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Cerebrosides of human aorta: isolation, identification of the hexose, and fatty acid distribution

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ABSTRACT Cerebrosides have been isolated from adult human aortic tissue. Each aorta was divided into portions classified as normal, fatty streaks, fibrous plaques, or complicated lesions. The cerebrosides were isolated by Florisil column chromatography, mild alkaline methanolysis, a second Florisil column, and preparative thin-layer chromatography.

The concentration of cerebrosides was higher in fatty streaks than in the more advanced plaques; apparently normal tissue gave the same cerebroside content as plaques found in the same aorta. The quantities of cerebrosides ranged from 0.01 to 0.73% of the total lipid.

Of the 16 cerebroside samples isolated, 10 contained glucosyl ceramide, 1 contained galactosyl ceramide, and 5 were not analyzed for specific hexose. The fatty acid distribution was determined for 11 of the samples; it was similar to that of spleen cerebrosides. We suggest that aortic cerebrosides originate in the plasma. "Normal tissue" cerebrosides contained less unsaturated fatty acid than cerebrosides from a diseased area of the same aorta.

Preparative thin-layer chromatography, the last step of cerebroside isolation, always separated at least two unidentified substances. One of these substances yielded both glucose and galactose on acid hydrolysis. Their removal from the cerebrosides accounts for the lower values for cerebroside compared to other authors' determinations.

KEY WORDS cerebrosides · atherosclerosis · human aorta · normal tissue · fatty streaks · fibrous plaques · complicated lesions · glucose · galactose · thin-layer chromatography · fatty acid composition · cerebroside companions

THE PRESENCE OF CEREBROSIDES in human aortic tissue has been reported (1–8). Hausheer and Bernhard found that cerebrosides increase with the severity of atherosclerosis from 2.2 to 3.4% of the total lipid (5). The sclerotic aorta had a ratio of galactose to glucose of 8:3. However, the IR spectrum shown (5) corresponds to that of glucosyl ceramide (9). Böttcher (6) has studied various portions of human aorta and finds the greatest concentration of cerebrosides in normal tissue after lesions have been removed. This concentration decreases with increased atherosclerosis from 6.5 to 4.8% of total lipid. Of the lesions examined, streaks and spots contained the highest concentration of cerebrosides, 4.8% of total lipid, and fibrous plaques had the lowest concentration, 2.4%. These values were calculated from data given by Böttcher (6). Our findings are in general qualitative agreement with Böttcher. However, the amounts of cerebrosides reported here are much less. The objectives of this investigation were to identify the carbohydrate portion of human aortic cerebrosides and to determine the fatty acid distribution of these cerebrosides.

MATERIAL AND METHODS

All reagents not specifically noted were analytical reagent grade. All solvents were distilled before use. Solutions are expressed by volume.

Tissue Classification

Aortas were obtained directly from autopsy and were kept immersed in 0.9% sodium chloride at −20°C until dissected just before extraction. The inner portions of the aortas were divided into four classes: apparently normal tissue (N), fatty streaks (S), fibrous plaques (P), and complicated lesions (L). The classification was made according to the definitions of the World Health Organization (10), but the tissue was not stained. The identified

Abbreviations: GLC, gas–liquid chromatography; TLC, thin-layer chromatography. Fatty acids are designated by chain length:number of double bonds; h, hydroxy.

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areas were dissected separately from the aortas. Each sample is identified by two letters: the tissue type classification is indicated by the second letter, while the first letter identifies the aorta from which it came. All samples were from adults with no known defects of lipid metabolism, except B, from a diabetic woman who died unexpectedly at the age of 65.

Isolation of Cerebrosides

Lipids were extracted (11) from the tissue and the extract was filtered through sintered glass. An additional amount of chloroform–methanol 2:1, equal to one-sixth the original volume, was used to rinse the homogenizer and filter through the residue. An equal volume of chloroform–methanol 1:2 was also washed through the residue (12). The same volume of chloroform was added to the combined filtrate to restore the 2:1 chloroform–methanol ratio. The filtrate was washed with one-fifth volume of 0.1 M KCl and the lower layer was washed again with equilibrated upper phase (11). Solvent was removed from the lower layer in a rotary evaporator. Anhydrous benzene–ethanol 1:1 was added near the end; this prevented splashing and aided in the removal of water. Evaporation from benzene–ethanol was repeated to constant weight of the crude lipid.

Cerebrosides were isolated from the crude lipid extract by a four-step procedure: (*a*) Florisil (Fisher Scientific Company, Chicago, Ill.) column chromatography, with hexane–ether 8:2 to remove triglycerides and cholesterol, and chloroform–methanol 3:1 (13) to elute cerebrosides; (*b*) mild alkaline methanolysis of the chloroform–methanol eluate (14) for removal of glycerophosphatides; (*c*) Florisil column chromatography as in (*a*) for removal of methyl esters formed in step (*b*); and (*d*) preparative TLC of the chloroform–methanol fraction. The Florisil contained 8% water by weight and was 40 times the weight of lipid. Eluting volumes were 30 ml/g of Florisil for the first fraction and 35 ml/g for the second fraction. Separations after steps (*a*) and (*c*) were checked by TLC. No cerebroside could be found in the hexane–ether fractions. Although most of the cholesterol was in the hexane–ether fractions, some was often present in the chloroform–methanol fractions. Passing the lipid recovered from the first hexane–ether eluate through a second Florisil column failed to recover any additional cerebroside. After finding that a Florisil to lipid ratio of 10:1 for step (*a*) gave the same results as 40:1, we used this amount for samples I and J. Step (*c*) was omitted for sample J.

The preparative TLC was done on Silica Gel HR (Brinkmann Instruments Inc., Westbury, L.I., N.Y.), 0.6 mm thick, in chloroform–methanol–water 24:7:1 (15). Cerebrosides, galactosyl ceramide from bovine brain (Applied Science Laboratories Inc., State College, Pa.), and(or) *N*-stearyl-1-*O*-D-glucosyl ceramide (Custom Synthesis, Miles Laboratories, Inc., Elkhart, Ind.) were used as markers. Bands were made visible with bromothymol blue; we recovered the lipid by scraping off the bands and packing the powder, suspended in chloroform–methanol 2:1, into small glass columns and eluting with chloroform–methanol, 40 ml/g of silica gel (16). Bromothymol blue was removed from the eluate by washing with one-fifth volume of water. The lower layer was washed again with equilibrated upper phase (11). Amounts of cerebrosides present were determined from hexose values or by GLC of the fatty acid methyl esters together with an internal standard.

To test the over-all efficiency of the cerebroside isolation, 1 mg of glucosyl ceramide was added to 1 g of hexane–ether eluate from the first Florisil column. This mixture was then subjected to the entire isolation procedure and finally analyzed by GLC. The recovery was 78%.

Hexose Determinations

Anthrone determinations were done on aliquots of the isolated cerebrosides (17). Glucose and galactose were determined after hydrolysis (16) with the aid of "Glucostat" and "Galactostat" reagents (Worthington Biochemical Corporation, Freehold, N.J.). Standard cerebrosides were subjected simultaneously to the hexose determinations and appropriate corrections were made for less than theoretical values. Glucose assays were very reproducible and gave the correction factor reported by Suomi and Agranoff (16). Galactose assays were somewhat erratic but were close to the theoretical value. Hexose was determined in substances other than cerebrosides after the same hydrolysis procedure.

Preparation of Fatty Acid Methyl Esters

Cerebrosides were mixed with 1 ml of 5% dry HCl in methanol, and heated in a tightly closed tube for 17 hr at 75°C. The resultant methyl esters were extracted with four 1 ml portions of hexane (18). Methyl esters were further purified by TLC on a Silica Gel G plate with hexane–diethyl ether 3:1 as solvent. The methyl esters of 23:0 and 18h:0 were used as markers. Bands were made visible with bromothymol blue; we recovered the esters by scraping off the bands and packing the powder, suspended in diethyl ether, into small glass columns and eluting with diethyl ether, 10 ml/g of silica gel. Plates were scraped within an hour after development so that short-chain esters should not be lost by evaporation.

The 2-hydroxy esters were acetylated with 0.05 ml of a solution containing 4 mg of *p*-toluenesulfonic acid in isopropenyl acetate (Eastman Organic Chemicals, Rochester, N.Y.; redistilled) for 30 min at 60°C in a tightly closed tube (19).

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GLC of Fatty Acid Esters

GLC was performed in an F & M model 402R-00 gas chromatograph (dual column; flame ionization detector) with U-shaped glass columns. All ester samples were chromatographed on a pair of columns 6 ft × 3 mm packed with 6% diethylene glycol succinate polyester on 80–100 mesh Diatoport S (F & M Scientific Corp., Avondale, Pa.). Some ester samples were also chromatographed on a pair of columns 4 ft × 3 mm packed with 17% Apiezon L on 60–80 mesh Gas-Chrom Z (Applied Science Laboratories). The unsubstituted methyl esters were chromatographed between 160 and 220°C with a temperature gradient of 2°C/min. The 2-acetoxy methyl esters were chromatographed between 200 and 220°C with a temperature gradient of 1°C/min. Standards KD and KF (Applied Science Laboratories) were chromatographed periodically; the composition found agreed with the stated values with a relative error of 3% or less. Duplicate analyses of esters had relative errors of 5% or less for major components (>10%).

A measured quantity of methyl 20:0 was added to the unsubstituted esters after one chromatogram. This served as an internal standard and corrections were made for the amount of 20:0 in the original sample. The preliminary chromatogram consumed between 1 and 5% of the sample.

Esters were identified by comparison with standards KD and KF as well as with the 2-acetoxy methyl esters of 18h:0 and 22h:0. Plots were made of relative retention time versus carbon number as an aid in the identification of substances not represented in the standards. The early analyses were performed on both types of column for further identification, and the acid identified as 18:2 was shown to be a C_{18} acid by hydrogenation followed again by GLC.

RESULTS AND DISCUSSION

Cerebrosides have been isolated from 16 tissue samples taken from 9 human aortas (Table 1). "Normal tissue" was taken by stripping the intima from apparently normal areas of an aorta whenever a sufficient quantity for analysis was obtainable. It is probable that small lipid deposits, visible only in stained tissue or with a microscope, were incorporated as part of the normal tissue (20). Of the fatty streak (S) samples, J was about 20% covered with streaks while A and B were about 40 and 70% covered, respectively. The tissue overlying some of the streaks in A was partially calcified. Aorta I had many scarred areas in the adventitia as well as in the intima. These areas contained a very granular yellow material. Extraction of this material yielded lipid that weighed less than 1% of the tissue wet weight. The complicated lesion (L) samples were all very similar, with D containing the largest lesion. Of these aortas we obtained only a portion that contained one large lesion.

Cerebroside Content

The amounts of lipid, based on wet weight of tissue, and the percentage of lipid that was cerebroside are shown in Table 1. Cerebrosides were considerably more prominent in the fatty streaks than in the larger plaques. This may simply mean that cerebrosides are diluted by large increases of other lipids as the plaques become larger. Another interpretation is that the fatty streaks are substantially different types of plaques rather than being an early stage of larger plaques. It is interesting to speculate that aortas are different from each other with respect to the cerebrosides present and therefore develop plaques of different types. "Normal tissue" shows considerable variation and tends to be similar in cerebroside content to the plaques found in the same aorta. Two S samples came from aortas (A, J) with a low percentage of crude lipid in the "normal" tissue. These N samples are probably the most nearly normal of the N samples. Small deposits of lipid in apparently normal aortic tissue may be similar in many ways to those in adjacent plaques.

The amounts of cerebrosides are considerably lower than reported previously (5–7). These lower values result, partially at least, from the last step in the purification of the cerebrosides. At least two bands, besides the cerebroside band, were always seen in preparative TLC. One of these bands, R_f = 0.8–0.85, contains carbohydrate, comparable in amount to that in the cerebroside band for the two samples analyzed, AS and CL. These substances are being investigated further. Our chloro-

TABLE 1 CONCENTRATION OF LIPID AND CEREBROSIDE IN HUMAN AORTA

	Normal Tissue						Fatty Streaks			Fibrous Plaques		Complicated Lesions		
Sample*	AN	DN	EN	FN	IN	JN	AS	BS	JS	FP	IP	CL	DL	EL
Crude lipid (% of wet tissue)	4.5	40.0	14.0	14.6	26.6	3.6	8.7	10.6	19.9	52.8	57.3	36.5	51.0	24.4
Cerebrosides (% of crude lipid)	0.26	0.10	0.06	0.04	0.01	0.26	0.37	0.73	0.22	0.01	0.02	0.02	0.02	0.03

* Each aorta was given an identifying letter which appears as the first sample letter. The second sample letter represents the tissue classification: N, normal tissue; S, fatty streaks; P, plaques; L, complicated lesions.

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form–methanol fractions from the second Florisil columns contained weights similar to those in the earlier reports—0.9% of crude lipid, average of L samples, to 4.8% for S samples. With these considerations it seems probable that previously reported cerebroside values for the aorta have included the substances that have here been separated by preparative TLC.

Hexose Composition

TLC on silica gel impregnated with sodium tetraborate (21) has shown that the cerebrosides consist predominantly of glucosyl ceramide. All samples chromatographed in this system except IP gave a spot corresponding to standard glucosyl ceramide. Samples EN and DL also had faint spots corresponding to the lower edge of galactosyl ceramide. These spots were probably contaminants and not galactosyl ceramide, for the slower-moving spot of this standard consists of cerebrosides containing hydroxy fatty acids and no hydroxy esters could be found in the aortic samples after acid methanolysis except in IP. DL has two additional faint spots near the solvent front. Sample IP showed two spots for galactosyl ceramide, corresponding to the two spots of this standard.

Samples AS, BS, JS, and IP, the only samples of which sufficient quantities were available, were also investigated for glucose and galactose content. The S samples had zero values for galactose and positive values for glucose. The glucose values represented 114, 93, and 105% for AS, BS, and JS, respectively, of the hexose values obtained with anthrone. The IP galactose value was 98% of its hexose value, as obtained with anthrone, and the glucose value was zero.

The apparent anomalous composition of IP cannot be explained. Sample FP was checked again by borate TLC after the unexpected results from IP, and again gave only one spot corresponding to glucosyl ceramide. Also, JS had been run on the same plate with IP. As discussed above, aorta I was very scarred. The patient had bronchogenic carcinoma of the right upper lobe with extension through the chest wall into the right breast and partial destruction of the fifth and sixth ribs. With this extensive carcinoma it is possible that some cancer had spread to the aorta and altered the lipid composition.

Hausheer and Bernhard (5) found more galactose than glucose in aortic cerebrosides. The TLC band reported here at R_f = 0.8–0.85 did contain galactose as well as glucose. Sample AS had 27% as much hexose in this band

TABLE 2 Percentage Composition of Normal Fatty Acids in Aorta Cerebrosides

	Normal Tissue*				Fatty Streaks	Fibrous Plaques		Complicated Lesions			
Fatty Acid†	DN	GN	IN	JN	JS	FP	IP	CL	DL	EL	GL
14:0	3.7	1.8	2.4	1.3	2.3	1.6	1.0	2.7	2.7	3.8	tr.
14:1	3.0	tr.	2.1	tr.	tr.	1.9	tr.	tr.	tr.	tr.	tr.
15:0	3.5	tr.	1.9	1.1	tr.	1.6	tr.	1.4	tr.	tr.	tr.
15:1	2.2	tr.	1.6	tr.	tr.	2.4	tr.	tr.	tr.	tr.	tr.
16:0	23.0	22.4	23.5	20.7	20.8	21.5	12.1	22.0	23.0	22.2	9.8
16:1	9.3	7.8	6.7	1.3	3.4	6.7	1.9	3.6	4.9	4.3	5.7
17:0	tr.	tr.	1.5	tr.	tr.	1.7	tr.	18.7	tr.	tr.	tr.
17:1	2.4	tr.	tr.	tr.	tr.	2.4	tr.	tr.	tr.	tr.	tr.
18:0	8.7	9.5	15.3	12.6	7.2	19.0	19.0	11.4	11.1	15.4	5.7
18:1	22.0	48.5	13.7	5.9	25.0	23.6	17.8	20.8	39.8	21.1	61.8
18:2	6.9	8.9			3.8	2.8	tr.	3.3	6.5	1.4	13.8
19:0	tr.	tr.	3.4	tr.	tr.	tr.	tr.	tr.	tr.	tr.	
20:0	1.6	1.1	4.9	1.5	tr.	2.9	1.1	tr.	tr.	tr.	tr.
20:1	tr.	tr.	tr.	tr.	tr.	tr.	3.0	tr.	tr.	2.1	1.9
21:0	tr.	tr.		tr.	tr.	1.7	tr.	tr.	tr.		tr.
21:1	tr.	tr.	tr.	tr.	tr.	2.4	tr.		tr.	tr.	tr.
22:0	1.7	tr.	1.0	10.4	6.6	tr.	1.1	tr.	tr.		tr.
22:1	tr.	tr.	2.0	tr.	tr.	2.4	2.6	tr.	tr.	6.2	1.4
23:0	tr.	tr.	3.8	9.8	4.2	tr.	13.5	tr.	1.4		tr.
23:1	tr.	tr.	tr.	1.3	tr.	5.4	1.6	tr.	tr.	4.0	tr.
24:0	8.9	tr.	8.8	17.3	10.6	tr.	5.4	16.0	10.6	12.6	tr.
24:1	tr.	tr.	2.7	11.6	12.6		8.4	tr.		2.5	tr.
25:0	tr.	tr.		tr.	tr.		3.5	tr.	tr.	tr.	tr.
25:1	tr.	tr.		2.0	2.1		2.1	tr.		tr.	tr.
26:0	3.2		4.6	3.2	1.4		3.6	tr.	tr.	4.4	tr.
26:1					tr.		2.5	tr.		tr.	tr.
Total unsaturated	45.8	65.2	28.8	22.1	46.9	47.2	39.9	27.7	51.2	41.6	84.6
18:0/18:1	0.40	0.20	1.15	2.14	0.29	0.81	1.07	0.55	0.28	0.73	0.09

* Identifying letters are given in Table 1.

† Traces (<1%) of 12:0, 12:1, 13:0, and 13:1 were found in all samples. Traces of 19:1 were present in DN, IN, JN, JS, and IP. DL contained a trace of 27:0.

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as in cerebrosides; of this hexose, 19% was galactose. The same figures for CL are 216 and 54%. While this amount of galactose does not account for the 8:3 ratio found by Hausheer and Bernhard (5), it does show the presence of other glycolipids which contain galactose. The presence of glucosyl ceramide might well be expected in the aorta wall since this is the predominant cerebroside in plasma (22, 23) and serum lipoproteins (24) and has now been found in erythrocytes (23) and leukocytes (25) as well.

Fatty Acids

The fatty acids of the aortic cerebrosides were almost exclusively nonhydroxy fatty acids. Only sample IP had hydroxy fatty acid sufficient for identification. Sample DL appeared by TLC to have some hydroxy cerebrosides, but no hydroxy acids were found after methanolysis. The hydroxy acids found in IP were 22h:0 (79.1%), 23h:0 (5.6%), and 24h:0 (15.3%). An internal standard was not added since after the first good chromatogram an estimate of how much of the sample had been used would have been subject to a large error. The hydroxy acids were estimated to be not more than 5% of the total fatty acids.

Table 2 gives the distribution of nonhydroxy fatty acids for those samples analyzed. Probably the most striking observation is the considerable individual variation. There are, however, some trends where "normal tissue" can be compared with a diseased area from the same aorta. The "normal tissue" has cerebrosides with less unsaturated fatty acid than the diseased tissue. The greatest difference is seen in aorta J which was the least diseased of the aortas analyzed and probably contained the "most normal" tissue of any of the N samples studied. The aorta with the least difference in unsaturation between samples is aorta D. In contrast to JN, DN was probably the "least normal" of the N samples. This difference in extent of unsaturation is most expressed in the C_{18} acids. The "normal tissues" have a higher 18:0/18:1 ratio than the corresponding diseased tissue, and a lower percentage of 18:2, except for DN. Sample IP showed, by contrast, higher unsaturation in the longer-chain fatty acids.

The fatty acid distribution of aortic cerebrosides, especially sample JN, is similar to cerebrosides of human spleen (16). This similarity, together with the hexose similarity, suggests a common origin for these cerebrosides, and since glucosyl ceramides are found in the blood (22–25), there is a strong implication that aortic cerebrosides are absorbed from the blood.

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