Lipid Droplets in Atherosclerotic Fatty Streaks of Human Aorta

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Abstract Preparations of lipid droplets and droplet-free tissue residue (cytoplasm + membranes + nuclei) were obtained by homogenization and centrifugal separation from intimal fatty streak lesions of aortic atherosclerosis of 21 adults who had died suddenly. Neutral lipids and phospholipids were analyzed by quantitative thin-layer chromatography and cholesteryl ester fatty acids by gas-liquid chromatography. Optical properties of droplets were evaluated by differential counting and sizing procedures with the polarizing microscope. The droplets occurred in mixtures of two forms distinguished by their optical properties, anisotropic (i.e. liquid crystals) and isotropic (true liquids). Both forms had average diameters of about 1.8 μ, with a range of 0.5–5 μ. The proportions of the two forms varied with temperature as individual droplets changed their form; anisotropic forms averaged 83.7% at 22°C and 37.8% at 37°C, with isotropic forms being 16.3 and 62.2% respectively. The proportions of anisotropic forms at 22°C decreased with age. These forms were not separated for chemical analysis. The droplets contained about half the lipid in the lesions. The composition of the lipids of the droplet mixture was remarkably uniform and strikingly different from that of the droplet-free residue, respectively: cholesteryl esters 94.9% vs. 38.7%, free cholesterol 1.7% vs. 18.6%, total phospholipids 1.0% vs. 38.6%, and triglycerides 2.4% vs. 4.0%. The proportions of individual phospholipids, with the exception of lysolecithin, were also different between the preparations. In the droplets only the proportions of lecithin correlated positively with the proportion of anisotropic forms (at 22°C). Droplet cholesteryl esters were particularly rich in oleic acid and when compared to residue esters had more palmitoleic (+0.7%), oleic (+12.3%), and eicosatrienoic (+2.4%) and less palmitic (−2.2%), linoleic (−12.4%), and arachidonic (−1.6%) acids. The proportions of most individual fatty acids of droplets and residue correlated positively. The lipids of the residue closely resemble those reported for the normal intima.

The observations that these droplets are prominent in the morphology of the fatty streak lesions, and that their high content of oleate-rich cholesteryl esters is similar to that reported for analysis of the whole lesions, suggest that the droplets may be involved in the pathogenesis of the fatty streak lesions of atherosclerosis in man.

INTRODUCTION

In fatty streaks, the initial lesion of human atherosclerosis, lipid accumulates prominently in the form of intracellular droplets (1, 2). The lipid composition of these droplets is inadequately described, although the composition of the whole lesion has been extensively studied (3–7). Study of these droplets may advance our understanding of the pathogenesis of arteriosclerosis.

Kaiserling and Orgler (8) were the first to observe that intracellular droplets from superficial lesions in human intima polarize light. This is a characteristic of liquid crystals, whose physical form combines the molecular orientation of a crystal with the fluidity of a liquid. Chalatow (9) noted these birefringent droplets also in the aortas of rabbits fed cholesterol, and thought them to consist mainly of cholesteryl esters. In recent years Stewart (10) studied liquid crystals of normal and atheromatous tissue and later identified free cholesterol, cholesteryl esters, and possible triglycerides in a crude droplet preparation from superficial fatty streaks (11). Weller (12) found that the number of liquid crystals

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(anisotropic droplets) were highest in fatty streaks, while in more advanced lesions the droplets were predominently in an isotropic, nonpolarizing, true liquid form. Histochemical studies in aortas of humans and arteriosclerotic rabbits led him to the conclusion that anisotropic droplets contain mainly a mixture of phospholipid and cholesterol, whereas isotropic droplets consist predominantly of cholesteryl esters. Later (13) it was observed that a mixture of anisotropic and isotropic droplets isolated from fatty streaks of two human aortas had a phospholipid: free cholesterol ratio of approximately 25:1.

We report here a study of the lipid droplets from fatty streaks of 21 human aortas, their optical properties, lipid composition, and relationship of their lipids to the other lipids in this lesion. The droplets, a variable proportion of which showed the characteristics of liquid crystals, had a remarkably uniform lipid composition, were rich in cholesteryl esters, poor in other lipids, and had a lipid composition quite different from that of the other lipids of the lesion.

METHODS

Tissue specimens. The thoracic and upper abdominal part of aortas with extensive fatty streaking were collected at autopsy from 15 male and 6 female subjects, 20–49 yr of age, dying suddenly and unexpectedly. Seven males and three females were Caucasian, while eight males and three females were Negro. Differences among subjects by sex and race were not considered significant since fatty streak involvement of aortas appears to be the same in both sexes, and the same in Negroes and Caucasians (14). The average age of the subjects was 30.9 ±10.1 yr (mean ±SD); two were in the 2nd decade, nine in the 3rd, six in the 4th, and four in the 5th. None had metabolic disorders, debilitating diseases, or cachexia known to distort tissue lipids. The autopsy diagnoses of the cause of death were: acute trauma, 12 cases; primary pneumonia, 3 cases; acute idiopathic myocarditis, 2 cases; and one case each of ruptured cerebral aneurysm, pulmonary embolism, idiopathic epilepsy, and coronary sclerotic heart disease. Using per cent standard body weight as an index of adequate nutrition before death, a value of 104.5 ±17.5% was observed for our subjects. The reference standard was the average weight recently observed for men or women at age 25–29 yr, corrected for shoe heel height, 1 inch for men and 2 inches for women, and 3 lbs. of clothing (15). The average time between death and necropsy was 10 hr (range 1–18 hr). A specimen consisting of the total circumference of the aorta at mid-thoracic level, representative of fatty streaks and undiseased intima, was fixed in formalin for histological examination.

Tissue fractionation and isolation of droplets. Immediately after autopsy the fresh aortas were processed to obtain the tissue fractions. They were rinsed thoroughly with distilled water and examined with a dissecting microscope at × 10–30 magnification. Intimal fatty streak lesions were identified as pale yellow, slightly raised areas of opaque spots or streaks covered by a delicate transparent membrane. All fatty streak lesions were confined to the intima, as their underlying media was of normal color and consistency. Blocks of aortic wall with typical intimal fatty streaks were fixed in formalin and sectioned by a freezing microtome. Microscopic examination of sections stained for fat with oil red 0 in propylene glycol (16) and with hematoxylin showed the fatty streak lesion confined to the intima. Lipid appeared in the lesions as fine droplets of various sizes largely grouped in oblong clusters. The clusters frequently contained a round plump nucleus. No free lakes of lipid were observed. Although the location of these droplets as either extra- or intracellular could not be conclusively demonstrated, the bulk of the droplets appeared to be intracellular. No signs of fibrosis were observed. The fatty streaks of the thoracic aorta were almost invariably located posteriorly between the orifices of the intercostal arteries, those of the abdominal aorta were distributed randomly proximal to the orifice of the superior mesenteric artery. With the aid of opthalmological surgical instruments, the fatty streaks were dissected off the internal elastic lamina, leaving the latter *in situ* attached to the underlying media. Care was taken to avoid contamination with lipid from periadventitial adipose tissue or from more advanced lesions. All fatty streaks present in each aorta were dissected and pooled to obtain 200–500 mg of tissue, the amount needed for analysis.

The dissected fatty streaks were thoroughly minced with fine scissors. Examination of the mince by polarizing microscopy typically demonstrated isotropic and anisotropic droplets occurring in spindle shaped clusters within the tissue fragments or floating free in the water. The mince was homogenized at 22°C in about 6 ml of distilled water with a tissue grinder having a pestle-wall clearance of 0.004–0.006 inch and the Teflon pestle driven at approximately 1200 rpm for 2 min.[1]

The homogenate was transferred to 15 ml round-bottom centrifuge tubes, brought to a volume of about 10 ml with distilled water, and centrifuged in a swinging bucket head in a refrigerated centrifuge[2] at 3800 g for 45 min at 22°C. The pale yellow fragile layer of droplets that floated on the top of the homogenate was carefully drawn off with 0.1–0.2 ml of supernatant into a 1 ml glass syringe through a blunt, 22 gauge needle and then immediately suspended in 15 ml of distilled water. The sedimented residue was thoroughly shaken with the supernatant fraction and recentrifuged to recover lipid droplets that might be trapped in the residue. Any second floating layer was aspirated and added to the tube with the first droplet preparation. The pooled droplet preparations were subsequently washed by centrifugation as before. This washing procedure was repeated two times for a total of three washes in order to remove all traces of the supernatant fraction of the original homogenate. The floating layer from the final wash was aspirated, yielding 0.1–0.2 ml of final droplet preparation consisting of a mixture of isotropic and anisotropic droplets of various sizes, and which appeared similar to the droplets seen in the tissue mince. The first wash of the droplet preparation was added to the original sedimented residue and supernatant fraction. This fraction with a volume of about 25 ml is hereafter referred to as residue preparation. The second and third washes of the droplet preparation were discarded. All final preparations were checked for purity by phase contrast microscopy,[3] the droplet suspension for particulate contaminants, the residue for intact cells

[1] Arthur H. Thomas Co., Philadelphia, No. 4288-B, size A.

[2] Ivan Sorvall, Inc., Norwalk, Conn. Model RC-2 with Type HB-4 head.

[3] Nippon Kogaku, K. K. Tokyo, Japan, dark contrast optics.

filled with lipid droplets and the supernatant for droplets. Additional homogenization of the residue and centrifugation to recover droplets were occasionally required.

Analysis of physical properties of droplets. A small aliquot of the final droplet preparation (approximately 0.02 ml) was mixed with four volumes of 95% glycerol. This mixture was used for differential counting and sizing of the droplets on a counting chamber [4] with a calibrated eyepiece micrometer disc having 100 graduations in 5 mm. The chamber was used solely for its grid to define areas of examination. To distinguish between anisotropic droplets, i. e. liquid crystals, and isotropic droplets during counting, the procedure was carried out using polarizing filters [5] and a first-order red quartz accessory plate [6] positioned in the standard way (17). This plate enables the determination of the crystallographic sign of anisotropic droplets and the visualization of isotropic droplets when polarizer and analyzer are completely crossed. The microscopic examination was performed at ×600 magnification.

The counting and sizing procedure was done in a manner to assure random performance. The 25 0.2 × 0.2 mm squares on the grid of the counting chamber were assigned numbers, and the sequence of squares to be examined was selected randomly. Sufficient squares were completely scanned until at least 600 droplets had been evaluated. In a preliminary analysis of variance of the proportions of anisotropic droplets the coefficient of variation was 1.9% at a count of 850 droplets, 2.4% at a count of 600 droplets, and 3.3% at a count of 350 droplets. On the basis of this and the time required for counting, a routine count of 600 droplets was judged acceptable.

Each droplet was classified according to the presence of anisotropy or isotropy, and size. Anisotropic droplets appeared divided by a symmetrical dark cross into quadrants of alternating blue and yellow color. In all the anisotropic droplets we observed, the left upper quadrant was yellow, classifying these droplets as crystallographically positive (by U. S. A. convention). In preliminary studies the symmetrical arrangement of the colored areas became severely distorted if the droplet preparation was allowed to dry, but could be restored through addition of water. These anisotropic droplets could therefore be categorized as lyotropic liquid crystals, that is, crystals formed through addition of a controlled amount of water or other polar solvents to certain compounds (18). Isotropic droplets appeared as pale yellow rings on the red background. The diameters of both kinds of droplets ranged from 0.5 to 5.0 μ with means of 1.8 μ. Their size was determined with the eyepiece micrometer and classified in four ranges: 0.50–1.25 μ; 1.25–2.50 μ; 2.50–3.75 μ; and 3.75–5.00 μ. The results of the counting and sizing procedure were recorded with a multiple unit tally counter, each register of which represented one class of size. For convenience the squares were examined first to count and size anisotropic droplets, and then reexamined for isotropic droplets.

To compute the volumes of anisotropic and isotropic droplets, the volume of each size class was calculated as the product of the number of droplets and the average volume of the respective size class. The latter was estimated from the observed diameters (x) by the formula

$$\int_{x_1}^{x_2} \frac{\pi x^3}{6(x_2 - x_1)}\, dx,$$

which gives the average volume integrated from the smallest (x_1) to the largest (x_2) diameter for each size class as defined above. The volumes of the four size classes were summed to give the total volume. Based on this the proportions of the volumes of anisotropic and isotropic droplets in the total volume of droplets were calculated. All data are expressed as the proportion of anisotropic droplet volume in the total volume of droplets; the proportion of isotropic droplets is simply complimentary.

Preliminary experiments showed the proportion of anisotropic droplets to be significantly temperature dependent. While there was a constant proportion between 4° and 25°C, it decreased continuously between 25° and 37°C. The counting chamber therefore was placed in a warming stage [7] in order to control the temperature. Routinely the droplets were evaluated at 22° and 37°C after the temperature of the suspensions was allowed to equilibrate for 20 min.

The optical properties of the droplets remain constant in the glycerol-water mixture for at least 72 hr. No difference in the proportions of anisotropic and isotropic droplets was observed for up to 4 hr in a fresh preparation suspended in either distilled water, physiological saline, or glycerol-water. The last was preferred because of the long-term stability of the droplets in this medium and the higher viscosity which caused the droplets to be stationary in the counting chamber. When the droplets counted at 22°C were warmed to 37°C, then cooled to 22°C and counted again, no change in proportions was found. The transformation, therefore, seemed to be reversible.

This method of isolation appeared to give uniform preparations of droplets with high yield and least distortion. Since the lipid composition of the droplets was relatively uniform while their size and proportions of anisotropy were variable, these latter were used as sensitive indicators of alteration of droplets. Evidence of destruction of droplets was not found. All isolated anisotropic droplets showed clearly the Maltese cross image of birefringence seen in droplets in fresh tissue. None showed distortion of the image and its replacement of swirls of birefringence as seen after droplets are deformed by being forced through a constriction narrower than their diameter, nor the centrifugally progressive loss of the image and birefringence seen extensively after droplets have stood in water for several days. No fragments of anisotropic material were observed in the droplets or residue preparations. Homogenization did not generate droplets *de novo* as 5 min homogenization of grossly normal intima, containing rare droplets, did not cause an increase in droplets. Washing did not appear to affect the droplets judging from the constancy of their anisotropy in water for up to 4 hr. Detailed studies of the physical properties of the droplets with Dr. Yoshiya Hata in this laboratory showed that some physical characteristics may change during isolation. Measuring droplet diameter more accurately by a split-image micrometer, droplets, freed by teasing fresh tissue bathed in a variety of media, increased in diameter within an hour about 19% over their diameters *in situ*. We have been unsuccessful in preventing or controlling the increase in size upon freeing the droplets. During 10 min of homogenization at 22°C the proportion of anisotropic forms

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[4] Petroff-Hausser bacteria counter, C. A. Hausser & Son, Philadelphia, Pa.

[5] Nippon Kogaku, K. K., Tokyo, Japan.

[6] Carl Zeiss, Inc., New York.

[7] Chicago Surgical & Electrical Div., Lab-Line Instruments, Inc., Melrose Park, Ill.

progressively decreased, e.g. from 87% initially to 72% at 2 min and 58% at 10 min. However, there were no significant changes in the distribution of the sizes of droplets of either form using the split-image micrometer and statistical analysis of the distribution of sizes by the Kolgomorov-Smirnov test. The apparent loss of anisotropic forms during homogenization remains unexplained. It could be attributed to conversion of anisotropic forms to isotropic forms of the same size, or to loss of anisotropic forms either by conversion to particles too small to be detected and sized by the light microscope, or by dissolution in the supernatant. However neither change would affect the chemical analyses of the droplet mixture. Anisotropic droplets dissolved in the supernatant would make the composition of the residue preparation closer to that of the droplet preparation. 2 min homogenization at 37°C compared to homogenization at 22°C and to unhomogenized controls showed a 12% increase in the diameter of isotropic forms. To minimize any differences between specimens due solely to differences in these isolation techniques, all specimens were prepared at 22°C by the uniform procedure described above.

To look for effects of tissue storage, half of one aorta was dissected immediately after autopsy, processed as described, and the proportions of anisotropic and isotropic droplets determined in the droplet preparation. The other half of the aorta was stored at 4°C in physiological saline solution for 24 hr, and then the same procedure was carried out. No difference in the proportions was observed, suggesting that no postmortem changes occurred in the optical properties of droplets remaining in the tissue during 24 hr, a time considerably longer than the average 10 hr interval between death and autopsy in our subjects.

Chemical analysis. Lipid was extracted from the droplet and the residue preparations by 20 volumes of a 2:1 chloroform:methanol mixture. The extracts were evaporated to dryness *in vacuo* at 40°C, while iso-octane-butanol 1:1 and sufficient 100% ethanol were added to remove the water without formation of insoluble residues. The lipids were dissolved in chloroform and transferred to screw-cap tubes. The procedure was carried out without interruption to this point. The lipid extracts were stored at 4°C up to 4 days before further analysis.

Separate determinations for cholesteryl esters, triglycerides, free cholesterol, total phospholipids, and individual phospholipids were carried out by quantitative thin-layer chromatography on Silica Gel HR[8] according to Amenta's method (19). All determinations were performed in duplicate, and quantitative standards of pure lipids were chromatographed adjacent to the lipid extracts on each plate. The total lipid standard contained cholesteryl stearate, tristearin, cholesterol, and α-L-lecithin. The phospholipid standard contained α-L-lecithin, sphingomyelin, and lysolecithin.[9] Lecithin was chosen as representative of total phospholipids in the total lipid standard because it had the largest proportion in both preparations and a response to the acid dichromate reagent midway between that of cephalin and sphingomyelin, the other major phospholipids. Typical reductions in the optical density of the acid-dichromate reagent for 100 μg of phospholipid were: sphingomyelin 0.345, lecithin 0.313, cephalin 0.291, and lysolecithin 0.213. Preliminary experiments showed that this technique measured the mass of lipids independent of the molecular weight and degree of unsaturation of the constituent fatty acids. The plates for total lipid determination were developed twice, first with acetone-benzene 20:80, and second with iso-octane-ethyl acetate 100:1. The plates for the determination of the individual phospholipids were developed with chloroform-methanol-water 65:25:4.

The developed plates were dried for 30 min in a vacuum oven (50°C, 30 inches Hg) and sprayed lightly with rhodamine 6G (7.9 mg/liter H_2O). The plates were placed in a horizontal position, sprayed heavily with distilled water, and vacuum-dried as above. The heavy spraying with water and vacuum drying was repeated once more. This was necessary to romove all traces of solvents from the plates.

The lipid bands were located by visualization under ultraviolet light and scraped off the plate. Equal areas of adjacent silica gel were used as blanks. The scrapings were digested with the acid dichromate reagent and the reduction in optical density measured at 350 mμ. The average of the duplicate determinations was used for computations. As an indicator for the agreement between duplicate measurements the standard deviation of the differences between the two measurements was computed for each lipid over all determinations and standards according to the formula

$$\text{SD} = \sqrt{\frac{\Sigma d^2}{2n}},$$

where d is the difference between duplicates and n the number of sets to be examined (20). While our absorbance readings characteristically were in the order of 0.200, the standard deviation within sample and standard duplicates did not exceed 0.01 for any lipid measured.

Our analyses are recorded as the proportions of total lipids or of total phospholipids. Small but unavoidable losses occurring particularly during the preparation of the droplet suspension prevented accurate determination of lipid concentration in the tissue.

For gas-liquid chromatography of the cholesteryl ester fatty acids, the remainder of each extract was spotted in a wide band on a thin-layer chromatography plate which was developed with iso-octane-ethyl acetate 100:1 to isolate cholesteryl esters. These were interesterfied with super-dry methanol containing 5% HCl and the fatty acid methyl esters extracted with hexane. Gas-liquid chromatography of the methyl esters of lauric through arachidonic acids was carried out with a F & M 609 machine[10] with a hydrogen flame ionization detector. The column was packed with a polar organosilicone polymer on diatomaceous earth (10% EGGS-X on 100–120 mesh Gas-Chrom P[11]), and had a minimum column efficiency of 3000 theoretical plates at stearate at 210°C. The stearate-oleate spearation factor was 1.19. Nine daily sets of duplicate analyses of standards of pure fatty acid methyl esters showed for myristic acid 24.81 ±0.14% (mean ±SE of mean) vs. a known composition of 24.77%, palmitic acid 10.30 ±0.07% vs. 10.25%, and stearic acid 64.88 ±0.15% vs. 64.98%. This standard deviation within fatty acid duplicates, computed as outlined above, was for palmitic and stearic acid approximately 0.4 and for myristic acid 0.3. In a preliminary study the recovery of pure arachidonic acid methyl ester was found to be 97% complete. An acid with a relative retention time of 2.74 relative to stearate was tentatively identified as eicosatrienoic acid on the basis of its retention time and the failure to chromatograph after bromination.

Most of the computations were performed on an electronic computer. The method for calculating correlations between

[8] E. Merck, A. G. Darmstadt.

[9] All phospholipids were obtained from Nutritional Biochemical Corp., Cleveland, Ohio.

[10] F & M Scientific Corp., Avondale, Pa.

[11] Applied Science Labs., Inc., State College, Pa.

components of a lipid mixture, whose values are expressed in proportions, has been described earlier (21).

RESULTS

Lipid droplets, isolated from fatty streaks of 21 aortas, occurred as mixtures having variable proportions of anisotropic and isotropic forms. The proportion of anisotropic forms in the droplet mixtures was a function of temperature, being constant over the range 4°–25°C and decreasing upon increase of the temperature from 25° to 37°C. All preparations showed this phenomenon (Fig. 1). A significant difference ($P < 0.01$) was observed between the proportions of anisotropic forms at 22° and 37°C, respectively 83.70 ±10.87% (mean ±SD) and 37.84 ±22.43%.

The lipid compositions of the droplet mixture and of the tissue residue were strikingly different, but markedly uniform within each kind of preparation (Table I). The droplet lipids were constituted largely of cholesteryl esters, with minor proportions of triglycerides, free cholesterol, and phospholipids. In the residue the lipids consisted largely of equal proportions of cholesteryl esters and phospholipids, a moderate proportion of free cholesterol, and a small proportion of triglycerides.

The mixtures of the phospholipids in the two preparations differed greatly ($P < 0.01$) in their proportions of sphingomyelin, lecithin, and cephalins (Table II). Most of the phospholipids in the droplets were lecithin

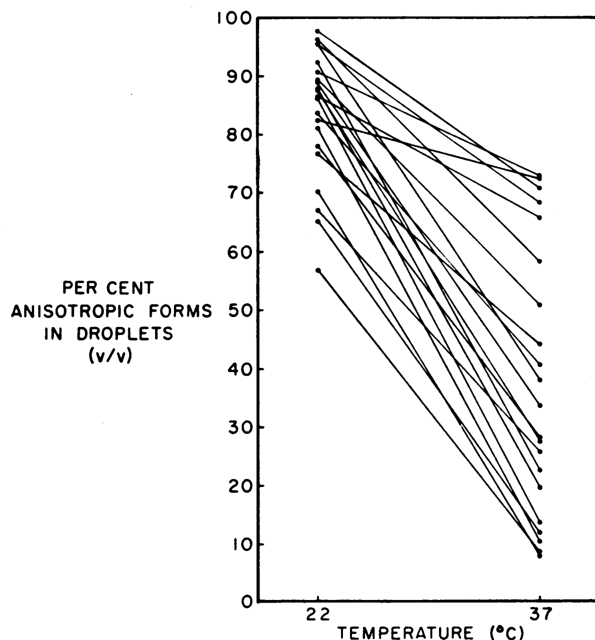

FIGURE 1 Relationship between the proportions of anisotropic forms observed at 22° and at 37°C of droplets from fatty streaks of 21 aortas. The observations on a single aorta are connected by a line.

TABLE I
Proportions of Various Lipids in Total Lipid of Droplets and Residue of Fatty Streaks from 21 Aortas

Lipids	Per cent of total lipid by tissue preparation: Droplets	Residue	Significance of difference between droplets and residue
	%	%	
Cholesteryl esters	94.9 ±2.2*	38.7 ±4.7	$P < 0.01$
Free cholesterol	1.7 ±0.6	18.6 ±2.0	$P < 0.01$
Phospholipids	1.0 ±0.7	38.7 ±3.8	$P < 0.01$
Triglycerides	2.4 ±1.3	4.0 ±1.3	$P < 0.01$

* Mean ±SD.

and cephalins, and in the residue lecithin and sphingomyelin. Lysolecithin, the smallest component, was not significantly different in the two preparations.

The cholesteryl ester fatty acid mixture of both preparations consisted largely, 97%, of eight acids: myristic, palmitic, palmitoleic, stearic, oleic, linoleic, eicosatrienoic, and arachidonic acids (Table III). Traces of lauric acid were detected inconstantly. Traces of seven other acids were also observed. The proportions of most fatty acids were significantly ($P < 0.01$) different between the preparations, droplets containing more palmitoleic, oleic, and eicosatrienoic acids, while the residue had more palmitic, linoleic, and arachidonic acids.

A number of significant correlations were observed among the variables measured in this study. Age correlated negatively with the proportion of anisotropic forms at 22°C, but not at 37°C, respectively $r = -0.52$ with $P < 0.05$ and $r = -0.38$. The proportions of droplet lipids did not correlate with age. In contrast, in the residue fraction the proportions of cholesteryl ester

TABLE II
Proportions of Individual Phospholipids in Total Phospholipid of Droplets and Residue of Fatty Streaks from Aortas

Phospholipid	Per cent of total phospholipid by tissue preparation: Droplets (17 aortas)	Residue (20 aortas)	Significance of difference between droplets and residue
	%	%	
Lecithin	39.5 ±9.4*	46.9 ±4.9	$P < 0.01$
Lysolecithin	8.3 ±9.9	6.0 ±2.2	NS
Sphingomyelin	17.1 ±11.9	27.7 ±5.3	$P < 0.01$
Cephalins	35.1 ±9.6	19.4 ±5.0	$P < 0.01$

NS, not significant.
* Mean ±SD.

increased with age, $r = +0.62$ and $P < 0.01$, while those of the free cholesterol and total phospholipids decreased, respectively $r = -0.58$ and $P < 0.01$ and $r = -0.50$ at $P < 0.05$. None of the proportions of individual phospholipids and of major fatty acids of the cholesteryl esters of both preparations correlated with age. The per cent of standard body weight correlated positively with the proportion of cholesteryl esters in droplets ($P < 0.05$) and residue ($P < 0.01$) and negatively ($P < 0.05$) with droplet triglycerides and residue phospholipid. Two fatty acids of the cholesterol esters in the residue correlated at $P < 0.05$ with the per cent of standard body weight, palmitic acid negatively, and arachidonic acid positively.

The proportions of anisotropic forms at the two temperatures, 22° and 37°C, correlated positively ($r = +0.61$, $P < 0.01$). Significant positive correlations ($P < 0.05$) existed between the proportions of anisotropic forms at 22°C and the proportions of lecithin ($r = +0.52$), and between the proportions of anisotropic forms at 37°C and the proportions of cholesteryl ester myristic acid ($r = +0.52$). The difference between the proportions of anisotropic forms at 22° and 37°C did not correlate with the constituents of the lipid classes, but had weak negative correlations with cholesteryl ester myristic and palmitic acids.

The proportion of cholesteryl ester in the lipids of the droplets correlated negatively with their percentages of palmitic and stearic acids (respectively $r = -0.63$ and $r = -0.69$, $P < 0.01$), and positively with the percentages of linoleic acid ($r = +0.51$, $P < 0.05$). The cholesteryl ester proportion of the residue lipids did not correlate with its fatty acid composition. No correlations occurred between the proportions of any of the identical lipids in the lipid classes of the two preparations, although correlations were observed between their cholesteryl ester fatty acids. All correlation coefficients between identical fatty acids of cholesteryl esters in the droplets and the residue were positive, and with the exceptions of palmitoleic and stearic acid, significant at $P < 0.01$ or $P < 0.05$. Significant correlations also existed between different acids in droplets and residue respectively: positive for palmitoleic and oleic ($P < 0.05$), and stearic and palmitic acids ($P < 0.01$), and negative for palmitic and linoleic ($P < 0.05$), oleic and eicosatrienoic ($P < 0.81$), linoleic and palmitic ($P < 0.01$), and arachidonic and stearic acids ($P < 0.05$).

A number of correlations between lipids within each preparation were significant (Table IV). In the droplets, the triglycerides correlated positively with total phospholipids and free cholesterol and negatively with cholesteryl ester; in the residues, the total phospholipids correlated positively with free cholesterol and negatively with cholesteryl esters. Significant associations were observed among the individual phospholipids, in the droplets positive between cephalins and lecithin and in the residue negative between cephalins and sphingomyelin. Between pairs of cholesteryl ester fatty acids significant correlations were seen in the droplets: positive for palmitoleic and stearic, palmitic and oleic, palmitoleic and oleic, and eicosatrienoic and arachidonic acids; negative for palmitic and linoleic, stearic and linoleic, and oleic and eicosatrienoic acids. In the residue a positive correlation occurred between myristic and stearic acids, and a negative correlation between oleic and linoleic acids.

DISCUSSION

Lipid droplets have been separated from the tissues of atherosclerotic fatty streaks, some of their physical properties characterized and their lipids compared with the lipids of the tissue residue. The method of prepara-

TABLE III

Proportions of Various Fatty Acids in Cholesteryl Esters of Droplets and Residue of Fatty Streaks from 21 Aortas

Fatty acid: Common name	Fatty acid: Short hand*	Per cent of total fatty acids by tissue preparation: Droplets	Per cent of total fatty acids by tissue preparation: Residue	Significance of difference between droplets and residue
Myristic	14:0	1.06 ±0.36‡	0.96 ±0.22	NS
Palmitic	16:0	8.93 ±1.76	11.15 ±1.91	$P < 0.01$
Palmitoleic	16:1	5.91 ±0.67	5.26 ±1.13	$P < 0.05$
Stearic	18:0	1.96 ±0.84	1.60 ±0.57	NS
Oleic	18:1	50.37 ±3.51	38.04 ±3.98	$P < 0.01$
Linoleic	18:2	15.18 ±3.24	27.61 ±4.54	$P < 0.01$
Eicosatrienoic	20:3	7.04 ±2.01	4.69 ±1.49	$P < 0.01$
Arachidonic	20:4	6.55 ±1.88	8.15 ±2.06	$P < 0.01$

NS, not significant.
* Number of carbon atoms: number of double bonds, per molecule.
‡ Mean ±SD.

TABLE IV

Significant Interlipid Correlations within Each Class of Lipid for Both Preparations ($P < 0.01$)

Droplets		Residue	
Neutral lipids and total phospholipids:			
Triglyceride and phospholipid	+0.624 (21)*	Phospholipid and free cholesterol	+0.685 (21)
Triglyceride and free cholesterol	+0.563 (21)	Phospholipid and cholesterol esters	−0.639 (21)
Triglyceride and cholesteryl ester	−0.714 (21)		
Individual phospholipids:			
Cephalin and lecithin	+0.749 (17)	Cephalin and sphingomyelin	−0.597 (20)
Cholesteryl ester fatty acids:			
16:0 and 18:0	+0.860 (21)	14:0 and 18:0	+0.594 (21)
16:0 and 18:1	+0.616 (21)	18:1 and 18:2	−0.606 (21)
16:0 and 18:2	−0.637 (21)		
16:1 and 18:1	+0.632 (21)		
18:0 and 18:2	−0.673 (21)		
18:1 and 20:3	−0.625 (21)		
20:3 and 20:4	+0.646 (21)		

* Coefficients of correlation with number of subjects in parentheses.

tion did not appear to have significantly altered the droplets except for swelling and a mild reduction in the proportion of anisotropic forms. The mean diameter of 1.8 μ we observed agrees reasonably well with that reported for intracellular droplets in morphological studies of atherosclerotic lesions (1–3, *22–25*).

Lipid droplets have long been recognized as a prominent part of fatty streak lesions of arteriosclerosis, but their precise composition has been only sketchily described. The prominence of cholesteryl esters, particularly cholesteryl oleate, noted previously in whole lesions (3–*7*), is emphasized by our observations, as the droplet lipids consisted almost exclusively of cholesteryl esters rich in oleate. These droplets appear to be richer in cholesteryl esters than lipid mixtures of any other tissue or tissue fraction. Other classes of lipids contributed only minor proportions. The contributions of phospholipids and free cholesterol appear to have been overestimated in previous studies using qualitative techniques (11, 13). The other part of the fatty streak, the residue preparation, had a lipid composition strikingly different from that of the droplets in every variable we measured. Its proportions of the various lipids resembled very closely that of normal intima (3–6, 26). The accurate measurement of the absolute concentrations of lipids in these tissue preparation, particularly in the droplets, was not possible in the present study due to a loss of about 10% of the droplet lipids during preparation. Although there was a considerable variation among the aortas, we estimate that droplets and residue each supply on the average about 50% of the total lipid in the fatty streak tissue with a minimum of 70% of the cholesteryl esters (76% of the cholesteryl oleate) and one-third of the triglycerides located in the droplets. Almost all the phospholipids and free cholesterol were in the residue. The varying droplet content of fatty streaks probably explains the rather large variation in the lipid composition of whole lesions.

In the fatty acid composition of cholesteryl esters the sum of the proportions of oleic and linoleic acids was the same in both preparations, while the droplets had more oleic and less linoleic acids than did the residue. This difference between residue and droplets in their ratios of these two acids is the same difference as has been observed between normal and arteriosclerotic pigeon aortas (*27*), and between normal intima and fatty streaks of human aortas (3, 4). This suggests that the differences between normal and diseased tissue is due to the presence of these droplets in the lesion. The acid which is tentatively identified as eicosatrienoic acid was prominent in both preparations, in droplets even exceeding arachidonic acid. It has been observed previously in arterial tissues (3, 4, 6, *7*) and also in increased amounts in liver lipids of rats fed cholesterol (28). These observations indicate that this acid may be important in cholesterol esterification for droplets. Significant correlations among the proportions of lipids, and among the proportions of fatty acids of the cholesteryl esters in each tissue preparation may provide clues to the metabolism of these tissues. These correlations may indicate links among the metabolism of the various lipids, or fatty acids. The droplets and residue had different patterns of their correlations, suggesting differences in their metabolism, or the requirements for spacial arrangements of lipid molecules in the droplets.

Although the mixtures of fatty acids in cholesteryl esters were different in droplets and residue, they seem closely linked. All eight ester fatty acids in the residue had positive correlation coefficients with the same acids in the droplets, six of them significant. Conceivably these two ester mixtures in fatty streak tissue share a common precursor pool, but are distinguished in the selection of their specific compositions by processes occurring in the tissue. Simple infiltration and deposition of unaltered plasma lipids is an unlikely process, because the fatty acid mixture of cholesteryl esters of the droplets does not resemble the fatty acid mixtures found in cholesteryl esters of any of the lipoprotein classes of human plasma (29, 30). The major source of these complex lipids would appear to be by synthesis from lipid precursors in the lesion, as active esterification of cholesterol has been reported in metabolic studies on aortas of man (31, 32), rabbits (33, 34), and pigeons (35, 27). We postulate the presence at the interface between the droplets and the surrounding tissue of a mechanism which specifies fixed proportions of the various lipids for deposition in the droplets.

The relationship of anisotropy of droplets to their lipid composition is obscure. The variation in the proportions of anisotropic droplets among the mixtures from 21 aortas was not accompanied by major differences in the lipid composition of these mixtures. Except for trace amounts of lecithin, anisotropic and isotropic droplets seem closely similar. Studies on artificial systems of lipid and water (36) have shown that the physical structure and optical properties of a system can be altered by very small changes in the concentrations of lipid constituents and of solvent (water), by the length of the hydrocarbon chain of fatty acid components, and by their temperature. We have observed similar effects, i.e., differences in the anisotropy of mixtures with change in temperature and with small variations in lecithin, the only lipid whose proportion correlated with the proportion of anisotropic forms. Phospholipids can be decisive for the anisotropic state in lipid mixtures (37). Lecithin alone can form liquid crystals when dispersed in water, and appears to play a crucial role, as its addition to cholesterol can render this anisotropic in an aqueous system (38). Hence the 0.4% of lecithin we observed in droplets may be critical. The precise roles of cholesteryl esters and triglycerides in multicomponent mixtures similar to mixtures of biological origin are not known. Cholesterol or triglyceride alone do not form liquid crystals. The role of proteins in liquid crystals is ill defined. Proteins may play an important role in biological systems and can cause anisotropy in lyotropic systems (39). Weller, Clark, and Oswald (13) observed lipoprotein and proteolipids in droplets from arteriosclerotic lesions. In our preparation protein-like material was present in such small amounts that it could not be characterized. Additional components related or unrelated to the mesomorphic state can be incorporated in these complex liquid crystals (18). While a specific molecular configuration for these droplets cannot be postulated at present, their constant and unique lipid composition supports the idea that stoichiometric relationships may exist among the lipid components. Molar ratios of the droplet lipids estimated from our mass analysis give for cholesteryl ester, cholesterol, phospholipids, and triglycerides 94:1:1:3 using molecular weights respectively of 651, 387, 752, and 884, with oleic acid as the esterifying acid. The molar ratio of free cholesterol to lecithin is 2:1, which exceeds the limit of 1:1 observed as the maximum compatible with anisotropy in pure mixtures of these two lipids (40). This emphasizes the potential importance of other components for the anisotropic state of these droplets. It may be possible to translate the stoichiometric relationships into a specific structure for these droplets, as a lamellated ultrastructure has been reported (12).

Anisotropic lipid droplets are biologically significant, since they have been observed in human adrenal cortex and corpora lutea (8, 11). However they have not been characterized in the manner we have applied to aortic droplets.

A concept linking these droplets with the pathogenesis of arteriosclerosis can be formulated from consideration of the various chemical and physical characteristics of the droplets and their milieu. Lipid appears within cells in arteriosclerotic arterial intima in two forms of droplets, anisotropic and isotropic. The anisotropic form is the initial form, for it is predominant in younger individuals and in the early lesions. Isotropic forms become prominent in fatty streaks with increasing age and in more advanced lesions as fibrous plaques and complicated lesions (12, 11). This later appearance of isotropic forms may be due to modification of anisotropic droplets, or formation of true liquid droplets by a separate mechanism. Anisotropic droplets have a high concentration of cholesteryl ester in their lipids, and a unique structure, possibly as an ultra-structure of a lamellar array of alternating lipid and aqueous phases (12). Conceivably this structure is crucial for active metabolism at the interface between the droplets and the cytoplasm, and within the intersticies of the droplet. The mechanism synthesizing these droplets appears to be highly selective for the chemical composition of the precursor and, possibly, highly specific for the physical structure of the product. The further development or regression of the lesion may depend on the phsyical structure of the intracellular droplets, because the lipid composition near to the droplets, i.e. in the residue, does not appear to be

qualitatively different from that of undiseased intimal tissue.

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REFERENCES

1. Geer, J. C., H. C. McGill, Jr., and J. P. Strong. 1961. The fine structure of human atherosclerotic lesions. *Amer. J. Pathol.* **38**: 263.
2. Balis, J. V., M. D. Haust, and R. H. More. 1964. Electron-microscopic studies in human atherosclerosis. Cellular elements in aortic fatty streaks. *Exp. Mol. Pathol.* **3**: 511.
3. Geer, J. C., and G. T. Malcolm. 1965. Cholesterol ester fatty acid composition of human aorta fatty streak and normal intima. *Exp. Mol. Pathol.* **4**: 400.
4. Smith, E. B. 1965. The influence of age and atherosclerosis on the chemistry of aortic intima. I. The lipids. *J. Atheroscler. Res.* **5**: 224.
5. Insull, W., Jr., and G. E. Bartsch. 1966. Cholesterol, triglyceride, and phospholipid content of intima, media, and atherosclerotic fatty streak in human thoracic aorta. *J. Clin. Invest.* **45**: 513.
6. Smith, E. B., P. H. Evans, and M. D. Downham. 1967. Lipid in the aortic intima. The correlation of morphological and chemical characteristics. *J. Atheroscler. Res.* **7**: 171.
7. Smith, E. B., R. S. Slater, and P. K. Chu. 1968. The lipids in raised fatty and fibrous lesions in human aorta. A comparison of the changes at different stages of development. *J. Atheroscler. Res.* **8**: 399.
8. Kaiserling, C., and A. Orgler. 1902. Uber das Auftreten von Myelin in Zellen und seine Beziehung zur Fettmetamorphose. *Virchows Arch. Abt. A Pathol. Anat.* **167**: 296.
9. Chalatow, S. S. 1913. Uber fluessige Kristalle in tierischen Organismus, deren Entstehungsbedingungen und Eigenschaften. *Frankfurt. Z. Pathol.* **13**: 189.
10. Stewart, G. T. 1959. Liquid crystals of lipid in normal and atheromatous tissue. *Nature* (*London*). **183**: 873.
11. Stewart, G. T. 1961. Mesomorphic forms of lipid in the structure of normal and atheromatous tissue. *J. Pathol. Bacteriol.* **81**: 385.
12. Weller, R. O. 1967. Cytochemistry of lipids in atherosclerosis. *J. Pathol. Bacteriol.* **94**: 171.
13. Weller, R. O., R. A. Clark, and W. B. Oswald. 1968. Stages in the formation and metabolism of intracellular lipid droplets in atherosclerosis. An electron microscopical and biochemical study. *J. Atheroscler. Res.* **8**: 249.
14. Eggen, D. A., and L. A. Solberg. 1968. Variation of atherosclerosis with age. *Lab. Invest.* **18**: 571.
15. Annon. 1959. New weight standards for men. *Statist. Bull. Metrop. Life Ins. Co.* **40**: 2.
16. Pearse, A. G. E. 1961. Histochemistry. 2nd edition. J. & A. Churchill Ltd., London. 855.
17. Bennett, H. S. 1950. *In* McClung's Handbook of Microscopical Technique. R. McClung Jones, editor. Hafner, New York. 3rd edition. 591.
18. Brown, G. H., and W. G. Shaw. 1957. The mesomorphic state. *Chem. Res.* **57**: 1049.
19. Amenta, J. S. 1964. A rapid chemical method for quantification of lipids separated by thin-layer chromatography. *J. Lipid Res.* **5**: 270.
20. Youden, W. J. 1951. Statistical Methods for Chemists. John Wiley & Sons, Inc., New York. 16.
21. Insull, W., Jr., and G. E. Bartsch. 1967. Fatty acid composition of human adipose tissue related to age, sex, and race. *Amer. J. Clin. Nutr.* **20**: 13.
22. Laitinen, E. 1963. Changes in the elemental structures of the aorta in human and experimental atherosclerosis. *Acta Pathol. Microbiol. Scand. Suppl.* 167.
23. Still, W. J. S., and P. R. Marriott. 1964. Comparative morphology of the early atherosclerotic lesion in man and cholesterol-atherosclerosis in the rabbit. An electron-microscopic study. *J. Atheroscler. Res.* **4**: 373.
24. Weller, R. O. 1965. The ultrastructure of intracellular lipid accumulations in atheroma. *J. Atheroscler. Res.* **6**: 184.
25. Marshall, J. R., J. G. Adams, R. M. O'Neil, and M. E. DeBakey. 1966. The ultrastructure of uncomplicated human atheroma in surgically resected aortas. *J. Atheroscler. Res.* **6**: 120.
26. Boettcher, C. J. F., and C. M. Van Gent. 1961. Changes in the composition of phospholipids and of phospholipid fatty acids associated with atherosclerosis in the human aortic wall. *J. Atheroscler. Res.* **1**: 36.
27. St. Clair, R. W., H. B. Lofland, Jr., and T. B. Clarkson. 1968. Composition and synthesis of fatty acids in atherosclerotic aortas of the pigeon. *J. Lipid Res.* **9**: 739.
28. Morin, R. J., S. Bernick, J. F. Mead, and R. B. Alfin-Slater. 1962. The influence of exogenous cholesterol on hepatic lipid composition of the rat. *J. Lipid. Res.* **3**: 432.
29. Lindgren, F. T., and A. V. Nichols. 1961. Fatty acid composition of the serum lipoproteins. *Ann. N. Y. Acad. Sci.* **94**: 55.
30. Goodman, D. S., and T. Shiratori. 1964. Fatty acid composition of human plasma lipoprotein fractions. *J. Lipid Res.* **5**: 307.
31. Wahlqvist, M. L., A. J. Day, and R. K. Tume. 1969. Incorporation of oleic acid into lipid by foam cells in human atherosclerotic lesions. *Circ. Res.* **24**: 123.
32. Abdulla, V. H., C. C. Orton, and C. W. M. Adams. 1968. Cholesterol esterification by transacylation in human and experimental atheromatous lesions. *J. Atheroscler. Res.* **8**: 967.
33. Newman, H. A. I., E. L. McCandless, and D. B. Zilversmit. 1961. The synthesis of C^{14} lipids in rabbit atheromatous lesions. *J. Biol. Chem.* **236**: 1264.
34. Whereat, A. F. 1964. Lipid biosynthesis in aortic intima from normal and cholesterol-fed rabbits. *J. Atheroscler. Res.* **4**: 272.
35. Lofland, H. B., Jr., D. M. Moury, C. W. Hoffman, and T. B. Clarkson. 1965. Lipid metabolism in pigeon aorta during atherogenesis. *J. Lipid Res.* **6**: 112.

36. Luzatti, V., and F. Husson. 1962. The structure of liquid-crystalline phases of lipid-water systems. *J. Cell Biol.* **12:** 207.
37. Chapman, D. 1967. Liquid crystalline nature of phospholipids. *In* Ordered Fluids and Liquid Crystals. Advances in Chemistry Series 63. R. F. Gould, editor. American Chemical Society, Washington, D. C. 157.
38. Bangham, A. D. 1963. Physical structure and behaviour of lipids and lipid enzymes. *In* Advances in Lipid Research. R. Paoletti and D. Kritchevsky, editors. Academic Press Inc., New York. **1:** 65.
39. Stewart, G. T. 1967. Liquid crystals as ordered components of living substance. *In* Ordered Fluids and Crystals. Advances in Chemistry Series 63. R. F. Gould, editor. American Chemical Society, Washington, D. C. 141.
40. Bourges, M., D. M. Small, and D. G. Dervichian. 1967. Biophysics of lipid association. II. The ternary systems cholesterol-lecithin-water. *Biochim. Biophys. Acta.* **137:** 157.