Glycosphingolipids from rabbit aorta, plasma, and red blood cells: effects of high cholesterol–high fat diets on fatty acid distribution and quantity of glycosphingolipids

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Abstract Four glycosphingolipids were isolated from rabbit aorta, plasma, and red blood cells. They were identified, by thin-layer chromatography and by quantitative analysis of hexose and fatty acid, as cerebroside, diglycosyl ceramide, triglycosyl ceramide, and globoside. The rabbits had been maintained on a normal diet or on one of three high cholesterol diets for 180 days. The quantities of the glycosphingolipids and their fatty acid distributions were determined, and comparisons were made between the control and experimental animals. Aorta and plasma glycosphingolipids were more affected by the high cholesterol diets than were those from red blood cells. The effects on aorta and plasma glycosphingolipids were similar. The amount of cerebroside was increased in aorta and plasma in all animals in the experimental groups. The amount was also increased in red blood cells in rabbits from two of the experimental groups. The average fatty acid chain length was greater in the lipids from the experimental animals than in those from the control animals for all measured glycosphingolipids from aorta. The average chain length was also greater in cerebrosides from the experimental animals from all three tissues. Probably the most notable differences in the experimental animals were the increased 24:1/24:0 ratios and the increased concentrations of 24:2. These increases occurred in nearly all samples from plasma and aorta, but not in red blood cells. There was also an increase of total unsaturated fatty acids in aorta cerebrosides from the experimental animals. Except for the increase in 24:2, lard generally caused more deviation from normal than did cottonseed oil when the level of cholesterol in the diet was 1%.

Supplementary key words fatty acid correlations · atherosclerosis · cerebroside · diglycosyl ceramide · triglycosyl ceramide · globoside

The present study is a result of continued interest in glycosphingolipids, especially as they may relate to atherosclerosis. Earlier reports (1, 2), directly related to atherosclerosis, dealt with cerebrosides from aorta only. Subsequently, the studies were expanded to include four glycosphingolipids from pig blood (3). The work reported here includes four glycosphingolipids each from aorta, blood plasma, and RBC from rabbits maintained on a normal diet or on one of three high cholesterol diets.

The primary objective of this study was to determine the effects of high cholesterol diets on the distribution of fatty acids in glycosphingolipids and quantities of the glycosphingolipids. A secondary objective was to ascertain possible metabolic relationships between any one of the lipids from two sources, e.g., aorta and plasma. Similarities in fatty acid composition are taken as an indication of metabolic relationships.

MATERIALS AND METHODS

All reagents were analytical grade unless specified otherwise. All solvents were distilled before use.

Rabbit diets, tissue, and blood

24 New Zealand albino male rabbits were divided into four groups of six. One group received plain Wayne rabbit chow, designated as diet I. Each of the three remaining groups received one of the following diets: II, 1.0 g of cholesterol and 2.8 g of cottonseed oil per 100 g of rabbit chow; III, 2.0 g of cholesterol and 2.8 g of cottonseed oil per 100 g of rabbit chow; and IV, 1.0 g of cholesterol and 2.8 g of lard per 100 g of rabbit chow. All animals were allowed water and chow ad lib. The rabbits were maintained on these diets for 180 days. During this period, the weights of the animals were recorded at 1-wk intervals. At

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography; GL-1, glycosyl ceramide; GL-2, diglycosyl ceramide; GL-3, triglycosyl ceramide; GL-4, globoside; ACL, average chain length; RBC, red blood cells. Fatty acids are indicated as number of carbon atoms:number of double bonds.

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the end of the experimental period, blood and aortas were obtained from the animals.

To obtain blood, the rabbits were first injected through the large ear vein with 5–10 mg of heparin. After 5 min, the hair was removed from the ear with a depilatory. The ear was first cut perpendicularly to the ear vein and then placed in a partial vacuum apparatus to facilitate bleeding. Fig. 1 is a photograph of this apparatus. This procedure yielded 80–120 ml of blood per animal. The blood was then separated into plasma and packed erythrocytes as described previously for pig blood (3). Aortas were removed from the bled animals and freed of adhering tissue. To obtain duplicate analyses, each group of six animals was further divided into two groups of three animals at the time they were killed. Blood, as well as aortas, within each subgroup was pooled.

Whole blood from mature rabbits (Pel-Freez Bio-Animals, Inc., Rogers, Ark.) was used for the isolation of the glycosphingolipids for purposes of identification. Red blood cells of mature rabbits from Pel-Freez were used for the isolation of GL-3, which was analyzed by GLC–mass spectroscopy.

Extraction of lipid and isolation of glycosphingolipids

Lipid was extracted from packed RBC as described by Vance and Sweeley (4). The plasma was first lyophilized, and an equal weight of water was added to the dried material. Lipid was extracted from this mixture, as well as from whole aorta, by the method of Folch, Lees, and Sloane Stanley (5).

Glycosphingolipids were isolated from all lipid samples as described by Vance and Sweeley (4). This included silicic acid column chromatography, alkaline methanolysis, and preparative silica gel TLC.

Preparation of fatty acid methyl esters; gas–liquid chromatography and related assays

Isolated glycosphingolipids were analyzed for fatty acids as previously described (1). This procedure included acid methanolysis (6), extraction of the resulting methyl esters with hexane and their purification by TLC on silica gel, and subsequent identification and quantitation by GLC. Vials with Teflon-lined caps were used for methanolysis (7). The methyl ester of 20:0 was added as internal standard before acid methanolysis. The quantities of glycosphingolipids were calculated using the amounts of methyl esters obtained. Preliminary studies indicated that less than 1% of the fatty acid was 20:0.

Sugars were analyzed as their trimethylsilyl derivatives by GLC as described by Vance and Sweeley (4).

GLC–mass spectroscopy was carried out with an LKB-9000 gas chromatograph–mass spectrometer (LKB Instruments, Inc., Stockholm, Sweden) using a 6-ft column packed with 3% OV-1 on 80–100 mesh Gas-Chrom Q (Applied Science Laboratories). The ionization chamber was set at 70 eV and the chromatography oven was temperature-programmed from 200 to 270°C at 2°C/min.

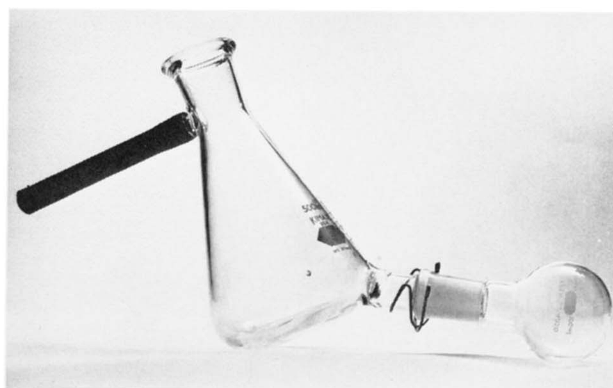

Fig. 1. Apparatus for bleeding rabbits. The rabbit ear was placed into the opening of the open flask. The vacuum tubing was attached to a water aspirator, and blood was collected in the round-bottomed flask.

Fatty acid distribution correlations

Correlations among fatty acid compositions of rabbit glycosphingolipids were determined using a PDP-10 computer (Digital Equipment Corp.). Pearson product-moment correlation coefficients were calculated.

RESULTS

Rabbit growth patterns and crude lipid from blood and aorta

Fig. 2 shows the growth curves of rabbits in each diet group over the course of the experiment. The points are the average weights for the animals in each group. Two animals, one each from groups II and III died during the experiment. The weights of these animals are not included in Fig. 2.

It can be seen from Fig. 2 that animals in the experimental groups grew faster than animals in the control

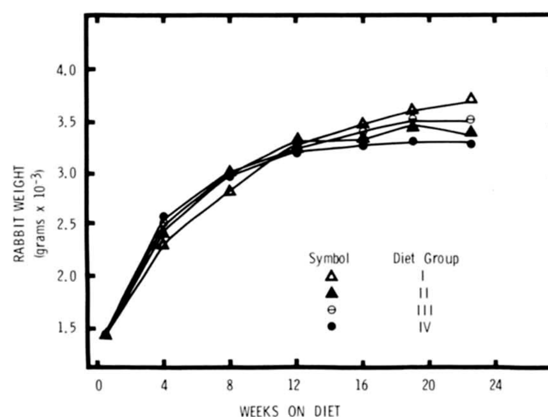

Fig. 2. Rabbit growth patterns.

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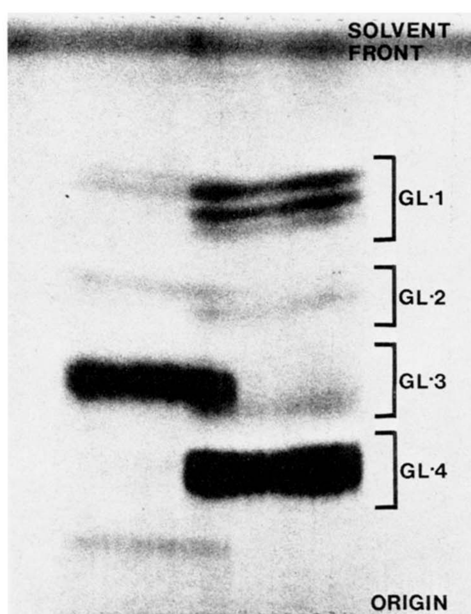

Fig. 3. TLC of glycosphingolipids. The sample on the left is whole blood from mature rabbits (Pel-Freez Bio-Animals, Inc.). Whole blood from pig is on the right. Samples were overlapped. Solvent was chloroform–methanol–water 100:42:6.

group through the first 8 wk of the experiment. At 12 wk the control group had caught up, and from this point to the 22nd wk the experimental groups (II, III, and IV) lagged behind the control group (I). The experimental animals attained a weight plateau by the 20th wk, but the control animals continued to grow to the end of the experiment and appeared still not to have attained their maximum weight.

Several observations were made during the dissection of the animals. The experimental animals had fatty livers and spleens as well as accumulations of fatty material in their aortas. The livers, spleens, and aortas of the control rabbits appeared normal, but these animals had larger amounts of depot fat than rabbits in the experimental groups.

The quantities of crude lipid obtained from the rabbits are presented in Table 1. Analyses for similar subgroups gave fairly similar results except for group II plasma.

TABLE 1. Crude lipids from rabbit blood and aorta

Diet	Aorta	Plasma	RBC
	mg lipid/g	*mg lipid/ml*	
I (control)	126[a]	1.6	8.5
	113	1.5	7.0
II (1% cholesterol +	116	20.1	7.9
2.8% cottonseed oil)	125	7.5	8.8
III (2% cholesterol +	113	34.0	12.4
2.8% cottonseed oil)	92	25.0	12.0
IV (1% cholesterol +	110	28.8	12.3
2.8% lard)	150	36.0	11.1

[a] Each value is for three rabbits.

TABLE 2. Hexose/fatty acid and galactose/glucose molar ratios for rabbit blood glycosphingolipids

Glyco-sphingolipid	Hexose/Fatty Acid	Galactose/Glucose
GL-1	0.98	1/4.7
	1.03	1/6.6
GL-2	1.90	1/1.3
	1.94	1/1.2
		1/1.2
GL-3	3.08	1.9
	3.02	2.0
		1.9

Identification of glycosphingolipids from rabbit blood

Analyses were performed on glycosphingolipids isolated from whole blood of mature rabbits. The lipids isolated were chromatographed by TLC (chloroform–methanol–water 100:42:6) (4) beside, with some overlap, the four glycosphingolipids isolated from pig blood (3). These latter lipids have been identified by Dawson and Sweeley (8). Fig. 3 is a picture of one TLC plate; all plates were essentially the same except for the relative intensities of the bands.

The glycosphingolipids were also subjected to acid methanolysis, which releases methyl esters and methyl glycosides. Glucose, galactose, and fatty acid were then determined by GLC for each lipid (except GL-4), and ratios of hexose to fatty acid and galactose to glucose were calculated. Not enough GL-4 was available for these studies. The results of these analyses are presented in Table 2. No peaks for hexosamines were seen.

Quantities of glycosphingolipids

The quantities of glycosphingolipids found in rabbit aorta, plasma, and RBC are presented in Table 3. The values given are the averages of analyses from the two sets

TABLE 3. Glycosphingolipids of rabbit

Tissue	Diet	GL-1	GL-2	GL-3	GL-4
		Glycosphingolipid			
		μmoles/10 g of tissue			
Aorta	I	1.14	NF[a]	0.97	0.41
	II	1.28	0.42	0.78	0.34
	III	1.78	0.63	0.68	0.36
	IV	1.37	0.58	0.78	0.44
		μmoles/100 ml			
Plasma	I	0.54	0.40	0.32	NF
	II	0.70	0.37	1.10	0.14
	III	1.73	1.36	1.96	0.78
	IV	1.09	0.34	0.91	0.20
RBC	I	1.62	0.55	3.03	0.46
	II	0.64	0.56	3.88	0.31
	III	2.04	0.74	4.54	0.63
	IV	2.44	0.70	4.08	0.46

[a] NF, not found.

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TABLE 4. Percentage distribution of major fatty acids in rabbit aortic glycosphingolipids

	Glycosphingolipid														
	GL-1				GL-2[a]			GL-3				GL-4			
Diet group	I	II	III	IV	II	III	IV	I	II	III	IV	I	II	III	IV
Fatty acid															
14:0	16.6	1.2	T[b]	T	2.1	1.9	2.3	3.0	2.1	1.4	1.3	5.5	4.2	3.3	2.2
16:0	26.7	18.4	18.7	16.5	21.0	19.0	26.6	16.9	14.2	14.2	14.3	31.6	20.2	18.9	20.6
16:1	5.8	T	T	T	T	2.4	1.9	3.8	T	2.8	1.7	7.2	3.3	6.0	4.3
18:0	14.5	8.8	5.9	7.2	7.3	6.4	12.9	10.7	7.1	6.6	6.5	14.6	10.7	9.2	9.3
18:1	16.1	5.9	3.9	10.0	4.4	4.1	4.0	5.5	2.0	3.4	2.3	7.3	6.8	7.6	6.1
18:2	4.8	5.0	2.6	3.4	2.3	T	T	T	T	T	T	T	2.5	2.1	2.4
22:0	3.7	12.8	13.8	12.8	12.3	13.1	10.6	21.9	18.2	16.3	16.1	11.0	10.5	10.9	10.6
22:1	2.1	2.3	1.2	1.8	T	T	T	T	T	T	T	T	T	T	T
23:0	T	5.4	5.7	4.4	3.8	4.5	3.0	1.8	5.9	5.0	4.4	12.0	3.0	2.6	2.2
24:0	9.6	13.1	12.9	11.4	14.2	12.2	10.5	26.3	22.1	20.0	17.6	8.1	13.6	12.4	13.8
24:1	T	16.5	22.5	24.5	11.3	17.4	17.6	8.2	12.4	12.9	19.4	2.6	4.7	7.0	7.0
24:2		5.7	8.8	3.5	4.3	6.2	2.2	T	4.9	5.0	3.0	T	1.8	2.6	2.9
26:0		3.3	2.3	2.6	9.4	5.5	5.0		8.6	9.5	10.4		11.2	9.0	10.2
ACL	17.5	20.9	21.4	21.1	20.6	20.8	20.2	20.6	21.8	21.6	21.8	18.6	20.0	20.0	20.2
24:1/24:0	0.1	1.3	1.7	2.4	0.8	1.4	1.7	0.3	0.6	0.6	1.1	0.3	0.3	0.6	0.5

[a] GL-2 was not found in rabbits fed diet I.
[b] T, trace (< 1%).

of animals in each group. The average deviation from the mean was 13%. This precision compares favorably with that of Vance and Sweeley (4). The lower limit of the assay is approximately 0.05 μmole of isolated glycosphingolipid. The pooled aortas from group I animals weighed 3.5 g. Therefore, using the units in Table 3 (μmoles/10 g of tissue), less than 0.14 μmole of a glycosphingolipid would not have been detected.

Comparing experimental animals with the controls, it is seen that GL-1 was greater in the aorta and plasma from the experimental animals, and in both instances, animals on diet III had the greatest concentration. The same pattern is seen for GL-2, but from aorta only. GL-3 was elevated in the plasma and RBC from experimental animals and again group III was most affected. Plasma and RBC GL-4 were also elevated for group III. GL-4 in aorta was barely affected by the diets.

Fatty acid distributions

Tables 4–6 contain the fatty acid compositions of glycosphingolipids from rabbit aorta, plasma, and RBC, respectively. Only major fatty acids are included, and hence some of the sums of the percentages in the tables are not 100%.

The effects of the high cholesterol diets on fatty acid distributions in the glycosphingolipids from aorta and

TABLE 5. Percentage distribution of major fatty acids in rabbit plasma glycosphingolipids

	Glycosphingolipid														
	GL-1				GL-2				GL-3				GL-4[a]		
Diet group	I	II	III	IV	I	II	III	IV	I	II	III	IV	II	III	IV
Fatty acid															
14:0	1.5	2.5	T[b]	T	1.5	4.2	T	5.1	1.7	T	T	T	4.8	2.8	2.2
16:0	23.9	16.0	13.9	13.5	8.1	22.5	29.8	18.1	15.8	9.0	8.5	10.0	20.0	22.2	26.7
16:1	6.2	T	T	T	1.0	2.5	1.4	T	3.3	T	T	T	6.2	1.3	2.2
18:0	15.8	6.1	5.6	5.4	5.5	11.1	8.1	5.5	9.0	3.9	5.2	5.6	9.4	17.5	11.4
18:1	19.5	3.2	T	T	2.7	6.7	4.0	2.1	8.6	T	T	1.0	6.1	4.6	5.5
18:2	13.1	1.3	T	T	1.7	4.6	2.2	T	2.0	T	T	1.0	3.3	5.7	2.7
22:0	5.0	17.5	18.4	17.6	16.1	7.5	9.7	11.0	15.4	19.5	15.3	17.6	11.1	12.1	11.2
22:1	T	T	T	T	T	1.6	3.8	T	1.0	T	3.6	T	1.7	T	T
23:0	5.5	11.4	11.1	11.3	17.5	5.1	6.1	6.8	12.3	13.7	10.0	11.4	6.9	6.6	8.5
24:0	3.9	16.0	15.7	14.0	19.8	6.9	9.4	11.2	11.1	19.6	14.7	13.3	8.2	7.6	7.2
24:1	3.0	16.9	24.3	31.4	18.7	7.5	12.3	25.5	13.6	22.9	28.1	32.7	8.2	11.7	17.3
24:2		7.1	9.2	5.0	3.5	3.2	4.9	4.2	2.7	9.5	13.1	5.6	4.2	4.8	3.4
26:0	2.5	T	T	T	2.0	1.2	T	T	1.7	T	T	T	4.3	1.7	T
ACL	18.6	21.2	22.0	22.1	22.0	18.6	19.5	20.4	20.6	22.4	22.4	22.2	19.6	19.9	19.9
24:1/24:0	0.8	1.1	1.5	2.2	0.9	1.1	1.3	2.3	1.2	1.2	1.9	2.5	1.0	1.5	2.4

[a] GL-4 was not found in rabbits fed diet I.
[b] T, trace (< 1%).

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TABLE 6. Percentage distribution of major fatty acids in rabbit RBC glycosphingolipids

	Glycosphingolipid															
	GL-1				GL-2				GL-3				GL-4			
Diet group	I	II	III	IV	I	II	III	IV	I	II	III	IV	I	II	III	IV
Fatty acid																
14:0	T[a]	T	1.3	T	1.7	1.6	3.2	2.9	T		T	T	T	1.9	3.8	T
16:0	16.5	21.4	17.6	18.2	11.6	12.6	17.7	16.0	4.4	4.3	5.9	7.1	10.6	14.8	18.9	15.3
16:1	2.1	T	1.6	T	T	T	3.6	2.5	T	T	T	T	T	2.5	5.8	2.5
18:0	14.7	23.2	25.5	30.8	3.0	6.9	6.6	6.4	1.4	1.3	2.1	1.7	3.4	7.4	5.8	5.0
18:1	14.5	8.2	9.8	12.8	1.7	2.4	5.6	5.7	T	T	1.7	T	2.5	5.3	7.3	5.7
18:2	19.4	6.8	5.4	5.5	T	1.3	T	1.5	T	T	T	T	T	T	T	T
22:0	8.8	9.9	7.9	7.0	5.9	8.1	6.3	7.6	5.2	6.8	7.1	7.3	5.3	7.3	5.3	6.6
22:1	2.5	2.0	2.1	3.9	14.1	3.1	1.7	3.0	2.2	1.5	3.3	2.0	2.6	4.6	1.2	4.8
23:0	2.4	4.3	3.6	2.3	3.4	1.9	2.3	4.3	3.3	3.1	3.2	2.6	3.6	1.9	T	1.1
24:0	2.6	7.6	6.2	4.0	5.1	7.4	6.3	5.9	4.6	5.8	7.3	7.3	5.8	5.0	5.1	5.3
24:1	5.1	9.8	13.0	10.9	34.8	26.7	25.1	35.5	56.2	49.2	50.4	63.1	43.6	23.7	26.5	33.9
24:2	3.0	4.6	4.8	T	12.1	13.3	9.8	5.2	18.4	24.0	17.3	6.6	16.0	11.9	10.4	6.1
26:0	2.2	T	T	1.3	2.8	1.9	4.7	2.3	1.4	2.6	1.7	2.1	3.0	6.2	5.1	5.3
ACL	19.0	19.6	19.6	19.2	21.8	21.1	20.8	21.0	23.2	23.4	23.1	23.1	22.3	21.2	20.6	21.4
24:1/24.0	1.9	1.3	2.2	2.7	6.8	3.6	4.0	6.0	12.2	8.5	6.9	8.6	7.5	7.7	5.2	6.4

[a] T, trace (< 1%).

plasma were evident in three ways. First, there were fewer shorter-chain fatty acids, generally 16:0 and the 18-carbon acids, but there was an increase in the longer-chain fatty acids, mainly those with more than 22 carbons. This change was reflected in the increased average chain lengths for the experimental groups. The only exception to this was plasma GL-2. No comparison could be made for aorta GL-2 and plasma GL-4. Second, the ratio 24:1/24:0 was nearly always increased and never lower in the samples from the experimental animals. Finally, the concentration of 24:2 was increased for all experimental samples except plasma GL-2 in animals fed diet II. These trends were either not seen or were small or inconsistent in RBC glycosphingolipids. For aorta GL-1 there was also an increase in total percentage of unsaturated fatty acids for the experimental rabbits. The values were 28.8, 35.4, 39.0, and 43.2 for diets I, II, III, and IV, respectively.

Correlations of the fatty acid distributions in glycosphingolipids

An attempt was made to compare fatty acid distributions in a different manner. Correlations were determined between all pairs of fatty acids in the two distributions to be compared, and an average was taken of these correla-

TABLE 7. Correlations among glycosphingolipid fatty acid distributions among the four diets

	Aorta			Plasma			RBC		
Diet	II	III	IV	II	III	IV	II	III	IV
					GL-1				
I	0.55	0.40	0.44	0.40	0.26	0.23	0.80	0.76	0.76
II	1	0.97	0.95	1	0.97	0.92	1	0.98	0.93
III		1	0.96		1	0.98		1	0.96
					GL-2				
I	0[a]	0	0	0.48	0.58	0.79	0.92	0.86	0.91
II	1	0.95	0.93	1	0.93	0.72	1	0.95	0.93
III		1	0.94		1	0.81		1	0.95
					GL-3				
I	0.92	0.90	0.83	0.84	0.76	0.81	0.99	1.0	0.97
II	1	0.99	0.95	1	0.96	0.94	1	0.99	0.93
III		1	0.97		1	0.96		1	0.97
					GL-4				
I	0.82	0.82	0.80	0	0	0	0.90	0.88	0.93
II	1	0.98	0.99	1	0.74	0.75	1	0.96	0.95
III		1	0.99		1	0.95		1	0.94

[a] Zeros indicate one of the lipids was not found.

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TABLE 8. Correlations of glycosphingolipid fatty acid distributions between aorta and plasma and aorta and RBC within a diet group

	Diet			
	I	II	III	IV
		GL-1		
Aorta to plasma	0.83	0.93	0.96	0.94
Aorta to RBC	0.65	0.82	0.68	0.49
		GL-2		
Aorta to plasma	0[a]	0.75	0.89	0.87
Aorta to RBC	0	0.50	0.88	0.78
		GL-3		
Aorta to plasma	0.81	0.86	0.77	0.86
Aorta to RBC	0.18	0.42	0.48	0.65
		GL-4		
Aorta to plasma	0	0.74	0.77	0.81
Aorta to RBC	0.16	0.54	0.64	0.51

[a] Zeros indicate one of the lipids was not found.

tions. This average is the correlation of the fatty acid distributions in any two lipids.

Table 7 presents the correlations among the fatty acid distributions in a particular glycosphingolipid from a particular tissue. These correlations indicate changes in the fatty acid brought about by the various high cholesterol diets.

Correlations among the glycosphingolipid fatty acids from animals fed diet I and the experimental groups were always lower for aorta and plasma than for RBC. This is consistent with the specific trends noted above. The correlations among the three experimental groups were higher than any correlation with the control group with one exception, plasma GL-2 of group IV.

Table 8 presents the correlations between the glycosphingolipid fatty acid distributions in aorta and plasma along with aorta and RBC, within a given group. All correlations between aorta and plasma are higher than between aorta and RBC except for GL-4, which was not found in plasma.

GLC–mass spectroscopy of 24:2

In order to further confirm the identity of 24:2, a sample of methyl esters of GL-3 was prepared from commercially available RBC. This sample was subjected to GLC–mass spectroscopy using an OV-1 column. Since an OV-1 column does not effectively resolve 24:2 and 24:1 methyl esters, the mass spectra were obtained at two positions on the leading edge of the GLC peak. The relative quantities of *m/e* values important to the identification of 24:2 as well as 24:1 are listed in Table 9. The base peak of both spectra was *m/e* 55, which was given the relative intensity of 999. Both mass spectra have *m/e* peaks expected of both esters. The first spectrum has a relative greater abundance for 24:2 (parent peak, 378).

TABLE 9. Relative abundance of some ions from a GLC peak expected to be a mixture of 24:1 and 24:2 methyl esters

m/e			Relative Quantities	
24:1		24:2	1st Spectrum	2nd Spectrum
	74		317	406
	87		196	268
	91		65	32
348 (M − 32)			178	309
		346 (M − 32)	51	26
349 (M − 31)			85	156
		347 (M − 31)	69	41
380 (M)			17	24
		378 (M)	111	53

DISCUSSION

The glycosphingolipids were identified on the basis of four criteria: The methods of extraction and purification were the same as used for human (4) and pig blood glycosphingolipids (3, 8); TLC comparison was made with pig glycosphingolipids (3); hexoses were identified; and finally, hexose to fatty acid ratios were determined. All data are consistent with the substances being mono-, di-, and triglycosyl ceramides and globoside.

Differences in TLC patterns between rabbit and pig glycosphingolipids are probably due to the presence of hydroxy acids in pig and the different relative concentrations of long- and short-chain normal fatty acids. Rabbit glycosphingolipids have more longer-chain fatty acids which cause them to migrate slightly faster on TLC plates. GL-3 from pig blood migrated as far as rabbit GL-3 but was very faint. The GL-4 band from rabbit blood was also very faint, but discernable, and corresponded to the GL-4 band of pig blood. The TLC band from rabbit glycosphingolipids that moved slower than GL-4 did not correspond to any lipid present in the pig sample. Identification of this substance was not satisfactory, and therefore data for it were not included in this paper. Eto et al. (9) have found a pentaglycosylceramide from stroma of RBC.

The decreases in the ratio 18:0/18:1, as seen previously for cerebrosides (1, 2), is distorted by the noticeable reduction in the 18-carbon acids in the lipids from the current experimental animals. This ratio change was transferred, however, to the 24-carbon acids as expressed in the increased 24:1/24:0 ratio observed for aorta and plasma glycosphingolipids of the experimental animals. This transfer to the 24-carbon acids is probably by an increased rate of chain elongation.

Increases in 24:2 in glycosphingolipids from rabbits fed the experimental diets are consistent with general increases in unsaturation and the increases in 18:2 seen previously (1, 2). The least deviation from normal in the concentration of 24:2 was for lipids from animals fed diet IV,

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whereas these same lipids had the greatest deviation from normal for the 24:1/24:0 ratio. This may well be a reflection of the high concentration of 18:2 in the cottonseed oil of diets II and III. The distributions of the major fatty acids are (*a*) cottonseed oil, 16:0, 27.3%; 18:0, 2.0%; 18:1, 18.3%; and 18.2, 50.5%; and (*b*) lard, 16:0, 4.0%; 18:0, 13.5%; 18:1, 42.9%; and 18:2, 9.0% (10). These differences may have affected the glycosphingolipid fatty acid distributions to some extent. However, the differences in the fatty acid compositions of the lipids are unlikely to be simply a reflection of the intake of different fatty acids. Thus, diet III generally caused a greater change than diet II with the same fatty acids.

It is interesting that the quantities of the various glycosphingolipids from aorta and plasma follow a somewhat different trend with diet than the fatty acids; i.e., animals fed diet III generally had the greatest deviation from normal with respect to quantity of glycosphingolipids, and animals fed diet II had the least deviation from normal.

The increased ACL for lipids from animals fed the high cholesterol diets was not observed in the previous study in which only cerebrosides were analyzed (2). However, the decrease in the ratio (16:0 + 16:1)/(18:0 + 18:1) previously observed was probably a limited form of ACL increase. The present study was for a longer duration and hence the ACL increase may have extended past 18:0 and 18:1 to longer acids.

The fatty acid distribution for GL-1 from aorta of control animals is similar to that reported earlier for rabbits (2), and it is also similar to "normal" human aorta (1) except that the current samples had substantially more 14:0. The increase in total unsaturated fatty acids from the experimental animals is also much the same as that seen in cerebrosides from human (1) aortic plaques and from atherosclerotic rabbit and pigeon (2).

Considering the correlations among the GL-1 fatty acids from each of the sources studied, a relationship is seen with the composition of the various diets; i.e., the greater the amount of cholesterol in the diet or the more saturated the fat in the diet, the lower the glycosphingolipid fatty acid correlation with the control animal fatty acids. For the lipids besides GL-1, the relationship holds only for the fatty acids of GL-3 from aorta and plasma.

The various trends and correlations point out that aorta and plasma glycosphingolipids were more affected by the experimental diets than were the lipids of RBC. Also, in general, the changes in aorta and plasma fatty acids brought about by the high cholesterol diets were similar. Similarities between aorta and plasma are reinforced by the correlations in Table 8. These data, coupled with the lack of evidence for cerebroside synthesis in aorta,[2] lead us to conclude tentatively that aorta glycosphingolipids are transferred there from the plasma.

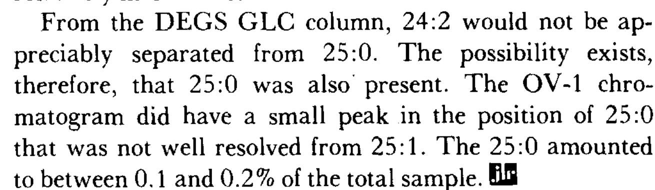
[2] Coles, E. Unpublished results.

Identification of 24:2 in blood glycosphingolipids was previously done by hydrogenation of a sample of methyl ester and observing the disappearance of peaks corresponding to the unsaturated fatty acids (3). As another means of confirming the identity of 24:2, a sample of GL-3 from rabbit RBC was analyzed by GLC–mass spectroscopy.

The GLC column liquid phase used for the fatty acid methyl ester analyses (DEGS) is not sufficiently stable for use with mass spectroscopy. Therefore, a different liquid phase, which was less satisfactory in terms of fatty acid methyl ester separation, had to be used. The chromatogram gave distinct peaks for all of the methyl esters identified using DEGS except 24:2. The 24:2 was not resolved from 24:1.

Since the elution of methyl esters from OV-1 depends primarily on their molecular weights, it was expected that if 24:2 were present it would be mostly at the leading edge of the very large 24:1 peak. Therefore, two mass spectra were obtained from about the first one-fourth of this peak. Major m/e peaks for methyl ester identification are given in Table 9. While the spectra were consistent with the presence of 24:1, it is concluded that 24:2 was also there because: (*a*) m/e peaks for the molecular ion of 24:2 were present in both spectra; (*b*) the expected m/e peaks for M − 31 and M − 32 were present; and (*c*) the relative intensities among these peaks were very similar to those observed and pointed out for 18:2 by Hallgren, Ryhage, and Stenhagen (11), i.e., a strong molecular ion peak and an M − 31 peak larger than for M − 32. These peaks, m/e = 378, 347, and 346, are all smaller in the second spectrum. Thus, the GLC peak became richer in 24:1. Corresponding m/e peaks can easily be found for 24:1, and the same relative intensities hold as were reported for 18:1 (11). In this case, the molecular ion was weak and M − 31 was about one-half of M − 32, as in methyl oleate. As expected, the peaks for m/e = 380, 349, and 348 were all larger in the second spectrum.

Consistent with GLC separation of 24:2 and 24:1, the first mass spectrum had relatively more 24:2 than the second, as can be seen by the differences in m/e intensities between the two spectra. Furthermore, it has been pointed out (12) that the peak at m/e = 91 increases in intensity with increasing unsaturation. The observed decrease for this peak in the second spectrum is an indication of a change in composition of this single GLC peak, i.e., from relatively more to less 24:2.



From the DEGS GLC column, 24:2 would not be appreciably separated from 25:0. The possibility exists, therefore, that 25:0 was also present. The OV-1 chromatogram did have a small peak in the position of 25:0 that was not well resolved from 25:1. The 25:0 amounted to between 0.1 and 0.2% of the total sample.

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