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The Isolation and Partial Characterization of Glycolipids of Normal Human Leucocytes

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1. The lipids of purified human leucocytes were extracted with chloroform–methanol and the extract was washed with water. Glycolipids, isolated by Florisil chromatography, were subjected to mild alkaline hydrolysis and the alkali-resistant fraction was fractionated on a silicic acid column. 2. Three classes of glycolipid were separated. The less polar, containing 3·6% of the total glycolipid hexose as galactose, was tentatively identified as ceramide monohexoside. The major glycolipid fraction was characterized as ceramide dihexosides. The more polar glycolipids comprised 1·6% of the total glycolipid hexose as galactose and glucose (in the molar ratio 2:1) and were non-acidic. This class was separated as a mixture containing ninhydrin-positive glycolipids. 3. The ceramide dihexosides taken from two leucocyte preparations accounted for 15·2% and 16·4% by weight of the total lipids. 4. The carbohydrate moiety of the ceramide dihexosides contained galactose and glucose in the molar ratio 2:1. Partial acid hydrolysis and paper chromatography indicated that the hexoses are present as disaccharides, lactose being identified as one of them. 5. Palmitic acid ($C_{16:0}$) and nervonic acid ($C_{24:1}$) were the major fatty acids of this glycolipid. Hydroxy fatty acids were not detected.

Cerebroside and cerebroside sulphate, containing in their molecule 1 hexose moiety/amino base, are the most common water-insoluble glycolipids of the mammalian nervous system, from which they have mostly been isolated and characterized.

Similar substances, with two hexose moieties/molecule, occur in small quantities in various tissues (Klenk & Rennkamp, 1942; Rapport, Graf, Skipski & Alonzo, 1959; Yamakawa, Kiso, Handa, Makita & Yokoyama, 1962; Svennerholm & Svennerholm, 1963; Gray, 1965). They have all been identified as ceramide dihexosides containing both glucose and galactose, usually in equal proportions, and they occur together with other water-insoluble glycolipids as minor components of the total lipids of the tissue. When examined immunologically, they show a biological (haptenic) activity that has been correlated with their molecular structure (Rapport, 1960). Some of the ceramide dihexosides contained hydroxy fatty acids as well as the normal straight-chain fatty acids.

As a continuation of our work on the lipid metabolism of normal and leukaemic leucocytes (Malamos, Miras, Levis & Mantzos, 1962; Miras, Mantzos & Levis, 1965), we have studied the water-insoluble glycolipids of these cells.

EXPERIMENTAL

Isolation of leucocytes. Blood from healthy male blood donors, who had not eaten for 12 hr., was used as a source of leucocytes. Blood (4 vol.) was aspirated into 1 vol. of anti-coagulant solution (0·48% citric acid hydrate–1·32% sodium citrate–1·47% dextrose).

Sedimentation of erythrocytes was carried out as described by Malamos *et al.* (1962), yielding leucocytes contaminated with fewer than 4 erythrocytes/leucocyte. To achieve purer preparations, the leucocytes were re-suspended in a small volume of plasma (free of platelets) and erythrocytes were added (taken from the first sediment). At this stage leucocytes were present in a concentration approx. 80 times that of the original blood, erythrocytes having half the normal concentration. Then polyvinyl-pyrrolidone solution was added to give a final concentration of 1·5% and the suspension was mixed gently. The leucocytes were then separated and washed twice with 10 ml. of 0·85% NaCl solution. The purified preparation contained approx. 75% of the blood leucocytes contaminated with 2–5 erythrocytes and 10–20 platelets/100 leucocytes. The differential count was not altered significantly.

Extraction of lipid. The lipid was extracted by a slight modification of the method of Folch, Lees & Sloane-Stanley (1957). To 1 vol. of leucocyte suspension in water, 6·3 vol. of methanol was added, and the mixture was shaken vigorously for 20 min.; 12·6 vol. of chloroform was then added and the mixture shaken for 40 min. The mixture was filtered and the procedure of Folch *et al.* (1957) was applied

to the filtrate with only one wash with the theoretical upper phase.

Isolation of the glycolipid fractions. The total lipids (30–40mg.) were dissolved in chloroform and placed on a Florisil (Floridin Co., Tallahasse, Fla., U.S.A.) column (5g.), the elution being carried out successively with chloroform, chloroform–methanol (19:1, v/v), chloroform–methanol (2:1, v/v) and chloroform–methanol (1:2, v/v). The anthrone test was performed on small samples of the eluted fractions to identify the glycolipids. Each of the two last fractions contained the glycolipids and was treated with a mixture containing equal volumes of chloroform and 0·6N-NaOH in methanol (Hübscher, Hawthorne & Kemp, 1960; as applied by Sweeley, 1963) for 1hr. at room temperature and was then acidified and extracted with chloroform. The chloroform phase was dried and placed on a silicic acid column (2g.) (special for lipid chromatography; Bio-Rad Laboratories, Richmond, Calif., U.S.A.). Methyl esters of fatty acids formed during the methanolysis were eluted from the column with chloroform. The elution was then carried out successively with the following mixtures of chloroform–methanol: (19:1, v/v), (18:2, v/v) and (16:4, v/v). Column chromatography on DEAE-cellulose was occasionally used according to the method of Rouser, Baumann, Kritchevsky, Heller & O'Brien (1961) for identification of acidic glycolipids.

Thin-layer and paper chromatography. Basic and neutral plates, prepared according to the method of Skipski, Peterson & Barclay (1962), were used for thin-layer chromatography, the first being developed with chloroform–methanol–acetic acid–water (70:12:3:2, by vol.) and the second with chloroform–methanol–aq. NH_3 (sp.gr. 0·88) (80:20:0·4, by vol.).

The spots were made visible by spraying with 50% (v/v) H_2SO_4 or with other reagents (Mangold, 1961). Thin-layer chromatography was also performed on plates with DEAE-cellulose (Mannex DEAE; Mann Research Laboratories Inc., New York, N.Y., U.S.A.) and developed with chloroform–methanol (2:1, v/v); spots were made visible by spraying with bromophenol blue. The lipid bases were chromatographed on thin-layer plates according to the method of Fujino & Zabin (1962). Fatty acid esters were chromatographed on neutral plates with light petroleum (b.p. 40–60°)–diethyl ether–acetic acid (40:10:1, by vol.).

Sugars were identified in the hydrolysate by descending paper chromatography on Whatman no. 3 paper in butan-1-ol–acetic acid–water (4:1:5, by vol.) and pyridine–butan-1-ol–water (1:2:1, by vol.). Spots were revealed with aniline hydrogen phthalate or $AgNO_3$ reagents.

Hexose determination. Hexose was determined with anthrone in two different non-hydrolysed samples, by the method of Radin, Lavin & Brown (1955), on 150 and 200μg. samples; a sample of brain cerebroside, purified by chromatography on a Florisil column and checked for purity by thin-layer chromatography and infrared analysis, was used as a standard. Analysis of total nitrogen and total hexose as galactose of this standard showed a galactose/nitrogen molar ratio 1:0·95.

Hexose was determined after hydrolysis as follows: the glycolipid (approx. 2mg.) was hydrolysed with 4ml. of 3N-HCl at 100° for 3hr. After removal of the HCl under reduced pressure, the residue was taken up in water and several samples were used for the estimation of the total hexose and of the galactose/glucose ratio by the carbazole method, as described by Seibert & Atno (1946). Hexose determination with anthrone was performed on the hydrolysate by the method of Radin, Brown & Lavin (1956).

Glucose was determined enzymically with glucose oxidase (Huggett & Nixon, 1957).

Identification of fatty acids. The glycolipid was hydrolysed by refluxing for 6hr. with methanol–conc. HCl (5:1, v/v). The fatty acids were extracted with light petroleum (b.p. 40–60°), esterified and analysed by gas–liquid chromatography. A Pye Argon gas chromatograph fitted with a polyethylene glycol adipate (10%, by wt., on Celite) column was used and the peaks were identified by comparison with standard methyl esters prepared from brain cerebrosides (Mead & Levis, 1963).

Determination of long-chain base. After the extraction of the fatty acids from the hydrolysate, most of the methanol was removed in a stream of N_2, water was added and the solution was made alkaline with conc. KOH. The long-chain bases were extracted into ethyl acetate and washed with water, and sphingosine was determined quantitatively by the method of Lauter & Trams (1962). The lipid base fraction of a brain cerebroside sample, with known nitrogen content, was used as a standard.

Partial hydrolysis. The glycolipid was heated in a boiling-water bath with 0·1N-HCl for 1hr. with continuous stirring. The hydrolysate was extracted with chloroform and the extract was subjected twice more to the same procedure, and then twice to the same procedure with 0·2N-HCl. The unaffected lipids were chromatographed on a silicic acid column with chloroform and chloroform–methanol (19·5:0·5, v/v), (19:1, v/v) and (18:2, v/v) as eluting solvents. The water phases from the hydrolysis steps after removal of the HCl under reduced pressure were first analysed individually for total hexose and were then combined and used for glucose estimation and paper-chromatographic analysis of the carbohydrates.

Nitrogen and phosphorus determination. Nitrogen was determined by direct nesslerization of the digested lipid by the method of King, Haslewood & Delory (1937). Phosphorus was determined by the method of King (1932) with the volumes adjusted to measure 0·4μg. of phosphorus.

Infrared analysis. Infrared analysis was performed on KBr pellets with a Beckam IR–5 spectrophotometer.

RESULTS

Two preparations, each extracted from the leucocytes of approx. 1·2l. of blood taken from three individuals, contained (I) 35mg. and (II) 28mg. of lipid.

The alkali-stable lipids, eluted from the Florisil column with chloroform–methanol (2:1, v/v), were chromatographed on a silicic acid column. A small glycolipid fraction was eluted with chloroform–methanol (19:1, v/v). The hexose content of the fraction, measured as galactose, accounted for 3·6% of the hexose of the total glycolipids. On thin-layer chromatography it showed the same mobility as brain ceramide galactoside; it was therefore tentatively identified as ceramide monohexoside. Further analysis of this fraction was not possible because of lack of material. Most of the glycolipid

was eluted as a single peak with chloroform–methanol (18:2, v/v). The yields were 5·3mg. and 4·6mg. of lipid from preparations I and II respectively.

The glycolipid was a white powder (phosphorus content less than 0·02%) that reacted strongly with anthrone reagent and did not show any reducing properties. On thin-layer chromatography it appeared as a double spot, negative to ninhydrin.

The glycolipid, when chromatographed on a DEAE-cellulose column, was eluted in the non-acidic fraction with chloroform–methanol (2:1, v/v). On thin-layer plates, coated with DEAE-cellulose, it moved with the solvent front, whereas a sample of brain cerebroside sulphate run simultaneously remained at the origin. The infrared spectrum showed features of the cerebroside molecule (Marinetti & Stotz, 1954), being almost identical with those presented for the blood serum ceramide dihexoside (Svennerholm & Svennerholm, 1963). The glycolipid did not contain any sulphate, as indicated by the absence of the characteristic bands for S–O and C–O–S at 1240 and 820cm.$^{-1}$ respectively (Lloyd & Dodgson, 1961). A weak band near 1720cm.$^{-1}$, possibly attributable to ester group, remained unchanged after mild alkaline hydrolysis and chromatography on silicic acid.

Paper chromatography of the carbohydrate fraction after complete acid hydrolysis revealed two spots with mobilities identical with those of glucose and galactose. After partial hydrolysis with 0·1N-hydrochloric acid two spots with the mobilities of galactose (main spot) and lactose were obtained; glucose could only be identified by the enzymic method and accounted for 3·4% of the total liberated hexose. After treatment with 0·2N-hydrochloric acid the chromatogram showed three spots with the mobilities of glucose, galactose and lactose, the third being faintly positive; glucose accounted for 24·5% of the total liberated hexose. The identity of the lactose spot was further established by two-dimensional paper chromatography. In this instance the hydrolysate was chromatographed after [1-^{14}C]lactose was added to it. Radioautographs taken after solvent development showed complete coincidence of the radioactive and colour spots of lactose. The lipid fraction was chromatographed on a silicic acid column after partial acid hydrolysis and, in addition to unchanged material, a new glycolipid fraction was eluted with chloroform–methanol (19:1, v/v). On thin-layer chromatography the new glycolipid fraction appeared as a double spot, with mobilities similar to those of ceramide monohexosides. These findings indicate the presence of both glucose and galactose bound in the form of dihexosides. The easier liberation of galactose compared with that of glucose indicates that the glucose-containing dihexoside is linked to the ceramide through glucose.

Table 1 shows the results of chemical analysis of the glycolipid. Determination of the fatty acid components was performed on different portions of one sample, derived by pooling together the remainders of each preparation after estimation of hexose and nitrogen.

The results of anthrone analysis on non-hydrolysed samples are expressed as the ratios of the extinctions/g.atom of nitrogen for the colours produced by the leucocyte glycolipid and the ceramide galactoside standard. The data of the first column of Table 1 could suggest that the leucocyte glycolipid contains more than 2 moles of galactose/g.atom of nitrogen, but since glucose with anthrone produce about 1·8 times more colour than galactose the presented ratios are close to those expected for a dihexoside containing galactose and glucose in the molar ratio 2:1. This finding correlates well with the glucose content of the glycolipid, which was determined in the hydrolysate by both the carbazole and glucose-oxidase methods.

Estimation of total hexose in the hydrolysates of the sample glycolipid and of the standard brain cerebroside by the anthrone method showed that the total hexose/nitrogen ratio of the hydrolysed

Table 1. *Chemical analysis of human leucocyte ceramide dihexoside*

The ratios of *E*(anthrone)/g.atom of nitrogen for leucocyte glycolipid to that for ceramide galactoside standard are averages of duplicate hexose determinations with anthrone on two different non-hydrolysed samples with known nitrogen content. Total hexose was determined by the carbazole method after hydrolysis; a mixture of galactose and glucose (molar ratio 2:1) was used as standard. Sphingosine was determined in a mixture of glycolipids of preparations I and II.

	Ratio of *E*(anthrone)/g.atom of N (leucocyte glycolipid/ceramide galactoside)	Total hexose (% dry wt.)	Glucose (% of total hexose)		Total N (% dry wt.)	Sphingosine/total N molar ratio
			Carbazole method	Enzymic method		
Preparation I	2·43	32·6	34	33	1·38	0·85
Preparation II	2·37	31·5	35·5	34·5	1·45	

sample was the same as that found in the non-hydrolysed one. This result indicates that both sample and standard were hydrolysed to the same extent. The actual recovery of total hexose, as judged from galactose estimation in triplicate hydrolysates of the standard, was $85 \pm 3\%$. When this recovery is taken into consideration, it is obvious that the data for total hexose presented in Table 1 are near to 38·8%, which is the value for hexose expected in a ceramide dihexoside containing fatty acids with an average mol.wt. of 320. The calculation of this value was based on the fatty acid composition derived from gas–liquid-chromatographic analysis.

The lipid base fraction has not been definitively identified, but it shows on thin-layer chromatography one major and one less intense ninhydrin-positive spot, with mobilities identical with those of a corresponding fraction from brain cerebrosides. The amount of sphingosine, estimated after hydrolysis, was less than 1 mole/g.atom of total nitrogen (Table 1). This may be due to partial removal of sphingosine into the fatty acid fraction.

Gas–liquid chromatography of the fatty acid methyl esters revealed the presence of only normal fatty acids (Table 2).

The leucocyte ceramide dihexoside appeared as a double spot on thin-layer chromatography. The presence of α-hydroxy fatty acids could probably explain a slower-running spot (Jatzkewitz, 1960), but as such fatty acids were not found in the hydrolysate of the glycolipids the two spots were separated by preparative thin-layer chromatography and analysed for glucose content, for fatty acid composition and for lipid base. The glucose content and the lipid base profiles were the same, but the fatty acid compositions of the two spots showed some differences (Table 2). The faster-running spot contained predominantly the longer-chain fatty acids, whereas palmitate was the predominant fatty acid in the slower spot. There was not a clear-cut distinction between the fatty acid compositions of the two spots, owing to a small overlap that occurred during the preparative thin-layer chromatographic separation. These differences are in accordance with the results of Suomi & Agranoff (1965) on the fatty acid composition of the double spot of spleen ceramide lactoside.

Table 2. *Fatty acid composition of human leucocyte ceramide dihexoside fraction before and after separation by thin-layer chromatography*

The compositions were determined by gas–liquid chromatography. Values are expressed as percentages of total methyl esters.

Fatty acid	Total dihexoside	Upper spot	Lower spot
$C_{14:1}$	1·3	1·4	4·5
$C_{16:0}$	27·4	6·2	55·0
$C_{17:0}$	1·3	—	1·0
$C_{18:0}$	3·9	1·2	3·4
$C_{18:1}$	5·6	4·3	6·4
$C_{19:0}$	0·9	—	—
$C_{20:0}$	0·6	3·0	2·1
$C_{20:1}$	0·3	1·5	0·9
$C_{21:0}$	1·2	2·7	3·0
$C_{21:1}$	0·9	1·7	0·7
$C_{22:0}$	4·3	5·3	2·5
$C_{22:1}$	1·1	7·2	—
$C_{23:0}$	—	—	—
$C_{24:0}$	12·9	12·2	2·9
$C_{24:1}$	38·3	53·0	17·6

The alkali-stable lipids eluted from the Florisil column with chloroform–methanol (1:2, v/v) were chromatographed on a silicic acid column and eluted with chloroform–methanol (16:4, v/v). From a DEAE-cellulose column they were eluted in the non-acidic fraction. The hexose content of this glycolipid measured as galactose–glucose (molar ratio 2:1) accounted for 1·6% of the hexose of the total glycolipids. On thin-layer chromatography four spots were obtained that moved slower than the ceramide dihexosides and it is possible that they consist of two double spots. The slower-moving of them gave a positive reaction with ninhydrin. Further analysis of these glycolipids was not possible owing to lack of material; nevertheless, their higher polarity compared with that of ceramide dihexoside and their non-acidic character suggest that these compounds contain more than two hexose moieties/molecule.

DISCUSSION

The isolated glycolipid that accounted for 15·2 and 16·4% of the total weight of the leucocyte lipid contained galactose and glucose in the molar ratio 2:1, and it was therefore a mixture of ceramide dihexosides. Partial acid hydrolysis indicated the presence of lactose, but the hexose ratio suggested that other disaccharides must also be present, like the galactosylgalactose found in ascites-sarcoma cells (Gray, 1965). Such a compound was not identified, however.

The carbohydrate composition of the major leucocyte glycolipid was similar to that of the ceramide dihexoside fraction isolated from tumour cells (Gray, 1965), since in both galactose was the predominant hexose. Nevertheless, it also resembled closely that of cytolipin H, the ceramide lactoside isolated from human epidermoid carcinoma (Rapport, Graf, & Yariv, 1961), and that of the ceramide dihexoside of erythrocytes and spleen (Yamakawa *et al.* 1962; Makita & Yamakawa, 1962). Ceramide dihexosides containing glucose and galactose in equal proportions have also been

isolated from human serum (Svennerholm & Svennerholm, 1963). The exceptional characteristic of leucocytes is that the ceramide dihexoside fraction comprised a high percentage of the total lipids. This may reflect a special role of this glycolipid in leucocyte structure and in their membrane function. The high content of ceramide dihexoside makes leucocytes unique among the tissues that have been shown to contain this glycolipid; therefore the presence of intact or destroyed leucocytes as contaminants in tissues analysed for glycolipids must be taken into consideration.

The isolated ceramide dihexosides were the major components of the extracted glycolipids. Another glycolipid fraction, presumably ceramide monohexoside, was present as a minor component. A similar pattern of glycolipid distribution has been reported for human spleen (Makita & Yamakawa, 1962), whereas the ceramide monohexoside content of rat tumour cells (Gray, 1965) was higher than that of the dihexoside. If this difference in the relative amounts of glycolipids is not due to species difference, it may reflect a functional or metabolic difference between normal and tumour cells. Gray (1963) found that different tumour cells contain relatively different quantities of glycolipids. Therefore the relative quantification of the glycolipids in leukaemic leucocytes may be useful in the study of the metabolism of leukaemic leucocytes.

The fatty acids of the leucocyte glycolipid, contrary to what has been reported for the ceramide dihexosides of spleen and tumour cells (Rapport, Skipski & Sweeley, 1961; Suomi & Agranoff, 1965; Gray, 1965), contain more $C_{24:0}$ acid. This is consistent with previous observations (Miras *et al.* 1965) according to which $C_{24:1}$ acids were synthesized *in vitro* from [^{14}C]acetate to much higher extent than $C_{24:0}$ acids. It is noteworthy that the leucocyte glycolipid did not contain either tricosanoic acid or α-hydroxytetracosanoic acid.

The leucocyte ceramide dihexoside will almost certainly possess haptenic activity (Rapport, 1960); it is therefore possible that this function is related to the immunological properties of the leucocytes, constituting part of their antigenic components. The more polar glycolipids, which under the experimental conditions used were isolated in very small quantities, have also been reported to possess blood-group activity (Hakomori & Jeanloz, 1961; Radin, 1957).

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