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Concentration and Fatty Acid Distribution of Aortic Cerebrosides in Cholesterol-Fed and Normal Rabbits

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CONCENTRATION AND FATTY ACID DISTRIBUTION OF
AORTIC CEREBROSIDES IN CHOLESTEROL-FED AND NORMAL RABBITS

by
Robert V. P. Tao

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INTRODUCTION

A number of studies have indicated the existence of phospholipids in atherosclerotic lesions of cholesterol-induced rabbits (1-9). However, the presence of aortic cerebroside has not yet been investigated. In order to establish an adequate base line for further studies on rabbit aortic cerebroside, a systematic study was carried out on the effects of feeding an atherogenic diet to rabbits for various lengths of time. The present thesis furnishes data on the concentration of aortic cerebroside and their fatty acid distribution obtained from rabbits fed normal or atherogenic diets.

The rabbit has, for some time, been the classical experimental animal for atherosclerosis research, and it was the first animal in which the fatty arterial lesions were experimentally induced. Early studies of Saltykow (10) and Ignatowski (11) indicated that diets of milk, meat and eggs could produce atherosclerosis in rabbit. Anitschkow (12) demonstrated that atherosclerosis was actually induced in rabbits by cholesterol feeding. Since then, the rabbit has been commonly and extensively used as the experimental animal for this purpose.

A dietary regimen of cholesterol given in amounts of 0.5 to 1.0 g per kg daily for 50 days is sufficient to induce moderate atheromatosis in rabbits (13). The common procedure consists of dissolving and suspending cholesterol and other additives in an oil and mixing this with a regular rabbit diet. Newman and

Zilversmit (14) used a diet, containing 1 g of cholesterol suspended in 2.8 g cottonseed oil per 100 g Purina rabbit chow, in their study of aortic phospholipids in rabbits. In the present study, this diet was followed since there are similarities between cerebroside and phospholipids.

Besides cholesterol, many substances have been implicated in atherosclerosis. Dietary carbohydrate has been shown to affect the severity of atherosclerosis of rabbits on cholesterol-fat diets. Cholesterol-lactose-fed rabbits become more atherosclerotic than cholesterol-sucrose-fed rabbits (15, 16). Cholesterol diet containing no fat will cause more severe atherosclerosis than the cholesterol plus fat diet (17-19). Kritchevsky has also demonstrated that increased atherogenicity can be induced by an increased level of free fatty acids in the diet (20).

Frequent criticisms indicate that the cholesterol-induced atherosclerosis of the rabbit does not resemble that of human atherosclerosis (21-23). However, Constantinides et al. (24) have produced humanlike arterial lesions of the advanced type (fibrosis, calcification, ulceration and thrombosis) by subjecting rabbits to repeated cholesterol feedings as well as by treating them with deoxycorticosterone, vitamin D and thrombin infusions before sacrificing. Humanlike lesions in rabbits have also been produced in 8 to 10 months by Hass et al. (25) using a combination of cholesterol feeding and viosterol administration.

Atherosclerosis affects man at the distal part of the aorta more than at the proximal part, while either end of the aorta may

be affected in animals. The proximal aorta is the site of atherogenesis in chicken, pigeon, cow and young pig. The distal part is more affected in the buzzard, turkey, dog, cat, elephant, older pig and man. The differences between human and animal atherosclerosis have been reviewed by Adams (26). In contrast to man, the primary site of aortic lesion formation in rabbits is at the thoracic aorta arch rather than at the abdominal aorta (27).

Atherogenesis in the cardio-vascular system of cholesterol-fed rabbits has been well discussed by Prior et al. (28). After being on the diet (1 g per day) for one month, the aorta does not show any lipid deposit; however, microscopic evidence indicates the accumulation of foam cells on the internal elastic membrane beneath an intact endothelial cell surface. After 2 months of feeding, yellow plaques, which develop from gross lesions, are noted along the aortic intima. Microscopically, this intimal change is produced by both intracellular and extracellular lipid suggesting degenerative changes within the intima. At the end of 3 months, all changes observed one month previously are exaggerated in the animals. The aortic intima is indicative of surface fibrosis and the extension of the fatty process into the media is apparent. At this stage, the aortic lesions are very similar to human-type plaques (thickened elevations over the intima, yellowish-grey in color, with an uneven nodulated surface). By the end of 4 months of cholesterol feeding, there is a progressive increase in the fibrous and smooth muscle elements of the plaque. No significant changes are observed at the end of 5 months. No complications (calcification, ulceration and

thrombus formation) are noted when this cholesterol-fat diet is continued for one year. However, Constantinides et al. (24) concluded from their studies that rabbit lesions could develop advanced features similar to those in man if they were allowed to age for 2 years after establishment. Moreover, the development of complications in addition to the advanced features could be achieved within a much shorter time if the rabbits were subjected to repeated lipemic or other treatments.

Prior et al. (28) stated that the distribution of these lesions in the rabbit is different from that in man since the small intramyocardial branches in the rabbit are affected and the large epicardial coronary arteries are spared. As the dietary regimen continues, the entire lumens of the intramyocardial arteries are transformed into a homogeneous lipid mass. According to Clarkson (27), the pulmonary artery is not involved in human atherosclerosis, but the pulmonary arteries and veins show marked luminal occlusion by masses of lipid-filled histocytes in the cholesterol-fat-fed rabbit.

The accumulation of cholesterol in the aorta of cholesterol-fed animals has been discussed by many investigators. However, the fact that a major part of the atheroma cholesterol infiltrated from the blood was unknown until Newman and Zilversmit (29) performed isotope studies in vivo on the aortic wall of the cholesterol-fed rabbit. Their studies showed that only a very small amount of cholesterol was synthesized within the arterial wall itself.

Unlike cholesterol, the major part of the phospholipid in atherosclerotic rabbit aorta appears to be synthesized by the aorta itself and is not deposited from the plasma (1). An in vitro study by Stein and Stein (2) shows that the major part of the fatty acid exposed to the aortic wall of the rabbit is incorporated into phospholipid. The major phospholipid is sphingomyelin, and lecithin is the next most abundant (14). Gas chromatography reveals pronounced differences in the pattern of phospholipid fatty acid composition of plasma and aortic lesions of the cholesterol-fed rabbits (3). These differences between plaque and plasma phospholipids further suggest that endogenous synthesis is the main source of phospholipid in the atherosclerotic artery. Histochemical studies show that phospholipid increases in the early stages of cholesterol-induced rabbit atheroma (4) and the phospholipid-cholesterol ratio remains above 1. This ratio falls to as low as 0.23 at the later stage of atherogenesis due to the increase of cholesterol. This suggests that synthesis of phospholipid is an early defense mechanism of the arterial wall against infiltration of cholesterol. According to Zilvermit (5), phospholipid may disperse infiltrated cholesterol of the arterial wall. Adams et al. (6) further demonstrated that the addition of phospholipid to a subcutaneous implant of cholesterol prevented fibrosis and giant cell formation and promoted rapid dispersion of cholesterol. The protective role of phospholipid in atherosclerosis may well be to facilitate the transport of high surface tension lipids, such as cholesterol and triglycerides, by solubilizing and suspending them as micelle or lipoprotein in plasma or tissue fluid (26).

Day (7) studied the histological distribution of phospholipid in aortic lesions and concluded that foam cells are involved in phospholipid synthesis. A direct study of lipid synthesis by the foam cells was made possible by Day et al. (8) with the development of a method for isolating foam cells from the atherosclerotic lesion. P^{32} -phosphate is incorporated into phospholipid, mainly phosphatidyl choline and phosphatidyl inositol, by these isolated foam cells when incubated in vitro. The pattern of incorporation of P^{32} -phosphate into the individual phospholipid by the foam cell is similar to the incorporation of P^{32} -phosphate into phospholipid by arterial intima incubated in vitro under the same conditions. This does not necessarily imply that the foam cells are completely responsible for the phospholipid synthesis in the arterial wall, although such possibility should not be overlooked. These results are indicative of a synthesis of phospholipid in the atherosclerotic artery.

Foam cells are round, mononuclear, wandering cells resembling large lymphocytes, monocytes or polyblasts (9). Their nuclei are dark and round at first, becoming pale later; the quantity of basophilic protoplasm increases with time and minute lipid drops accumulate in it. These lipid drops are stored until the cells are finally transformed into large macrophages, with all of their protoplasms being occupied by lipid droplets. According to Anitschkow (9), these foam cells appear during the first and second week of daily cholesterol feeding. After 2 to 3 months of feeding, these lipid macrophages become scarce. Either they have disintegrated and contributed

to the atheromatous mass, or they have undergone atrophy. Thus, a high phospholipid-cholesterol ratio observed at the beginning of atherogenesis in rabbits may well be ascribed to the formation of foam cells. As the atherogenesis progresses, more plaques are produced and fewer foam cells are present. This may impair the phospholipid synthesis (of the aortic wall) so that the quantity of cholesterol will eventually outstrip that of phospholipid and result in a low phospholipid-cholesterol ratio.

Although much research on the phospholipids of cholesterol-induced atherosclerotic lesions in rabbits (1-9) has been published, no study of cerebroside in rabbit aorta has been reported. As a polar lipid, cerebroside may play an important role similar to phospholipids in the defense mechanism of the atherosclerotic aorta. The presence of cerebroside in human aorta has been noted (30, 31). More cerebroside was found in the atherosclerotic aortas than in the normal aortas (31).

In this study the presence and amount of aortic cerebroside in both the normal and cholesterol-fed rabbits were investigated, and the fatty acid distributions of these cerebroside determined. The purpose was to find correlations between the fatty acid patterns or the amounts of cerebroside, and the degree of atherosclerosis.

MATERIALS AND METHODS

Materials

New Zealand albino male rabbits, weighing approximately 900 g each, were distributed into various groups as shown in Table 1. The rabbits were obtained at various times during the course of study. However, rabbits of batch I or II were always raised at the same time. Batch I and II were duplicates for each time point studied. The experimental rabbits received Wayne rabbit chow supplemented with 1 g of cholesterol suspended in 2.8 g cottonseed oil per 100 g chow. The control rabbits received plain Wayne rabbit chow. All animals were fed water and chow ad libitum. At time intervals of one, two and four months, rabbits were sacrificed and whole aortas removed. Aortas from the same group were pooled and stored at -20° in 0.9% sodium chloride solution until used.

Table 1

Experimental design showing distribution of rabbits among control and experimental groups

Time of Diet	No. of Aortas		Total
	Normal Diet	Expt. Diet	
1 month - I	4	4	8
II	3	3	6
2 months - I	3	3	6
II	3	3	6
4 months - I	4	6	10
II	2	3	5

All reagents were of analytical grade, and all solvents were redistilled before use. Solvent mixtures are expressed in terms of volume. Florisil (Fisher Scientific Company, Chicago) was used for column chromatography and Silica Gel G (Brinkman Instruments, Inc., Westbury, L.I., N.Y.) for thin-layer chromatography (TLC).

Extraction of Lipid

Lipids were extracted (32) from the pooled aortas of each group with chloroform-methanol 2:1, and the suspension was filtered through a sintered glass funnel. One-sixth of the original volume of chloroform-methanol 2:1 was utilized to rinse the homogenizer and filter through the glass funnel. The residue was then washed again with an equal volume of chloroform-methanol 1:2 (33). Another addition of chloroform to the filtrate was required to restore the chloroform-methanol ratio to 2:1. The filtrate was washed with one-fifth volume of 0.1 M KCl and the lipid solution (lower layer) was washed again with chloroform-methanol-0.1 M KCl 3:48:47 (32). Solvent was removed from the lipid solution on a rotatory evaporator. Near the end of the evaporation, anhydrous benzene-ethanol 1:1 was added to remove water and avoid splashing. The process was repeated until a constant weight of the crude lipid had been reached.

Isolation of Cerebrosides

Cerebrosides were isolated from the crude lipid extract in four steps: (1) Florisil column chromatography, (2) alkaline methanolysis, (3) Florisil column chromatography, and (4) preparative thin-layer chromatography-(TLC).

The Florisil contained 8% water by weight, and a 40:1 weight ratio of Florisil to lipid was used. A volume of 25 ml per g Florisil of hexane-ether 8:2 was used to remove the cholesterol and triglycerides in step one. This is designated as the first fraction. The second fraction from the Florisil column was eluted with chloroform-methanol 3:1 (34) in the amount of 35 ml per g Florisil which contained the cerebrosides. The alkaline methanolysis was performed on the chloroform-methanol eluate (35) to remove the glycerophosphatides. The resulting lipid solution was then passed through a second Florisil column to remove the methyl esters formed in the previous step. Elution was the same as in step one. Finally, the chloroform-methanol eluate was purified by preparative TLC using Silica Gel G, 0.6 mm thick with chloroform-methanol-water 24:7:1 (36) as developer. To render the bands visible, the plates were sprayed with bromthymol blue. Cerebrosides, galactosylceramide from bovine brain (Applied Science Laboratories, State College, Pa.) and/or N-stearyl-1-O-D-glycosylceramide (Custom Synthesis, Miles Laboratories, Elkhart, Indiana) acted as markers.

The bands on the TLC plate were scraped and the silica gel was packed into small glass columns. Lipids were recovered by passing chloroform-methanol 2:1 through the silica gel. A volume of 40 ml per g of silica gel was used (37). The eluate was washed with one-fifth volume of water to remove the bromthymol blue. This was accomplished by mixing on the Vortex mixer vigorously for a few minutes, and centrifuging to separate the two liquid phases. The lower layer was washed again with chloroform-methanol-water 3:48:47 (32). Solvent was

removed from the lower layer on a rotatory evaporator and the residue weighed. These were cerebrosides.

Preliminary experiments indicated that the cerebrosides of both the cholesterol-fed and the normal rabbits contained no appreciable amount of the fatty acid 22:1. Hence, methyl erucate (Applied Science Laboratories, State College, Pa.) was employed as an internal standard. A quantity equivalent to 8% of cerebroside weight of methyl erucate was added to the cerebrosides. The fatty acids are denoted by the carbon chain length, colon, and the number of double bonds. An h indicates a hydroxy fatty acid.

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 at 75° for 17 hours. The resultant methyl esters were extracted with five 1 ml aliquots of hexane (38). The methyl esters were purified by preparative TLC on Silica Gel G with hexane-diethyl ether 85:15 as the developing solvent. Methyl esters of 23:0 and 20h:0 fatty acids served as markers. Bands were made visible with bromthymol blue, and were subsequently scraped off the plate within one hour after developing to avoid loss of short chain esters by evaporation. The unsubstituted and 2-hydroxy esters had R_f values of 0.55 and 0.13 respectively. A suspension of the silica gel was made with diethyl ether, and packed into a small glass column. The esters were eluted with diethyl ether using 40 ml per g of silica gel.

The 2-hydroxy esters were acetylated with 0.025 ml of a solution containing 4 mg p-toluene sulfonic acid in 1 ml of iso-propenyl acetate (Eastman Organic Chemicals, Rochester, N.Y.) at 60° for 30 minutes in a tightly sealed tube.

Gas-Liquid Chromatography (GLC) of Fatty Acid Esters

An F and M model 402R-00, dual column, flame ionization detector, gas chromatograph was used for methyl ester analyses. The glass columns were U-shaped. All ester samples were chromatographed on a pair of 6 ft x 3 mm columns packed with 6% polydiethylene glycol succinate on 80-100 mesh Diotoport S (F and M Scientific, Avondale, Pa.). The unsubstituted methyl esters were chromatographed between 160° and 220° with a temperature gradient of 3°/min. The 2-acetoxy methyl esters were chromatographed between 200° and 220° with a temperature gradient of 1°/min. Standards KD and KF (Applied Science Laboratories, State College, Pa.) were chromatographed periodically. These standards, together with 2-acetoxy methyl esters of 18h:0, 19h:0 and 22h:0, served for identification. The composition of the standards corresponded to the stated values with a relative error of 5% or less. Duplicate chromatograms of esters had a relative error of 5% or less for major components (> 5%), except for 22:0 (14%) of the first 2-month control, 18:1 (10%) and 23:0 (10%) of the first 4-month control, 16:1 (8%) and 23:0 (6%) of second 4-month control, and 24:0 (6%) of the first 4-month experimental. Plots of relative retention time versus carbon number were utilized in the identification of substances not represented in the standards. Area calculations were based on measurements of peak height and width at half the peak height.

RESULTS AND DISCUSSION

New Zealand albino rabbits were distributed into various groups as shown in Table 1. The experimental group received Wayne rabbit chow with cholesterol suspended in cottonseed oil, and the control group received plain Wayne rabbit chow. Rabbits from both groups were kept for one, two or four months before they were sacrificed. Whole aortas were removed and pooled for each group in order to obtain a sufficient amount of lipids for analysis. Lipids were extracted from the tissue samples and cerebroside isolated according to the procedure listed under Materials and Methods.

Total Lipid

Total lipid values are expressed as weight percentages of both the dry and wet tissue (Table 2). Compared to the control levels, the amounts were found to be greater in the experimental groups of 4-month rabbits. On the contrary, the 2-month crude lipid values were essentially the same except that one of the control batches had somewhat more crude lipid. Comparisons should be made between animals raised at the same time; i.e. control I and experimental I. The 2-month animals were all raised at the same time, and therefore, the I and II designations are arbitrary. Significant variances in weights were recorded between the two batches of rabbits at four months. The average weight of batch-I rabbits was about 2000 g while those of batch-II weighed about 1250 g. This discrepancy cannot be explained at this time.

Table 2
 Concentration of Aortic Lipids and Cerebrosides from Cholesterol-Fed and Normal Rabbits

	1 Month				2 Months				4 Months			
	Control		Experiment		Control		Experiment		Control		Experiment	
	I	II	I	II	I	II	I	II	I	II	I	II
Crude Lipid Percent of Wet Tissue	10.7	6.3	12.6	6.0	11.7	15.9	10.4	11.8	16.2	12.2	20.1	15.3
Crude Lipid Percent of Dry Tissue	38.44	28.72	41.74	23.20	39.63	—	36.90	39.72	50.35	39.40	55.08	46.94
Cerebrosides Percent of Total Lipid	0.041	0.063	0.056	—	0.035	0.047	0.069	0.135	0.020	0.078	0.027	0.062
Cerebrosides Percent of Dry Tissue	0.015	0.018	0.023	—	0.014	—	0.025	0.053	0.010	0.030	0.015	0.029

Percent Cerebroside

The percentage of aortic cerebroside in normal and cholesterol-fed rabbits at various times can be seen in Table 2. An average molecular weight of 733.60 for cerebroside, which includes an average chain length of 18.40 for the fatty acid, was used to determine the amounts of cerebroside. The amount of fatty acid therefore represents 37.3% of the cerebroside weight. The cerebroside concentration varied from 0.020% to 0.135% of the total lipid. This amount is between 0.01% and 0.05% of the dry tissue weight. Dry tissue weight as used here was the dried residue after lipid extraction plus the weight of crude lipid. Because of the loss of the sample during acid methanolysis, figures were unavailable for the duplicate set (II) of the 1-month experimental group. Furthermore, due to the accidental loss of the protein residue after lipid extraction, the percent of cerebroside and total lipid in dry tissue could not be calculated for the duplicate set of 2-month control group.

The amounts of cerebroside found were low in comparison to those first reported (31) from human aortas (2.2% to 3.4% of the total lipid). The low values could in part at least be explained by the fact that three bands were always present ahead of the cerebroside on preparative TLC. Their R_f values were 0.70-0.75, 0.80-0.85 and 0.90-0.95. These substances have not been identified. Nevertheless, it is possible that these bands were included in the reported percentages of cerebroside by other investigators, which would result in a higher value than reported here. Recent results from human aorta (39, 40) give amounts of cerebroside similar to those reported here.

The main feature discernible for the amounts of cerebroside was the increased percentage of cerebroside for the 2-month experimental group. There was approximately 2 to 5 times more cerebroside content in the experimental group as compared to the controls at this time. The other time points showed little if any difference between the experimental and control groups. This suggests some sort of dose-response phenomenon whereby the highest increase of cerebroside percentage occurs at the end of two months on the atherogenic diet with subsequent decrease at four months. Of course, one should bear in mind that this study involves only three different time points during the course of atherogenesis. More points as well as better controlled animals are required for a close estimation of the peak response. It may be somewhere between one and two months or two and three months. This considerable change in the cerebroside with time on an atherogenic diet may represent an important feature in atherogenesis.

The amounts of cerebroside can be seen to increase from control group to experimental group throughout the study; except for the slightly lower value of the 4-month experimental group-II compared to its control. This may suggest that, at four months, cerebroside have become a smaller constituent of the aortic lipids, and the amount in control and experimental animals are about the same.

Fatty Acid Distribution

The normal fatty acid distribution of the rabbit aortic cerebroside is shown in Table 3. The fatty acid patterns have several,

Table 3
 Normal Fatty Acid Composition of Aortic Cerebrosides from Cholesterol-Fed and Normal Rabbits
 Fatty Acid Values in Percent of Total Normal Fatty Acids

Normal Fatty Acid	1 Month			2 Months				4 Months			
	Control		Experiment	Control		Experiment		Control		Experiment	
	I	II	I	I	II	I	II	I	II	I	II
14:0	1.3	1.9	1.0	2.1	3.0	3.1	2.6	2.0	3.2	0.8	2.8
14:1	—	—	—	—	—	0.8	0.6	2.9	5.0	—	0.6
15:0	1.2	1.3	1.5	1.2	1.1	1.2	1.1	2.1	2.0	0.8	0.9
15:1	—	—	—	—	—	0.3	0.4	1.2	0.6	—	0.4
16:0	22.4	25.5	26.9	32.7	32.8	24.3	31.6	27.8	23.4	22.4	23.3
16:1	2.9	4.3	3.0	3.3	4.4	4.3	4.8	4.7	5.8	2.0	4.3
17:0	1.1	1.3	2.0	1.2	1.4	0.8	1.3	1.3	1.2	1.1	1.3
17:1	—	0.6	—	—	—	0.6	0.5	2.4	0.7	0.6	0.7
18:0	13.4	16.4	17.8	18.8	17.9	12.1	13.0	14.2	14.1	8.5	19.1
18:1	11.7	14.6	17.4	14.5	17.7	24.2	26.3	21.9	22.1	9.9	30.3
18:2	3.0	2.9	6.9	3.0	7.5	3.9	7.2	—	2.6	3.8	3.2
20:0	6.0	2.2	2.0	2.4	1.6	1.6	—	1.9	2.2	4.1	1.2
20:1	3.1	—	—	—	—	1.0	1.3	—	—	—	0.7
21:0	—	—	—	—	—	0.9	1.0	1.0	2.0	1.0	0.5
21:1	—	—	—	—	—	1.2	0.6	—	—	—	0.3
22:0	4.0	2.7	3.2	5.5	3.5	3.9	1.1	3.9	1.7	9.0	2.0

Table 3 Continued

Normal Fatty Acid	1 Month			2 Months				4 Months			
	Control		Experiment	Control		Experiment		Control		Experiment	
	I	II	I	I	II	I	II	I	II	I	II
23:0	15.7	11.0	7.4	5.7	2.0	3.9	1.1	7.8	10.9	9.0	5.4
23:1	—	1.4	—	—	—	—	—	—	—	—	—
24:0	5.7	3.6	3.7	5.9	3.8	4.9	1.0	4.7	1.2	10.9	1.9
24:1	4.4	3.2	2.3	3.8	2.3	4.5	1.2	—	1.4	8.8	0.9
25:0	—	—	—	—	—	—	—	—	—	0.8	—
25:1	—	1.1	—	—	—	—	—	—	—	1.9	—
26:1	—	1.5	1.1	—	—	—	—	—	—	—	—
27:1	4.8	4.4	3.5	—	—	2.8	2.7	—	—	3.8	—
18:0/18:1	1.15	1.12	1.02	1.30	1.01	0.50	0.49	0.65	0.64	0.36	0.63
16:0/18:0	1.67	1.56	1.52	1.74	1.83	2.00	2.43	1.96	1.66	2.65	1.22
% Total Unsaturated Fatty Acid	29.7	34.0	34.7	24.6	31.8	43.7	45.9	33.1	38.1	30.7	41.4

common, characteristic features. There were almost no hydroxy fatty acids. Of the normal acids, 16:0, 18:0 and 18:1 generally predominate in all samples, and constitute together about 60% of the total normal acids. Most samples had little variation between the duplicates for the major acids. The 4-month experimental group-I was the most notable exception. The 18:0 and 18:1 values were low as compared to any other group. This group also had an unusually high percentage of longer chain fatty acids (22:0, 23:0, 24:0 and 24:1). Several changes were noted in the fatty acid patterns of cerebrosides with atherosclerosis.

The concentration of 15:0 was about the same (1.20) for both the control and experimental groups in 1-month and 2-month-fed rabbits. As for the 4-month rabbits, the controls had a slightly higher value (2.00) and the experimental group was slightly lower (0.90). These changes are small in comparison to other values, and may not be at all significant.

The most striking aspect of fatty acid analysis was the inverse ratio of 18:0/18:1 observed at two months. In the control group, there was less oleic acid than stearic acid while the converse was seen in the experimental group. The oleic acid was greater than the stearic acid by 2-fold in the experimental group. The ratios of 18:0/18:1 are listed in Table 3. This ratio remained rather constant (around 1.1) for all the 1-month rabbits and the 2-month control group, but dropped to 0.5 for the 2-month experimental group. This increased amount of oleic acid may account for the increased amount of cerebrosides observed for the same group during this period. At the end of

four months, this ratio had decreased somewhat to 0.65 for the controls, and increased to 0.86 for the experimental group. This suggests that the ratios became the same for both groups.

Total percents of unsaturated fatty acids are listed in Table 3. There was no significant difference between the controls and experimentals, except the 2-month animals. The experimental rabbits had more of the unsaturated fatty acids than the corresponding controls. This includes the increased oleic acid as well as several other mono-unsaturated fatty acids. The 2-month results are in general agreement with the observations made by Coles (39) and Foote and Coles (40) on human aortic tissue.

No appreciable differences were found for the ratio of 16:0/18:0 in all the 1-month rabbits. The 2-month experimental showed a higher ratio than the controls. Likewise, the ratio was higher in the 4-month experimental-I than in its controls. However, the duplicate set (II) failed to confirm this result. Again, the extreme differences of the body weights within the duplicate set could be the cause for such inconsistency. It is of interest to note that the ratio of 16:0/18:0 increases for both the control and experimental groups from one to two months, but the cholesterol-fed group shows the greater increase.

There was considerable variation in the distribution of linoleic acid (18:2) from all groups.

Some disparities were noticed among the longer chain fatty acids. If results are compared between the animals of the same time points, the controls contained about 20 to 110 percent as much of behenic acid (22:0), tricosanoic acid (23:0) and lignoceric acid (24:0) as the

experimentals in 1-month and 2-month rabbits. An average between the two batches of 2-month rabbits was used in this calculation. The duplicate results of 4-month rabbits did not confirm each other as far as 23:0 and 24:0 fatty acids are concerned. Yet, the experimental rabbits had 1.3 to 2.3 times more of 22:0 than the controls.

Since the hydroxy fatty acids were present in extremely small amounts (by TLC), attempts to determine their percent distribution were difficult and unreliable.

SUMMARY

Quantitative determinations have been made on the cerebroside isolated from whole aortas of cholesterol-fed and normal rabbits at various time points. The fatty acid patterns of cerebroside in the course of atherogenesis were also studied.

The amounts of cerebroside were similar to those reported for human aortas (39, 40). The 2-month experimental group showed the highest concentration of cerebroside, and generally speaking, the experimentals contain more cerebroside than the controls.

Rabbit aortic cerebroside contain principally 16:0, 18:0 and 18:1 with very long-chain and hydroxy fatty acids as relatively minor components. As a result of a two fold increase in oleic acid over that of the stearic acid, the ratio of 18:0/18:1 was inverted for the 2-month experimental group. It was also shown that this group contained more other unsaturated fatty acids than the rest.

Though the ratio of 16:0/18:0 increases from one to two months for both the controls and experimentals, the experimental group shows the greater increase.

For the longer chain fatty acids, the 1-month and 2-month controls contained about 20 to 110 percent as much of 22:0, 23:0 and 24:0 as the experimental rabbits.

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