Alterations in Aorta Lipids with Advancing Atherosclerosis.* (26218)

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Although it has long been possible to perform reasonable quantitative analyses of the lipids of normal and diseased arteries(1-4) only recently have such analyses become rapid, convenient, and accurate on a very small scale. This has been largely due to two advances in technic: silicic acid chromatography, which permits separation and analysis of relatively small samples of lipids into several classes; and gas-liquid chromatography, which permits separation and analysis of fatty acids derived from such lipids on a very small scale. These technics, permitting complete analyses on many more samples than was previously possible, should facilitate testing of the several hypotheses concerning the origin of the atherosclerotic lipids. For example, are lipids which accumulate in the arterial wall in this disease formed *in situ*(5) or do they result from deposition of a portion of the blood lipids(4,6); and, in either case, is their resistance to removal due to the saturated or other high melting properties of their fatty acids(7) or to the nature of the lipids in which they find themselves?

There have been a few attempts to apply these modern technics to these problems(8-11). In general, however, these have been preliminary reports on only a few subjects, and not concerned with alterations in the atherosclerotic lipids with progress of the disease. However, Böttcher and his coworkers(10) analyzed both lipids and fatty acids in 4 stages of atherosclerosis in aortas and also, in some cases, in coronary arteries and the circle of Willis.

Our experiments have been carried on for several years with cooperation of the Pathology Dept. of this University.† The aim has been to perform as complete an analysis as possible of lipids involved in development of the atherosclerotic deposits with the hope that such detailed information might serve as a basis for theories of the mechanism of lipid accumulation and, ultimately, in formulating measures for its prevention.

Four main lipid fractions (sterol esters, triglycerides, sterols and phospholipids) have been separated by silicic acid chromatography, and have been equated to the extent of the disease in the aortas from which they were extracted. Fatty acids of the sterol esters have been analyzed by gas-liquid chromatography.

Methods. Preparation of tissues. Aortas were graded from 0-4 depending on extent and severity of plaques at time of autopsy and were stored at −20°C until use.‡ After thawing, the adventitia was stripped from the media and intima and was discarded. No attempt was made to separate media and intima because of the impossibility of such a separation in advanced stages and the desire to maintain comparable conditions. For similar reasons, whole tissues were analyzed rather than plaques alone (By far the greatest part of the lipid was present in the plaques of the diseased aortas). The tissues were cut into small pieces, lyophylized and dried under high vacuum.

Extraction of tissues. The dried tissues

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‡ (See footnote †). Only fresh samples, taken from autopsies performed at Univ. of California Medical Center, Los Angeles, were used. Grading was performed on basis severity and extent of subintimal fibrosis and lipid deposition and, in advanced cases, calcification of plaques. Subjects were patients at the Medical Center before death. Average ages were: for grade 0, 32; grade 1, 61; grade 2, 64; grade 3, 67 and grade 4, 71 years. Although it is difficult to find adult grade 0 aortas, we were fortunate in this respect. One patient, a chronic alcoholic with liver cirrhosis, was 52. All patients with plaques of grade 2 or greater had moderate to severe arteriosclerotic symptoms.

were extracted in the Waring blendor with methylal-methanol (4:1) using approximately 60 ml of solvent per 5 g of tissue for the first extraction followed by 5 subsequent extractions with 30 ml. The solutions were clarified by centrifugation and evaporated almost to dryness at 40°C under a stream of nitrogen. Final drying was accomplished under reduced pressure. The lipid was generally a clear orange tacky grease unless the presence of considerable free cholesterol resulted in formation of crystals. The samples were dissolved in pentane, separated by centrifugation from any small amounts of insoluble material, and stored at –20°C until use.

Chromatography. All chromatograms were carried out in duplicate except for 2 grade 0 samples for which too little lipid was obtained. The method used was a modification of the system of Fillerup and Mead(12). Silicic acid (Mallinckrodt 100 mesh) was washed 7 times with acetone, allowing the denser particles to settle for a few minutes before decanting the supernatant suspension. A slurry of this silicic acid in acetone was poured into a 19 mm diam. column to a height of 95 mm. It was then washed with successive 100 ml portions of methanol, ethyl ether and pentane. The sample was transferred to the column with small volumes of pentane. Twenty ml fractions (1 col. vol.) were then collected using the following solvent mixtures and collecting 5 fractions for the first solvent and 10 thereafter: pentane, 2%, 5%, 20% ether in pentane, ether and methanol.§ The solvent was removed from the fractions in a warm water bath under a steam of nitrogen and each fraction was dried at reduced pressure and weighed. Estimation of per cent of each lipid was accomplished with the aid of a bar graph (Fig. 1) and with careful examination of intermediate fractions. Slight overlapping of the triglyceride with the sterol and the sterol with the partial glyceride fraction was noted. Average recovery from the columns was 94%.

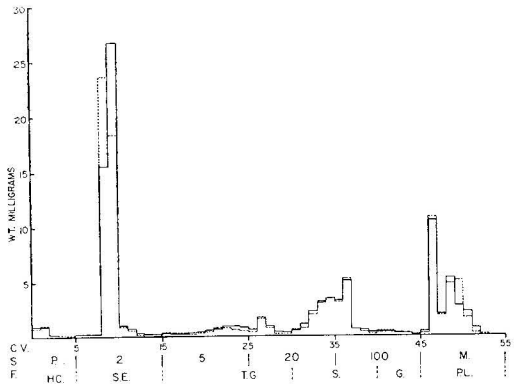

FIG. 1. Chromatographic separation of duplicate samples of aorta lipid. Abscissa: Small numbers designate column volumes of solvent (C.V.). Solvents (S.) are: pentane (P.), % ether in pentane (large numbers) and methanol (M.). Lipid fractions (F.) are: hydrocarbons (HC.), sterol esters (S.E.), triglycerides (T.G.), sterols (S.), partial glycerides (G.) and phospholipids (P.L.).

Sterol ester fractions were transmethylated using 1% methanolic sulfuric acid at 70°C for analysis of their component fatty acids by gas-liquid chromatography.‖

Results. In Table I are shown percentages of the several lipid fractions in the aortas of different stages. Salient points are: the proportion of triglyceride is low and does not change significantly with progress of the disease; sterol esters increase rapidly in the lower stages, then remain constant; free sterols increase irregularly throughout plaque development; phospholipids decrease rapidly from the 0 to 1 stages, then more slowly; ratio of total sterol to phospholipid is probably the best index of extent of the disease.

In Table II are shown the percentages of the major identifiable fatty acids of the sterol esters of the aortas of different stages. No significant changes are apparent with increased severity of atheromatosis. Linoleic acid appears to be the major fatty acid followed by oleic and palmitic.

§ Although the phospholipid fraction can be readily separated into subfractions, this was not attempted because of the added great complications of fatty acid analysis which would be required. A preliminary report of such an analysis has appeared in the literature(13).

‖ Gas-liquid chromatographic analyses were performed with the Wheelco Model 10 apparatus (Barber-Colman Co.) with a 6 ft. 6 mm I.D. column of ethylene glycol-succinate polyester on 60-100 mesh celite at 175-180°.

TABLE I. Composition of Lipids from Intima and Media of Aortas with Different Degrees of Atherosclerosis.

Grade	No. of subjects	Lipid, % of dry wt	% of total lipid ± stand. dev. where possible							Ratio: Total sterol/ phospholipid
			Hydrocarbon	Triglyceride	Di- & monoglyceride?	Sterol ester	Free sterol	Total sterol	Phospholipid	
0	5	6.5 ± 2.0	3.5	7.2 ± 1.8	1.5	15.5 ± 6.6	18.8 ± 2.6	34.3 ± 8.5	48.3 ±10.7	.8
1	3	16	1.0	9.3	1.2	35.2	23.4	58.6	22.6	2.6
2	4	10.5 ± 4.1	1.6	7.1 ± 2.1	.7	41.5 ± 2.9	21.5 ± 2.4	63.0 ± 4.0	20.1 ± 5.7	3.4
3	2	16	.8	8.6	1.6	42.4	25.1	67.5	15.7	4.3
4	5	22.6 ± 3.1	1.0	6.3 ± 1.8	.8	41.5 ± 6.2	30.2 ± 6.3	71.7 ± 2.8	15.2	4.8

Discussion. The results shown above indicate clearly that with progress of atheromatous disease, the nature of the deposited lipid undergoes a steady change in the direction of an increased proportion of total sterol and a decreased proportion of phospholipid. This is in general agreement with the results of Böttcher and his co-workers(10) although the absolute values are quite different. It is not clear whether this is the result of a long continued exposure to blood lipids in which the sterol/phospholipid ratio is too high, although this has been considered an attractive hypothesis(4,6).

The picture presented by these lipid changes is one of increasing metabolic inertness, and the types of lipids predominating in the later stages are typical of tissues with a very slow turnover rate regardless of their component fatty acids. This observation is also borne out by the finding of high peroxide values in lipids of advanced plaques.¶ It is difficult to see how such peroxides could accumulate if there were any reasonable turnover of fatty acids. This inertness is not inconsistent with the morphological changes occurring in the progressively necrotic plaques but may also be due to other causes. The origin of the lipid deposits, whether from blood lipids or by synthesis within the tissue, is not explained by this study, although some light may be shed by the fatty acid composition of the lipids. Nevertheless, it appears that, regardless of the route by which the lipids reach the intima of the arteries, consideration should be given to the enzyme pattern of a tissue which permits them to remain and accumulate.

The constancy of the fatty acid composition of the sterol esters with progressive atheromatous disease does not agree with the findings of Böttcher and co-workers(10) who reported an increase in the linoleate-saturated acid ratio nor does it support the hypothesis of Sinclair(7) that accumulation of lipid may be related to inclusion of saturated or other high-melting fatty acids. Further

¶ The high peroxide numbers of lipids of advanced atheromatous plaques(14) have been confirmed in this laboratory.

TABLE II. Fatty Acid Composition of Cholesterol Esters from Aortas with Different Degrees of Atherosclerosis.

Grade	No. of samples	% of major fatty acids					
		Palmitic	Palmitoleic	Oleic	Linoleic	Eicosatrienoic?	Arachidonic
0	4	12.4	3.3	30.6	41.2	3.0	6.5
1	3	13.4	4.8	29.2	42.7	1.1	6.8
2	4	10.6	3.4	27.2	47.6	2.4	7.5
3	2	12.6	3.2	26.3	49.3	1.0	5.6
4	4	13.1	5.6	27.4	42.1	1.6	7.3

study should reveal whether these differences may be explained by changes in diet.

Summary. Analysis of lipids of the intima and media of aortas with progressive stages of atherosclerosis reveals an increase in sterol and sterol ester fractions and a decrease in phospholipids. It is proposed that these changes reveal a very low rate of lipid turnover in this tissue. The fatty acid composition of the cholesterol esters did not change with advancing atherosclerosis.

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A Ventilatory Effect of Carbonic Anhydrase Inhibition in Man. (26219)

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An increase in resting ventilation has been a consistent finding in dogs given the carbonic anhydrase inhibitor acetazolamide(1, 2,3). In man, however, no increase in resting ventilation has been reported even though doses of the drug were well within the range which produced a marked effect in dogs(4,5, 6). In seeking a reason for the absence of an effect in man in the reports, it was noticed that the drug was usually given intravenously into the dogs but invariably was taken orally by the human subjects. If insufficient time during the experiment was allowed for absorption and attainment of a sufficient concentration of drug in the blood, the ventilatory response of man to carbonic anhydrase inhibition might well not have been seen. In the experiments described below, the results obtained in man with the same dose of drug given orally and intravenously are compared.