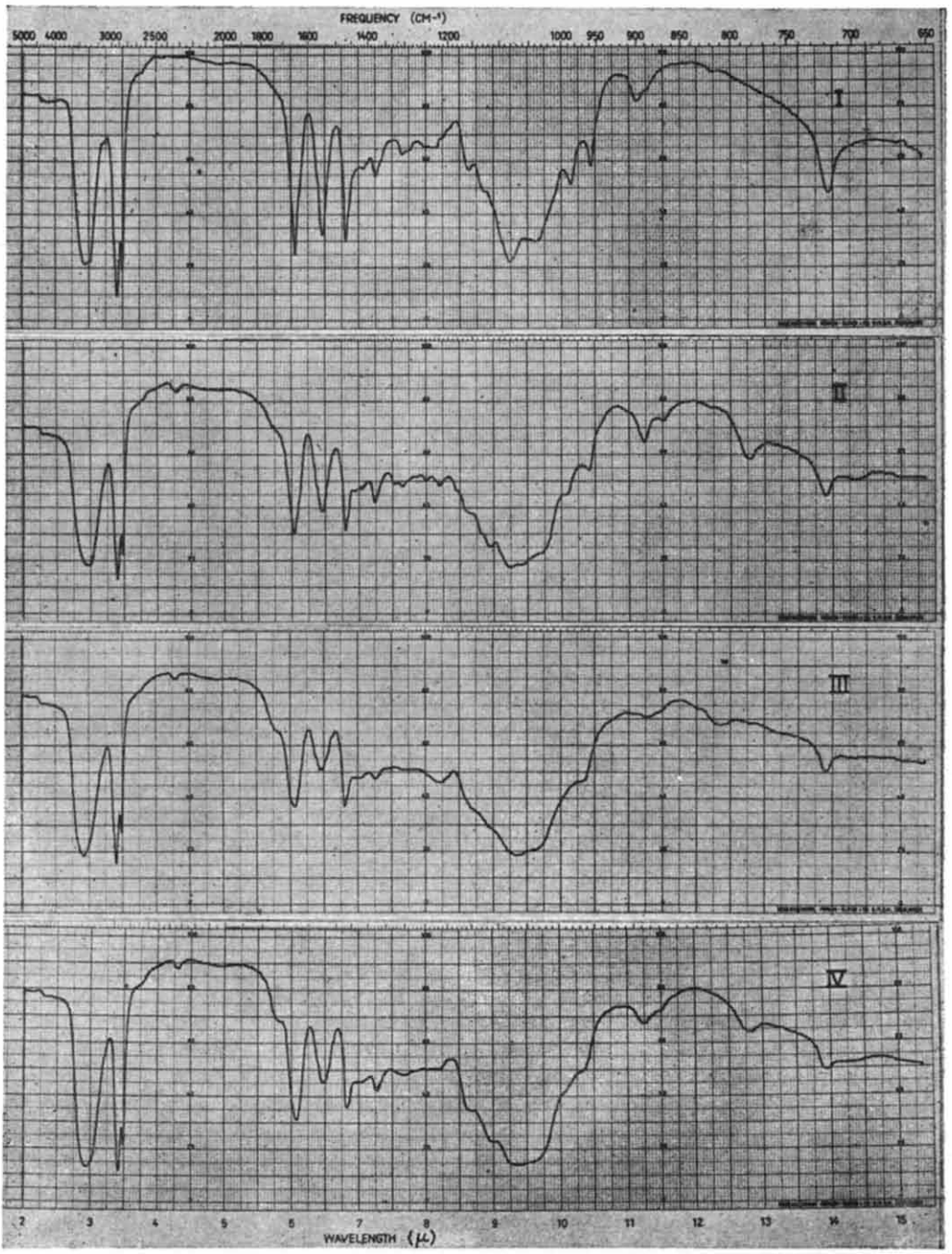

Fig. 3. Infrared spectra of isolated serum glycolipids. I, ceramide-monohexoside; II, ceramide-dihexoside; III, ceramide-trihexoside; IV, amino glycolipid, pressed in KBr.

broside. It is possible that there are also small amounts of galactocerebrosides (less than 5 %) as traces of galactose were detected at the borate electrophoresis. This small amount may as well be derived from an admixture of component A with component B, although we were not able to detect it at the analytical chromatography. MAKITA AND YAMAKAWA[19] have recently demonstrated that the cerebrosides of normal spleen contain only glucose. As we have found that serum and liver cerebrosides[22] also contain glucose instead of galactose it is reasonable to assume that glucocerebrosides are the physiological cerebroside outside the nervous system.

Component B

Hexose chromatography and electrophoresis showed two spots of the same size and with the same R_F and M_G values as glucose and galactose. No other sugar was detected. At quantitative analyses the hexose content was 33.0 % with an equimolar mixture of glucose and galactose as standard. Glucose oxidase showed 16.4 % glucose. Nitrogen was 1.46 %. Phosphorus was less than 0.01 %. The molar ratio nitrogen to hexose was 1:1.9.

An aliquot of component B was rechromatographed on a small column of silicic acid with chloroform–methanol (4:1, v/v). Hexose was then increased to 38.3 % and nitrogen to 1.50 %. The molar ratio nitrogen to hexose was in this preparation 1:2.0.

$$[\alpha]_{589}^{20} = -8.6° \text{ (}c\text{, 3.0 \% in pyridine).}$$

From 24.7 mg of component B (before rechromatography) 4.6 mg of normal and 1.2 mg of hydroxy fatty acid methyl esters were isolated.

Component B was converted to component A under release of galactose at mild acid hydrolysis. Therefore, it is considered to be a ceramide-dihexoside. A compound with the same composition and characteristics in chromatography has been isolated by us from liver and spleen. It seems also to be very similar to cytolipin H isolated from tumour tissue and normal bovine spleen[20,21] and the ceramide-dihexosides isolated from spleen of several species[19].

Component C

As component C was admixtured with component B, a rechromatography was run on a small column of silicic acid. The column was developed with chloroform–methanol (4:1, v/v). On the rechromatographed sample, hexose chromatography and electrophoresis showed only galactose and glucose in a molar ratio of about 2:1. The hexose content was 42 % in the orcinol- and 45 % in the anthrone-method (standard galactose:glucose = 2:1). Glucose oxidase showed 14 % glucose. Nitrogen was 1.23 % and phosphorus 0 %. The molar ratio nitrogen to hexose was 1:2.7. Only traces of hexosamine could be indicated.

Component C is considered to be a ceramide-trihexoside though the ratio nitrogen to hexose is 1 to 2.7 instead of 1 to 3. This assumption is supported by the fact that a glycolipid of human liver[25] with identical R_F value and very similar infrared spectrum of serum component C contained theoretical amounts of sugar and nitrogen for a ceramide-trihexoside. Further, component C of serum was converted to ceramide-monohexosides and -dihexosides after weak acid hydrolysis.

There was not sufficient material for determination of optical rotation and isolation of fatty acid methyl esters.

A ceramide-trihexoside has been isolated from bovine spleen by MAKITA AND YAMAKAWA[19].

Component D

Sugar chromatography and paper electrophoresis after acid hydrolysis for 6 h showed galactose, glucose and galactosamine. The ratio galactose to glucose was about 2:1. After treatment with ninhydrin in pyridine the galactosamine was converted to lyxose. No arabinose was detected.

After recrystallization of component D from CH_3OH the hexose content was 41 % in the orcinol- and 44 % in the anthrone-method (standard galactose:glucose = 2:1). Glucose oxidase showed 13.5 % glucose. Nitrogen was 2.20 %. The ratio nitrogen to hexose was 2:2.9. There was a trace of phosphorus but no sialic acid. After acid hydrolysis there appeared material with the same R_F values as components A, B and C with normal fatty acids.

At chromatography after methylation only methyl esters of normal fatty acids were detected.

There was not sufficient material for determination of optical rotation.

Relative distribution of neutral glycolipids

The purpose of the present investigation was to isolate and characterize the neutral glycolipids of blood serum and to determine their relative percentage distribution. These results are given in Table II.

TABLE II

RELATIVE DISTRIBUTION OF GLYCOLIPIDS IN BLOOD SERUM

All figures are expressed in per cent of total neutral glycolipids.

Material	*Expt. 1 (17 l serum)*	*Expt. 2 (4 l serum)*	*Expt. 3 (4 l plasma)*	*Calc. distribution*
Ceramide-monohexoside	41	44	46	40–50
Ceramide-dihexoside	47	51	47	45–55
Ceramide-trihexoside	9	4	5.5	5–10
Amino glycolipid	3	1	1.5	2–5

In order to determine whether plasma and serum had different profiles, two small-scale experiments with 4 l of plasma and 4 l of serum were elaborated with the method described. The results are given in Table II. No corrections have been done for the amounts used for the analytical control of the different isolation steps. It is likely that more trihexosides and amino glycolipids were lost than of the other two glycolipids. It is, however, evident that there is no difference between plasma and serum. In the last column of Table II corrected figures for the percentage distribution of neutral glycolipids of blood serum are given. In this column the amount of amino glycolipids has been calculated from hexosamine determinations instead of gravimetric analysis of isolated glycolipid.

DISCUSSION

Thin-layer chromatography has been applied for the quantitative separation of neutral glycolipids from blood serum. As the total recovery of hexose was more than 95 % and the recovery of component D was low, the recovery of ceramide-monohexosides and -dihexosides can be considered excellent. The fractions were also rather free from impurities as most of the silica gel could be removed from the hexosides by the partition between a chloroform–methanol and a methanol–water phase. The recovery of amino glycolipids was not satisfactory, as less than 1/3 of hexosamine was recovered. The low recovery of this glycolipid was due to at least three factors: the low percentage of amino glycolipid in the lipid mixture, incomplete extraction from the silica gel, and losses to the methanol–water phase at the partition. It is possible that the low recovery from the gel is partly due to the $CaSO_4$ of the carrier which is added for increasing the adhesiveness of the carrier to the glass plates. It is also likely that the value found for lipid-hexosamine (0.32 %) in Fraction IA was falsely too high as no correction was done for unspecific chromogens from amino acids-hexoses[15]. The latter assumption is supported by the finding of 50 % recovery of hexosamine from Fraction IIA and by recovery experiments with pure amino glycolipids of spleen at which recoveries of 60–70 % were obtained. Nevertheless, until new carriers of silica gel are commercially available, amino sugar-containing glycolipids with relatively large carbohydrate moieties can be better separated on silicic acid columns than on thin-layer plates of silica gel.

In Table II the relative distribution of the neutral serum glycolipids is given. It is evident that glucocerebrosides and ceramide-dihexosides constitute more than 4/5 of the total amount. It has earlier been shown that the dihexosides and the amino glycolipids occur in relatively large concentrations in human erythrocytes[19]. To exclude that the glycolipid profile was modified by release of glycolipids from the cells at the blood coagulation, plasma and serum were run simultaneously. As can be seen from Table II there was no distinct difference between plasma and serum. In these small-scale experiments very low amounts of trihexosides and amino glycolipids were found. This finding further supports the suggestion that the recovery of strongly polar glycolipids from thin-layer plates is far from quantitative when these lipids occur in low concentrations.

The present investigation demonstrates that a determination of only total lipid-hexose in plasma will be of limited value. It is, however, impossible to apply the technique described for an individual patient. Work is now in progress to elaborate a method by which a semiquantitative estimation of total serum glycolipids on thin-layer plates can be performed on 10 ml of plasma. In Gaucher's disease there seems to be a selective increase of the ceramide-monohexosides (glucocerebrosides) while the other three neutral glycolipids occur in normal amounts.

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