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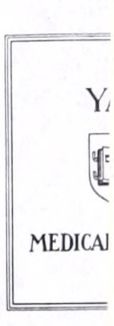

ARTERIAL PERMEABILITY TO NATIVE AND TO SUCCINYLATED PLASMA BETA-LIPOPROTEINS

by

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A Thesis

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"No man can reveal to you aught but that which already lies half asleep in the dawning of your knowledge. The teacher who walks in the shadow of the temple, among his followers, gives not of his wisdom, but of his faith and lovingness. If he is indeed wise, he does not bid you to enter the house of his wisdom, but rather leads you to the threshold of your own mind."

-Kahlil Gibran

The Prophet

This thesis is dedicated to those who have inspired me in the spirit of the above passage.

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INTRODUCTION

The blood vessels of man serve to transport cellular elements, electrolytes, proteins, carbohydrates, lipids, and water to the tissues. Since over 94% of the noncellular component of blood is water, such an aqueous medium would not be especially suitable to carry water-insoluble lipids. Yet these lipids are essential in metabolism as substrates for energy and also as building blocks for highly specific biological membranes. Man and other animals have developed a water-soluble, protein-bound form of these lipids for transportation in the blood which have been appropriately called plasma lipoproteins.

Within the past 20 years extensive research has been carried out on lipoproteins and their interaction with vessel walls, because lipoproteins have been implicated as being a causal factor in the pathogenesis of arteriosclerosis. While arteriosclerosis is not completely understood, certain facts and observations have resulted in the "lipid filtration-injury theory" or "combination

theory" to explain its pathogenesis.[1,2] This theory assumes that only small molecules such as water, glucose, free fatty acids, electrolytes, etc. are able to penetrate the intact endothelial surface of the vessel wall either by pinocytosis or by squeezing through endothelial junctions. Because of their large size, lipoproteins can not penetrate the intact endothelial surface of the artery. If, however, the endothelial surface of the vessel is subjected to an injury, physically or chemically induced, the integrity of this barrier to the passage of large proteins and lipoproteins is lost. These large molecules can then find passage into the subendothelial layers of the vessel wall. Here the lipoproteins encounter the structural elements, fibrillar proteins, and the ground substance of the vessel wall with which they may physically or chemically interact. The cells in this compartment may be active in phagocytizing the lipoprotein. Such cells are the well-known lipophages or "foam cells" of the early arteriosclerotic lesion. There follows a proliferative process of fibrosis with deposition of collagen, hemorrhage, calcification, ulceration, and thrombosis. This process can eventually lead to occlusion of the vessel wall.

As a theory the "combination theory" may explain many observations in the pathogenesis of arteriosclerosis. However, evidence for the molecular basis upon which

lipoproteins might interact with arterial wall connective tissue, including its ground substance, is nebulous. Whether such interactions are crucial in initiating the arteriosclerotic process is also not known. The question arises as to why lipoproteins are unique in their abilty to initiate it. Is it the size of the lipoprotein molecule that gives it this property, or could it be the physiochemical nature of the molecule which accounts for its behavior in injured arterial vessels?

It has been suggested that the large size of the lipoprotein molecule is responsible for its being trapped in the vessel wall. However, it is also known that lipoproteins will form insoluble precipitates with the mucopolysaccharides extracted from the ground substance of arterial walls.[3] There is no concrete evidence to refute the idea that there exist critically charged species of lipoprotein molecules which allow them to bind to the ground substance of the vessel wall, thus keeping them in the intima to be subsequently phagocytized.

The present study examines the effect of altering the surface charge of the lipoprotein molecule in relation to its permeation through the artery wall. If the permeability of the unmodified lipoprotein molecule through the vessel wall is the same as that of the modified

lipoprotein molecule, which is essentially the same size and shape as the unmodified lipoprotein molecule but with an altered surface electrical charge, it could be concluded that the size and shape of the molecule are important in retarding the passage of the lipoprotein through the vessel wall. However, if the modified lipoprotein with its uncharged surface has a markedly different permeability through the vessel wall than the unmodofied lipoprotein, then it can be postulated that, at least in the conditions of the experiment, the charged character of the lipoprotein molecule may be critical in determining the permeability of these large proteins through vessel walls.

CHOLESTEROL AND BETA-LIPOPROTEINS IN ARTERIOSCLEROSIS

There is a large body of evidence that cholesterol and its primary plasma transporting protein, beta-lipoprotein or low density lipoprotein, play a primary role in the development of arteriosclerosis. This evidence has come from a number of different sources- epidemiological, clinical, experimental, and pathological.

The Framingham Study, an epidemiological study of risk factors in coronary heart disease, showed that elevated serum cholesterol levels were the most important determining factor as to who would develop ischemic heart disease.[4] It was assumed that the vast majority of the ischemic heart disease was on an arteriosclerotic basis. The Frederickson classification of the hyperlipidemias on a genetic basis has provided further clinical evidence that hypercholesterolemia and hyperbeta-lipoproteinemia are important predisposing factors in coronary artery disease.[5]

Analytical studies of the chemical composition of arteriosclerotic lesions, foam cells, and fatty streaks have in some instances shown a striking similarity to the

chemical composition of beta-lipoproteins.[6] It seems likely that it is only the beta-lipoproteins which are agents in initiating a proliferative response in the intima of the vessel wall. Watts used flourescent labeled antibodies against beta-lipoproteins to show that these were the compounds found in foam cells and arteriosclerotic lesions.[7] Getz et al. demonstrated a localization of beta-lipoprotein antigen in foam cells.[8]

That most of the lipid found in these lesions is cholesterol suggests faulty beta-lipoprotein metabolism, since beta-lipoproteins transport 75% of the plasma cholesterol. The reason why cholesterol is the predominant lipid that accumulates, and not triglycerides or some other lipid, may be explained by the enzymes present in the vessel wall. Lipases are known to exist in the vessel wall which clear triglycerides from lipoproteins.[9] This might allow individual triglyceride molecules to diffuse freely through the vessel wall. The triglyceride-carrying lipoprotein, now free of its lipid, could readily diffuse through the vessel wall. However, the arterial vessel wall does not seem to have the enzymes needed to clear cholesterol and cholesterol esters from beta-lipoproteins. Should the beta-lipoproteins bind to some component of the ground substance, the beta-lipoprotein with its cholesterol and cholesterol esters would be rendered immobile in the intima. Defense mechanisms of the body may then be called upon to react with these entrapped

molecules. Phagocytosis of the lipoproteins by cells in the vessel wall starts the reactive proliferative response seen in arteriosclerosis.

In some experimental animals arteriosclerosis can be produced by elevating the animal's serum beta-lipoproteins. In these animals the rate of formation of these lesions is proportional to the increase in the serum lipoprotein level.[10]

THE CONNECTIVE TISSUE OF THE VESSEL WALL

The connective tissue of the arterial intima is compartmentalized between the endothelial cells that line the vessel wall and the musculature of the media, as shown in figure 1.

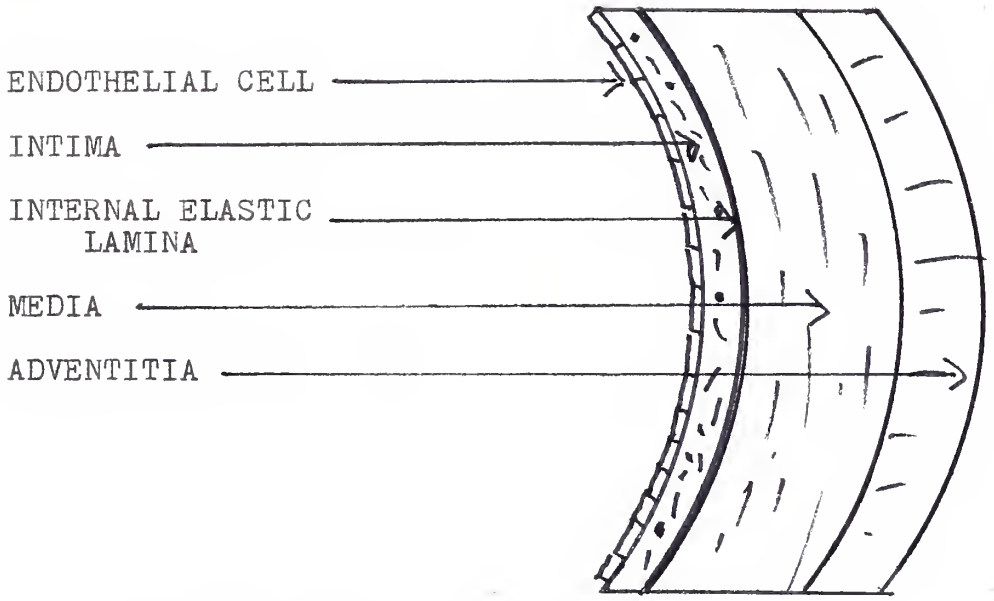


FIGURE 1- The arterial vessel wall

There are three types of structural elements in the arterial connective tissue- cells, fibrillar proteins, and compounds in the amorphous ground substance. These fibers and compounds in the amorphous ground substance could conceivably interact chemically with beta-lipoproteins

to form insoluble precipitates. Some of the cells are also capable of phagocytizing lipoproteins and lipids. Thus if the beta-lipoprotein can penetrate the endothelial cells and find passage into the intima, there is potential for both cellular and chemical interaction with the connective tissue of the intima. It is likely that some of these interactions are involved in the pathogenesis of arteriosclerosis.

Cells of the Intima

Fibroblasts, macrophages, wandering cells (probably capable of differentiating into smooth muscle cells or phagocytes), fat cells, and blood elements are the cells that inhabit the intima of a large artery of man. In terms of material production the fibroblast is by far the most important, since this versatile cell has the enzymatic machinery to produce the fibrillar proteins as well as the compounds of the ground substance.[11]

One of the enigmas in the investigation of arteriosclerosis is in defining which cells are responsible for phagocytizing lipid which has infiltrated into the intima. Evidence suggests that smooth muscle cells (wandering myoblasts of multipotential differentiation), migrate from the media into the intima where they are capable of differentiating into phagocytes which can ingest lipid and lipoproteins that have accumulated in

the intima. It is believed that these cells can also differentiate into fibroblasts and lay down collagen in response to the insult of lipid accumulation.[12]

Fibrillar Proteins of the Intima

There are three distinct fibrillar proteins in the connective tissue of the human artery- collagen, elastin, and reticulin. Collagen and elastin are the most important, since they each comprise about 25% of the dry weight of large arteries.[13] Gerontologists have shown great interest in these proteins, because molecular changes in them are believed to be important in the ageing of organisms. Since arteriosclerosis is a prominent disease in the aged, the question arises as to whether the changes in the fibrillar proteins that occur with age predispose to the development of arteriosclerosis.

Collagen is the major supporting structure of the body, and its role as such can be attributed to its high tensile strength. The molecular make-up of this molecule is helpful in explaining this important property.

Collagen is composed of a number of subunits which have an inherent proclivity to aggregate with each other. These subunits are the tropocollagen molecule which itself is composed of three peptide strands. Each of these strands is about 1,000 amino acids, and two of the three strands are identical. The three peptide strands

are cross-linked by intramolecular covalent bonds.

The amino acid hydroxylysine is unique to collagen, and along with lysine these two amino acids are responsible for the intramolecular cross-linkings between the peptide strands in the tropocollagen molecule and the intermolecular cross-linkings between tropocollagen molecules. Oxidative deamination of lysine and hydroxylysine yield the semialdehyde of α-aminoadipic acid α-aminohydroxyadipic acid, respectively. Both of these compounds can undergo aldol condensations to form these covalent cross-linkages.[14] It is interesting to note that collagen has a variable carbohydrate content depending on its age and location in the body. Sugar groups attached to hydroxylysine in ester linkage prevent the formation of the aldol bond needed to form the cross-links.[15]

Experimental lathyrism has demonstrated that the intermolecular cross-links of the collagen molecule are primarily responsible for the structural stability of the molecule.[16] However there are other contributing forces. Hydrogen bonds, Van der Waal's forces, and ionic bonds between side chains of polar amino acids all strengthen the mechanical stability of the individual tropocollagen molecules. Additional intermolecular stabilization is provided by water molecules and the amorphous ground substance.[17]

Unlike collagen fibers elastin fibers show a high degree of extensibility. Large arterial conduits contain more elastin than arterioles. The high elastin content of large arteries gives them a snap-like action which pushes blood forward in diastole in the absence of any contractile forces of the heart.

The resiliency of elastin can be appreciated by examining its molecular structure. Unique to the elastin molecule are the amino acids desmosine and isodesmosine. Containing a pyridine nucleus alkylated at the nitrogen atom and three of the ring carbon atoms, both amino acids form cross-links at each of these branched chains.[18]

Amino acid analysis of elastin shows a high percentage of apolar amino acids. It is thought that the many apolar amino acids form stable hydrophobic interactions when the elastin molecule is stretched. Thus to re-establish the more stable configuration of maximal hydrophobic interactions, the molecule spontaneously returns to its resting state when stretched.[19]

The highly apolar nature of the elastin molecule explains the affinity that elastin has for lipids and lipoproteins. The yellow streaking that is so often found in the elastin of elderly people is from lipid deposition. It is also noteworthy that elastin has the property of calcifying with age. That mucopolysaccharides with their anionic sulfate groups may be important in

attracting calcium ions to the vicinity of the elastin molecule has been postulated.[20]

Reticulin, found only in small quantities in the arterial intima, branches characteristically to form a mesh of fine reticular fibers. The fibers are 85% protein- the amino acid sequence being similar to that in collagen, 10% fatty acids, and 5% glucides. Controversy exists as to whether these fibers originate from collagen and collagen precursors, or whether their synthesis is totally independent of collagen.[21]

Ground Substance of the Intima

The ground substance of the intimal connective tissue consists of two groups of complex macromolecules, the mucopolysaccharides and the glycoproteins.

The mucopolysaccharides are a heterogeneous group of repeating disaccharide units of a hexosamine, which may or may not be sulfated, and a hexuronic acid. These polymeric chains are linked to a core protein and have a molecular weight of 1-4 x 10^6 grams.[22] Much has been done in characterizing the carbohydrate portion of these complexes, but little is known about the core protein. It appears that more than one different mucopolysaccharide can attach to the same core protein molecule. The linkage of the carbohydrate to the protein is through serine and xylose.[23]

The hexosamine and hexuronic acids are synthesized from glucose and then attached to growing chains of mucopolysaccharides by uridine nucleotides. The hexosamine molecules are formed by the amination of hexose groups, the amine groups being donated by glutamine.[24] Sulfation occurs through a donor 3-phosphoadenosine-5-phosphosulfate molecule. The sulfur atom comes from sulfur containing amino acids.[25]

Table 1 lists the mucopolysaccharides that are found in the ground substance of human arterial connective tissue.

TABLE 1-Mucopolysaccharides of the human artery

1. Chondroitin sulfate A
2. Chondroitin sulfate B
3. Chondroitin sulfate C
4. Hyaluronic acid
5. Heparin
6. Heparitin sulfate
7. Keratosulfate

Chondroitin sulfate B and heparitin sulfate are the predominant mucopolysaccharides of the vessel intima. About 0.5% of the dry weight of aorta is mucopolysaccharides.[26]

The metabolism of the mucopolysaccharides appears to be under hormonal control. Hypothyroid states cause an increase in hyaluronic acid formation and a decrease in chondroitin sulfate formation. Corticosteroids depress all mucopolysaccharide production.[27] Sex hormones have

a marked influence on the metabolism of mucopolysaccharides in rat aorta. Testosterone caused a 20% increased synthesis of rat aortic mucopolysaccharides in incubation media; whereas estradiol caused a marked inhibition of mucopolysaccharide synthesis.[28]

Mullinger and Manley correlated vessel wall mucopolysaccharide profiles of various animal species based on uronic acid content to the susceptibility of the animal to arteriosclerosis. They found that in the most susceptible animal the uronic acid content was 7.6 ug./mg. of dry weight aorta, and in the least susceptible 1.07 ug./mg. of dry weight aorta.[29] Rats and hamsters had the lowest mucopolysaccharide content per unit weight of arterial tissue, and these are two animals in which it is extremely difficult to induce arteriosclerotic changes through prolonged lipemia. Likewise, the chicken and the pig had high levels of mucopolysaccharides in their intimal ground substance, and these are animals in which it is quite easy to produce experimental arteriosclerotic changes through lipemia.[30]

Up to the age of 30 there is a progressive increase in the mucopolysaccharide content of the ground substance in human arteries, most of which is heparitin sulfate and chondroitin sulfate. In the early lesions of arteriosclerosis there is an increase in the quantity of mucopolysaccharides, but with the onset of fibrous plaques

and more extensive lesions, there is a decrease in the quantity of mucopolysaccharides.[31]

Forman and his coworkers demonstrated that sulfated mucopolysaccharide production was increased at sites of arteriosclerotic lesions. They gave sulfate labelled with S^{35} to a group of 15 rabbits. Arteriosclerosis was then induced in 7 of the rabbits by cholesterol feeding. After 12 weeks the sulfate concentration in the arterial tissue of the arteriosclerotic animals was greater than in the controls. Arterial autoradiograms showed that the greatest localization of sulfur was in arteriosclerotic lesions.[32]

Much less is known about the glycoproteins of the connective tissue matrix. Structurally they contain no uronic acids or sulfate esters. They consist of proteins linked to acetylhexosamines, neutral sugars, various derivatives of N-acetylhexuronic acid, and sialic acid. Unlike other connective tissue components, the glycoproteins are highly antigenic.[33] Murata and Kink compared sialic acid content of normal and arteriosclerotic aortas. They found that in the arteriosclerotic aortas there is a decrease in the amount of sialic acid compared to the uninvolved aortas.[34]

THE INTERACTION OF BETA-LIPOPROTEINS WITH SULFATED MUCOPOLYSACCHARIDES

In 1949 Faber showed that cholesterol was deposited in regions of human aortas that had a high sulfate-containing polyuronidecontent (sulfated mucopolysaccharides).[35]

In 1952 Walton demonstrated that sulfated mucopolysaccharides such as heparin and dextran sulfate formed insoluble complexes with fibrinogen.[36] Oncley thought that this precipitation was the result of an electrostatic interaction between the negatively charged sulfate groups of the sulfated mucopolysaccharides and the positively charged surface groups of the large fibrinogen molecule.

Oncley was interested in finding a technique for separating beta-lipoproteins from other plasma proteins. Since the beta-lipoprotein molecule is also large and has a positively charged surface at physiologic pH, he investigated whether beta-lipoproteins also share the property of precipitating mucopolysaccharides. In 1957 Oncley made the observation that low molecular

weight dextran sulfate formed soluble complexes with beta-lipoproteins, and high molecular weight dextran sulfate formed insoluble precipitates with beta-lipoproteins.[37]

The observation that beta-lipoproteins could precipitate with sulfated mucopolysaccharides was of signifigant interest to those studying arteriosclerosis. Since the arterial intima contains high molecular weight , sulfated mucopolysaccharides, it was postulated that these sulfated compounds might precipitate beta-lipoproteins in the vessel wall should the beta-lipoprotein gain access to the subendothelial space. If this were true, the entrapment of the beta-lipoprotein molecule in the intima by this mechanism might be an important consideration in the arteriosclerotic process.[38,39]

Further work on the nature of the interaction between beta-lipoproteins and sulfated mucopolysaccharides was done by Nishida and Cogan in 1970. They showed that succinylation or acetylation of beta-lipoproteins would render the modified beta-lipoprotein unable to complex with or precipitate dextran sulfate.[40]

Succinylation of beta-lipoproteins is thought to change the charge of the molecule by reacting succinate groups at positively charged amino groups of the peptide portion of the molecule. These positively charged amino groups are on the surface of the molecule, and because

of this sterically favorable location, it is no surprise that large negatively charged species such as sulfated mucopolysaccharides will precipitate the parent molecule by electrostatic forces. (see figure 2)

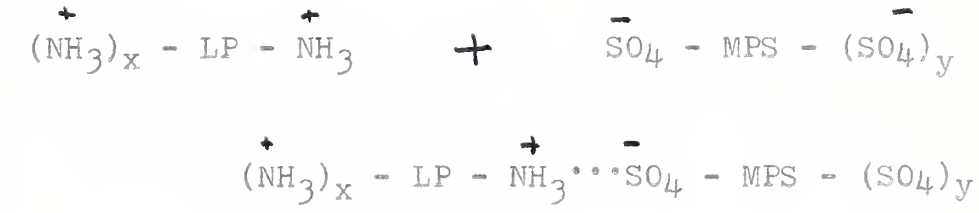


FIGURE 2- The electrostatic interaction of beta-lipoproteins (LP) and sulfated mucopolysaccharides (MPS)

The many positively charged amino groups on the lipoprotein molecule and the many negatively charged sulfate groups on the sulfated mucopolysaccharide allows for both intermolecular and intramolecular interactions.

However, succinylation of the beta-lipoprotein drastically changes this favorable charged environment for the precipitation of sulfated mucopolysaccharides by binding to the nitrogen of the reactive amino groups.[41] (see figure 3)

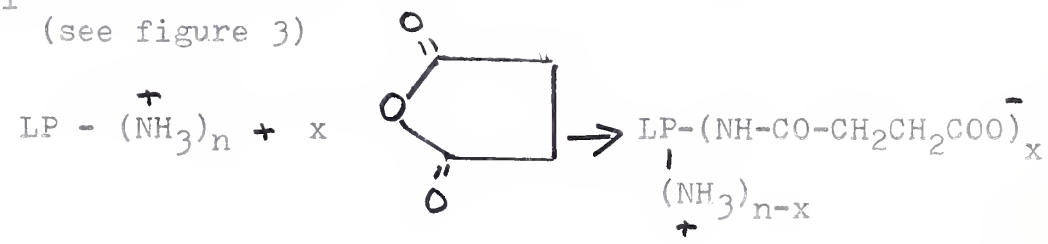


Figure 3- Succinylation of beta-lipoprotein

The nitrogen of the amino group in the modified beta-lipoprotein is now uncharged, and thus the electrostatic attraction between it and a positively charged species is decreased. This would explain why the modified beta-lipoprotein does not react with sulfated mucopolysaccharides in the test tube.

Margolis and Langdon estimated that a beta-lipoprotein molecule, having a molecular weight of about 2.5×10^6 grams, has 1200 positively charged groups and 1160 negatively charged groups at its isoelectric point. This would mean that at a pH of 7.40 there would be about 1100 positively charged amino groups.[42] Nishida and Cogan showed that by succinylating only 5% of these positively charged amino groups of the beta-lipoprotein molecule, complex and precipitate formation with dextran sulfate, and presumably other sulfated mucopolysaccharides, could be prevented.[40]

Modification of the beta-lipoprotein by succinylation only slightly increases its molecular weight. Assuming all 1200 positively charged amino groups were succinylated at pH 7.40, the molecular weight would increase by $.12 \times 10^6$ grams/mole, a 5% increase. Thus it is unlikely that any differences in the behavior of these two molecules could be attributed to differences in their size or molecular weight.

EXPERIMENTAL DESIGN

In 1950 Wilens performed a very simple experiment to determine the permeability of various plasma components through human arteries.[43] He took iliac vessels from autopsy cases within 24 hours of death, sealed one end of the vessel by inserting and tying a glass rod, and then cannulated the other end of the vessel with a glass tube. Human plasma was then added into the vessel lumen, and the composition of the filtrate that was filtered atvarious pressures was determined. Wilens discovered that while very little cholesterol (an indirect measurement of beta-lipoprotein) was found in the filtrate even at pressures as high as 320 mm. of Hg, as much as 30% of the cholesterol was unaccounted for in the filtrate and the remaining plasma in the lumen after 24 hours. This meant that the cholesterol and its transport protein were somewhere in the vessel wall.

To test the permeability of the succinylated (modified) and the native (unmodified) lipoprotein through the vessel wall, a method similar to that used

by Wilens was employed in the present experiments. Both common carotid arteries were taken from autopsies no more than 24 hours after death. The common carotid arteries were used because they have no branches and because they usually do not demonstrate far advanced arteriosclerotic lesions. The vessels were stripped of their adventitia and washed with 0.9% sodium chloride solution to assure that the vessels were devoid of any contaminating material. This process was quite satisfactory, for cholesterol determinations on these washings were zero.

A short glass rod of slightly smaller diameter than the vessel was inserted 5 mm. into one end of the vessel, and coarse thread was securely tied around the vessel upon the glass rod to make as tight a seal as possible.

The vessel was cut to 2.5 cm., and the other end of the vessel was cannulated with a 5 ml. pipette. Leaks were tested for by submerging the vessel in water and subjecting it to a pressure of 300 mm. Hg pressure. Only vessels which could maintain this pressure for 5 minutes were used.

The same procedure was then carried out on the other carotid segment. The open ends of the pipettes were connected to a T-tube, and the other end of the T-tube was connected to a bulb-operated sphygmomanometer. Both vessels were then put into glass tubes containing

0.9% sodium chloride solution. (see figure 4)

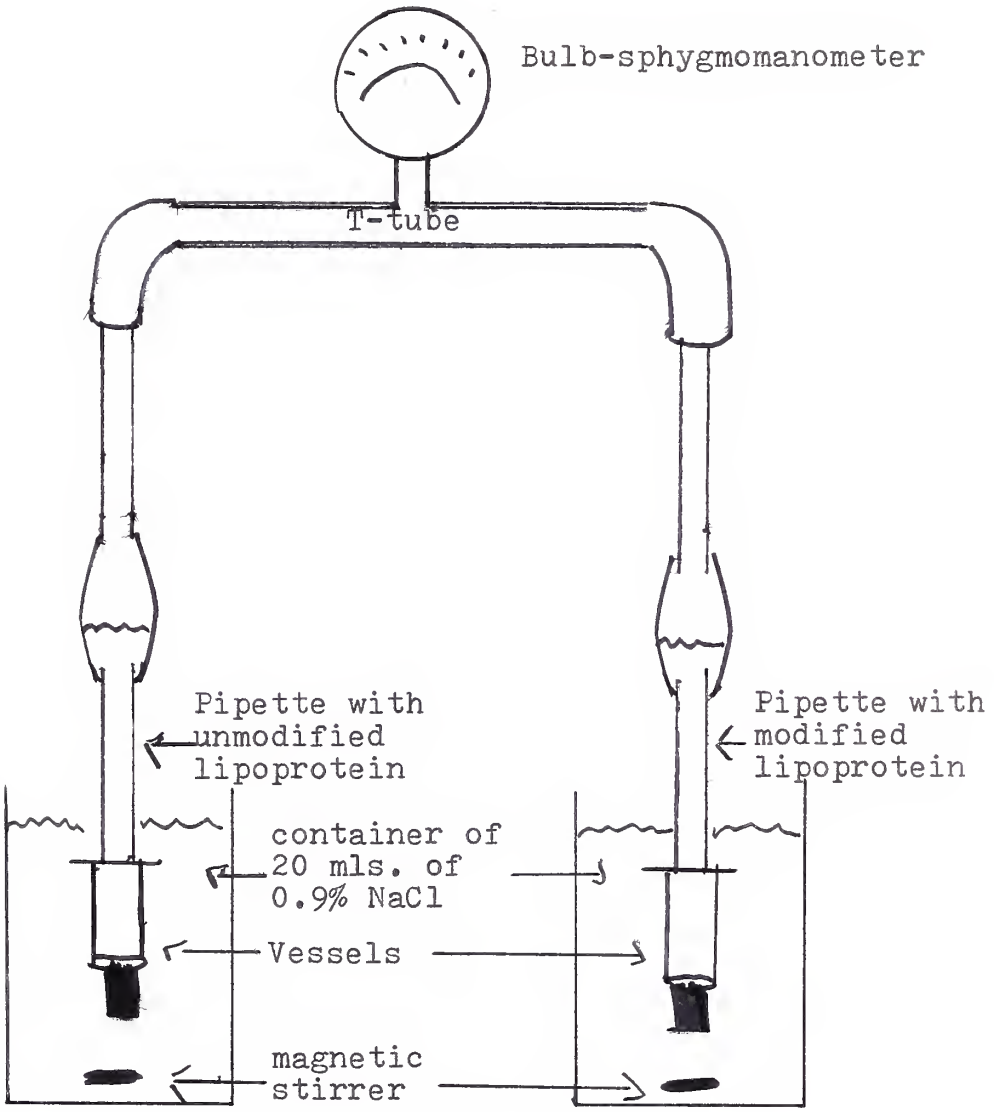
Three mls. of the unmodified lipoprotein solution were put into one pipette, and three mls. of the modified lipoprotein solution were put into the other pipette. Small magnetic stirrers were added to each glass container, and the bathing solutions were gently stirred. The pressure was then raised to 300 mm. Hg and was kept there for 24 hours. The experiment was carried out at room temperature.

All vessels were cut to 2.5 cm. and placed in 20 mls. of 0.9% sodium chloride solution except on trial 1. In this trial 30 mls. of distilled water were used as the bathing solution, and the vessels used were cut to 5 cm. in length. Also only 2 mls. of lipoprotein solution were put into each pipette in trial 1.

After 24 hours the pressure was released. The volume and cholesterol content in each of the vessels and in each of the bathing solutions was then determined. Frozen sections of each of the vessels following the 24 hour exposure to the lipoprotein solutions, as well as control sections on vessel segments not exposed to the solutions in the pressurized <u>in vitro</u> system, were obtained. These were cut at 15-20 u, after the vessels were fixed in formalin for 48 hours. The frozen sections were stained with Sudan IV by a modification of the technique used by Putt and Chiffelle.[44]

Thus the experiment utilized pieces of vessel from the same person, of the same dimensions, and with the same amount of arteriosclerotic change. The vessels were exposed to the same amount of lipoprotein at the same pressure for the same amount of time. The only difference was in the nature of the lipoprotein solution in each vessel.

Figure 4- EXPERIMENTAL APPARATUS



Preparation of beta-lipoprotein solutions

Beta-lipoproteins were separated from four liters of pooled, human A.C.D. plasma by the following method. Calcium chloride was added to precipitate the fibrinogen. The clotted fibrinogen was removed by filtration. Carbowax 45 was then added to precipitate the plasma globulins. The precipitate was redissolved, and the carbowax was removed by multiple washings in water at low ionic strength, leaving only the plasma proteins in solution. The low density, beta-lipoproteins were finally isolated by ultracentrifugation for 18 hours at 38,000 r.p.m. in 10% saline. (density-1.063)

Twenty-eight mls. of beta-lipoprotein were obtained by this technique. Electrophoresis of this fraction on cellulose acetate strips gave a single, sharp band.

Ten mls. of this beta-lipoprotein fraction were succinylated by the method suggested by Klotz.[41] One and a half grams (.01 moles) of succinic anhydride were slowly added to 10 mls. of the beta-lipoprotein in 25 mls. of distilled water. The pH of the solution was monitored electronically, and as the succinic anhydride was added the pH was kept between 7.0-8.0 by the addition of 10 M sodium hydroxide. When the succinylation was completed, the solution was dialyzed against cold, running water for 24 hours. The final volume was 54 mls.,

and the pH of the solution was adjusted to 7.40. Five hundred milligrams of sodium chloride were added to make the solution 0.9% sodium chloride.

Ten mls. of the unmodified stock solution were then diluted to 54 mls. with distilled water, and the pH of this solution was adjusted to 7,40. Five hundred milligrams of sodium chloride were added to this solution to make it also physiologic with respect to salt concentration.

Total cholesterol concentrations of the modified and unmodified solutions were done by the Sperry and Brand method.[46] The cholesterol concentrations of the prepared solutions were:

UNMODIFIED	BETA-LIPOPROTEIN	1175 mg.%
MODIFIED	BETA-LIPOPROTEIN	1175 mg.%

These two solutions were used on trials 1-7. After the seventh trial new solutions were made by the same procedure as described above. These solutions were used in trials 8-13. The total cholesterol concentrations of these solutions were:

UNMODIFIED	BETA-LIPOPROTEIN	1025 mg.%
MODIFIED	BETA-LIPOPROTEIN	1050 mg.%

Electrophoresis on cellulose acetate strips showed that the unmodified beta-lipoprotein migrated toward the anode; whereas the modified beta-lipoprotein migrated toward the cathode. (see figure 5)

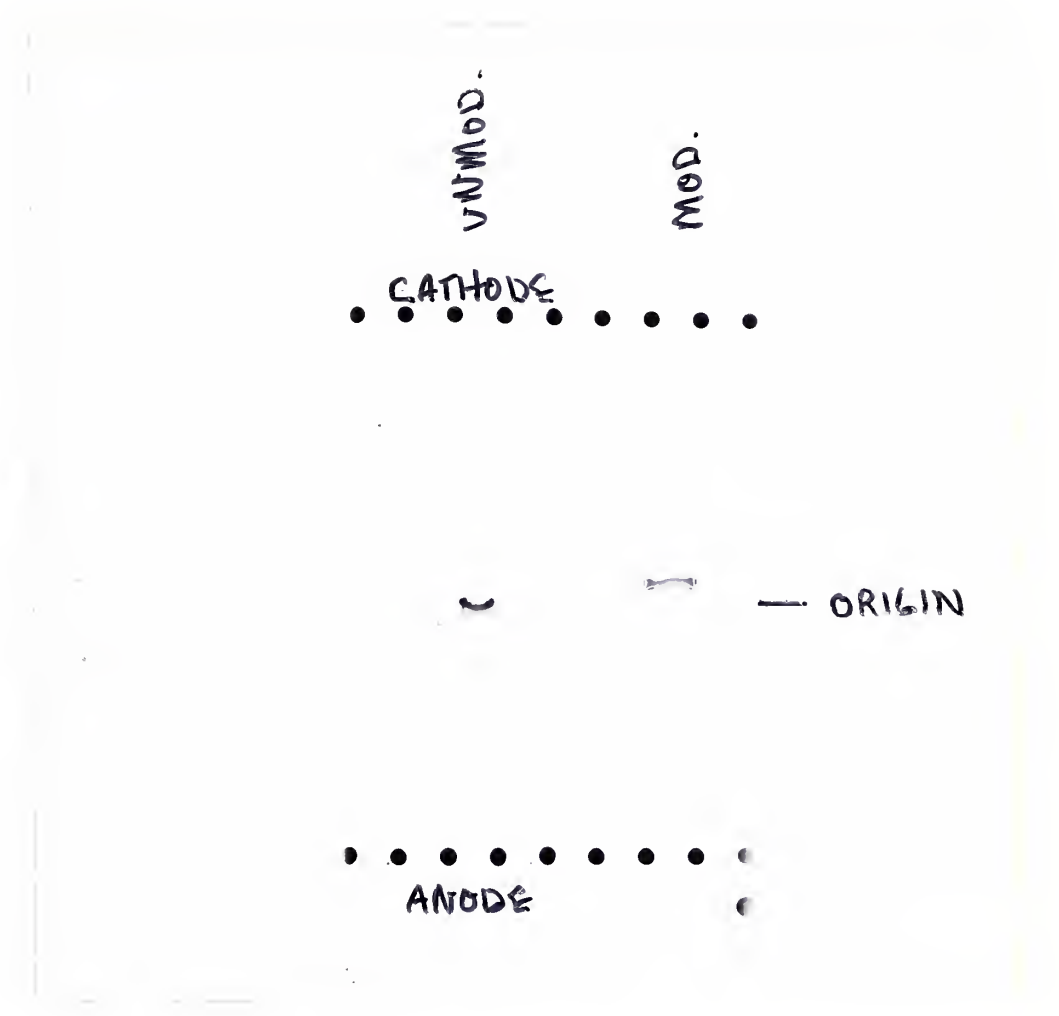


Figure 5- Electrophoresis of unmodified and modified beta-lipoprotein fraction on cellulose acetate strips.

When 0.1 mls. of the sulfated mucopolysaccharide heparin (1,000 units/ml.) were added to the unmodified beta-lipoprotein, a precipitate was observed; but no precipitate appeared when 0.1 mls. of heparin were added to 0.1 mls. of the modified beta-lipoprotein. This confirmed the expected behavior of the two beta-lipoproteins in the presence of sulfated mucopolysaccharides.

Nishida and Cogan found that if at least 5% of the free amino groups of the beta-lipoprotein molecule were succinylated at pH 7.40, this modified beta-lipoprotein would not precipitate dextran sulfate.[40] They correlated the ratio (moles of succinic anhydride/moles of lysine in the beta-lipoprotein molecule) to the per cent of free amino groups succinylated. They found that at a ratio of 5/1, 76% of the free amino groups were succinylated; at a ratio of 15/1, 96% of the free amino groups were succinylated; and at a ratio of 50/1, 97% of the free amino groups were succinylated.

In succinylating the 10 grams of beta-lipoprotein it was decided to use a ratio of 10/1, since this would succinylate about 85% of the free amino groups. This would be much greater than the minimum of 5% needed to prevent precipitation with dextran sulfate, and presumably other sulfated mucopolysaccharides.

If a ratio of 10/1 is used, then 10 grams of beta-lipoprotein require about 0.015 moles of succinic anhydride, or 1.5 grams.

Using the following:[47]

2.3 grams of beta-lipoprotein/mole
350 moles of lysine/ mole of beta-lipoprotein

$$\frac{\text{10 moles of succinic anhydride}}{\text{1 mole of lysine}} \times \frac{\text{350 moles of lysine}}{\text{1 mole of beta-lipoprotein}} \times \frac{\text{1 mole of beta-lipoprotein}}{2.3 \times 10^{6} \text{ grams}} \times \text{10 grams of beta-lipoprotein}$$

$$= 1.52 \times 10^{-2} \text{ moles of succinic anhydride}$$

$$= 1.5 \text{ grams}$$

RESULTS

The results of the experiment on 13 sets of vessels are shown in tables 2 and 3.

Table 2 indicates changes in volume of lipoprotein solutions in the vessels' lumens after they were subjected to a pressure of 300 mm. Hg for 24 hours. The amount of cholesterol in the bathing solution after this 24 hours is also given for each trial. Cholesterol and volume determinations were carried out on 12 of the 13 trials. The amount of cholesterol in the vessel wall was obtained by subtracting the cholesterol in the bathing solution and the cholesterol in the lumen at the end of the 24 hours from the initial quantity of cholesterol in the lumen of the arterial segments.

Of the 12 trials on which permeability parameters were obtained, 9 of these showed virtually no unmodified or modified beta-lipoprotein in their respective bathing solutions after 24 hours of exposure in the pressurized *in vitro* test system. Two of the 12 trials (9 and 10) showed increased permeability in both vessels, and in

one trial (8) there was increased permeability to the native beta-lipoprotein but not the succinylated one.

The volume changes in the lipoprotein solutions within the vessel lumen were quite variable. While as much as 1.7 mls. of the unmodified and 2.1 mls. of the modified samples remained within the lumen on certain trials, there were trials when no measurable volume was left.

There was no consistency as to the amount of cholesterol that was calculated to be in the vessel walls. In 5 of the 12 trials there was more cholesterol in the vessel wall exposed to the unmodified beta-lipoprotein solution, and in 4 of the 12 trials there was more cholesterol in the vessel wall exposed to the modified lipoprotein solution. In 3 of the 12 trials there was approximately the same quantity (within one milligram) of cholesterol in each of the vessel walls. The quantity of cholesterol in the vessel wall was also quite variable- from 8.0 to 28.5 mg. with vessels exposed to the native beta-lipoprotein and from 8.6 to 31.4 mg. with those exposed to the modified beta-lipoprotein.

On trials 7,8,9, and 10 none of the modified lipoprotein solution was left in the vessel lumen after the 24 hours at 300 mm. Hg pressure. It was noted that the formalin solutions in which these vessels were placed after the experiment became quite turbid. Even in those

trials where some of the lipoprotein solution remained in the vessel lumen after 24 hours, a slight cloudiness was noted in the formalin solutions fixing the vessels that were exposed to the modified beta-lipoprotein solution. In contrast the formalin solutions fixing the vessels that were exposed to the unmodified beta-lipoprotein solution remained clear in all of the trials.

Table 3 gives the histologic findings of frozen sections of control vessels and the vessels after exposure to the lipoprotein solutions in the pressurized, in vitro system. This table shows that there was a marked difference in the histologic appearance of the vessels exposed to the unmodified beta-lipoprotein solution and of the corresponding vessels exposed to the modified beta-lipoprotein solution. In all 13 trials the histology of the vessel subjected to the unmodified beta-lipoprotein solution revealed a massive accumulation of lipid at the intimal surface with varying degrees of penetration into the intima. In only two of the 13 trials (4 and 7) was there any lipid accumulation at the intimal surface with the modified lipoprotein solution. In these two instances the sudanophilia was not as intense or extensive as that seen with the vessels exposed to the unmodified beta-lipoprotein solution. In the other 11 trials the vessel exposed to the modified lipoprotein solution was histologically comparable in all respects to the control.

Hematoxylin and eosin stains were done on the vessels used in trials 10 and 13. These showed that the vessels exposed to the pressurized, *in vitro* system did not lose their cellular or compartmental integrity and were comparable to their corresponding control.

Photomicrographs of representative frozen sections stained for lipid from trials 8 and 13 are shown in the following pages.

Table 2- Cholesterol and Volume Changes in Vessel Lumen Solution and Bathing Solution After 24 Hours at 300 mm. Hg Pressure

	UNMODIFIED						MODIFIED					
Trial	Vol. in vessel lumen INITIAL (mls.)	Vol. in vessel lumen FINAL (mls.)	Chol. in vessel lumen INITIAL (mgs.)	Chol. in vessel lumen FINAL (mgs.)	Chol. in bathing solution FINAL (mgs.)	Chol. in vessel wall (mgs.)	Vol. in vessel lumen INITIAL (mls.)	Vol. in vessel lumen FINAL (mls.)	Chol. in vessel lumen INITIAL (mgs.)	Chol. in vessel lumen FINAL (mgs.)	Chol. in bathing solution FINAL (mgs.)	Chol. in vessel wall (mgs.)
1.	2.0	0.6	23.5	9.3	less than 0.05	14.1	2.0	0.6	23.5	13.0	less than 0.05	10.4
2.	3.0	1.7	35.3	24.0	less than 0.05	11.2	3.0	1.8	35.3	23.0	less than 0.05	12.2
3.	3.0	1.2	35.3	12.7	less than 0.05	22.5	3.0	1.4	35.3	18.3	less than 0.05	16.9
4	3.0	1.4	35.3	21.2	less than 0.05	14.0	3.0	1.0	35.3	19.3	1.2	14.8
5	3.0	1.6	35.3	22.4	less than 0.05	12.8	3.0	1.8	35.3	20.3	less than 0.05	14.9
6	3.0	0.6	35.3	14.4	0.40	20.5	3.0	1.1	35.3	26.5	less than 0.05	8.7
7	3.0	0.2	35.3	6.7	less than 0.05	28.5	3.0	0.0	35.3	0.0	less than 0.05	35.2

Table 2-continued

	UNMODIFIED						MODIFIED					
Trial	Vol. in vessel lumen INITIAL (mls.)	Vol. in vessel lumen FINAL (mls.)	Chol. in vessel lumen INITIAL (mgs.)	Chol. in vessel lumen FINAL (mgs.)	Chol. in bathing solution FINAL (mgs.)	Chol. in vessel wall (mgs.)	Vol. in vessel lumen INITIAL (mls.)	Vol. in vessel lumen FINAL (mls.)	Chol. in vessel lumen INITIAL (mgs.)	Chol. in vessel lumen FINAL (mgs.)	Chol. in bathing solution FINAL (mgs.)	Chol. in vessel wall (mgs.)
8.	3.0	0.0	30.8	0.0	3.8	27.0	3.0	0.0	31.5	0.0	less than 0.05	31.4
9.	3.0	0.0	30.8	0.0	8.3	22.5	3.0	0.0	31.5	0.0	8.3	23.2
10.	3.0	0.5	30.8	11.5	11.3	8.0	3.0	0.0	31.5	0.0	1.3	30.2
11.	3.0	1.0	30.8	12.1	less than 0.05	18.6	3.0	1.3	31.5	14.1	less than 0.05	17.3
12.	NOT OBTAINED											
13.	3.0	0.7	30.8	10.3	less than 0.05	20.4	3.0	2.1	31.5	22.8	less than 0.05	8.6

Table 3- Histology of Arterial Vessel Frozen Sections Stained with Sudan IV

Trial	Control	Unmodified	Modified
1. 60 year old white male diabetic	moderate intimal thickening focal lipid deposits in the intima and internal elastic lamina diffuse lipid deposits in the media	layer of sudanophilic material on endothelial surface penetrating the first few layers of intima	comparable to control
2. 50 year old white male post coronary artery by-pass surgery	large plaque with focal lipid deposits around it intima relatively thin with little lipid in it extensive, diffuse lipid in media	intense sudanophilic staining of endothelial surface and intima diffuse staining throughout vessel including media and adventitia	comparable to control
3. 45 year old white female metastatic GI cancer	relatively thin intima lipid staining of internal elastic lamina diffuse lipid staining of media in droplet form	intense sudanophilic staining of endothelial surface penetrating the first few layers of intima	comparable to control
4. 70 year old white male chronic obstructive pulmonary disease	plaque with accumulation of lipid around it scattered lipid deposition in droplet form in media	endothelial layer of sudanophilia with some penetration into intima	some area of endothelium comparable to control, others comparable to unmodified
5. 59 year old white male hypertension diabetes	large, atheromatous plaque with focal lipid staining around it diffuse lipid staining in droplet form in media focal lipid staining in intima	amorphous sudanophilia at intimal surface with little penetration into intima piling up of lipid around plaque	comparable to control much less lipid around plaque compared to unmodified

Table 3- continued

Trial	Control	Unmodified	Modified
6. 65 year old white female metastatic ovarian carcinoma	large plaque with focal lipid deposition around it internal elastic lamina stained scattered droplets of lipid in intima	accumulation of sudanophilic material at endothelial surface with little intimal penetration intense lipid accumulation around plaque	comparable to control
7. 36 year old white female Hodgkin's disease	very thin intima only lipid is found in the internal elastic lamina	lipid staining material at intimal surface with penetration into intima	some accumulation of lipid at endothelial surface, not as marked as unmodified
8. 33 year old white female breast cancer	very thin intima only lipid is found in the internal elastic lamina	lipid deposition at the endothelial surface with penetration of first few layers of intima	comparable to control
9. 31 year old black male sickle cell anemia	slight intimal thickening some diffuse lipid in media in droplet form	sudanophilic material at intimal surface which penetrates into the first few layers of intima	comparable to control
10. 52 year old white male suicide	moderate arteriosclerotic changes focal lipid accumulation around plaques	sudanophilic staining at the intimal surface, not as intense as in other unmodified sections	comparable to control

Table 3-continued

Trial	Control	Unmodified	Modified
11. 51 year old white male myocardial infarction	some intimal thickening only lipid is found in the internal elastic lamina	intense sudanophilic staining at endothelial surface penetrating the first few layers of the intima	comparable to control
12. 88 year old black male renal failure	very thick intima local lipid deposits in the intima lipid droplets in the media	accumulation of sudanophilic material at the intimal surface penetrating the first few layers of intima	comparable to control
13. 14 year old black male leukemia	small foam cell plaque rest of vessel wall is free of lipid	massive sudanophilic staining material at endothelial surface penetrating the first few layers of intima	comparable to control

DISCUSSION

The results of the experiment can be discussed in terms of differences in the permeability of the native and the modified lipoprotein molecules as measured by the movement of cholesterol through the vessel wall, and in terms of differences in the localization of lipid in the vessel wall as demonstrated by the histology of the fat stained frozen sections of the vessels. An explanation of the turbidity observed in the formalin solutions is advanced as an aid in correlating the major findings.

The majority of the trials showed the vessels to be quite impermeable to both lipoprotein solutions, as measured by the amount of cholesterol in the bathing solution. However, there were a few vessels which showed increased permeability to the solutions they contained. While preliminary testing for leaks was done on all vessels used, these instances of increased permeability probably arose from minute leaks that went undetected in the preliminary screening, or from leaks that

developed during the 24 hours in which the pressure was maintained. The histologic findings, which will subsequently be discussed in detail, are in agreement with this conclusion, as they show no lipid staining material beyond the intima into the media and adventitia, except in one instance (2-unmodified). Thus the intact arterial vessel wall proved to be an effective barrier to the passage of both the unmodified and modified beta-lipoproteins from the vessel lumen into the bathing solution.

As table 3 indicates the striking histologic accumulation of sudanophilic material at the intimal surface of the vessels exposed to the native lipoprotein solution, but not of those segments exposed to the modified lipoprotein solution is a consistent finding. This is the most important observation of the experiment, as it parallels the in vitro behavior of the modified and unmodified beta-lipoproteins in the presence of sulfated mucopolysaccharides. Since all other variables were the same for both vessels in each trial except for the nature of the lipoprotein solution that they contained, the histologic difference observed must be explained in terms of differences in the modified and unmodified lipoprotein solutions themselves. It is not possible from the present experiments to characterize the state of the lipid observed histologically at the intimal surface of vessels exposed to the native lipoprotein-

whether it is in the same unbound form as it is in the solution, or whether it has reacted with one or more components of the intimal connective tissue. Extraction and chemical analysis would be necessary.

However, the possibility exists, based on the known in vitro chemistry of these lipoprotein solutions, that the observed lipid accumulation is a result of binding in the intima or at the intimal surface between the native beta-lipoprotein and the sulfated mucopolysaccharides of the arterial connective tissue. Beta-lipoproteins modified by succinylation, which have lost their electrostatic potential to react with sulfated mucopolysaccharides, accordingly would not be expected to be bound at the intimal surface or in the underlying subendothelial space.

The ability of the native beta-lipoprotein to combine with the sulfated mucopolysaccharides of the intima may be the property which makes beta-lipoproteins unique in their ability to initiate the arteriosclerotic process. The vessel wall may be quite impermeable to large molecules such as the unmodified and modified beta-lipoproteins in the sense that these molecules can not easily traverse the wall's entire thickness. However, given the opportunity to penetrate into the intima by the application of extremely high pressure or previous vessel wall injury, the native beta-lipoprotein may enter the subendothelial compartment and become bound

by sulfated mucopolysaccharides. Cellular defense mechanisms may now come into play in this altered intimal state, and the chronic process of arteriosclerosis begins.

On the other hand, the modified beta-lipoprotein in such circumstances would remain in the vessel lumen, because there are no electrostatic forces between the lipoprotein and the sulfated mucopolysaccharides to attract the lipoprotein into the intima. Thus these proteins would not be expected to act as a stimulus for the initiation of the arteriosclerotic response.

Electron microscopic research on the vessel wall of of small blood vessels has revealed a narrow, electron dense band that lies between the endothelial cell and the vessel lumen.[48] It is not certain whether this represents a basement membrane, or whether it is just an artifact. With autoradiographic studies Curran demonstrated that endothelial cells of small capillaries accumulated radioactive S^{35}, suggesting that these cells might secret sulfated mucopolysaccharides; and that in fact this basement membrane may be composed of mucopolysaccharides similar to those in the intima.[49] Whether or not this situation holds true for larger vessels such as the aorta and the carotids is not known. However, if it were so, then this compartment of high sulfated mucopolysaccharide concentration may also be responsible for some of the lipoprotein binding

observed in our experiments.

Other possibilities could be entertained to explain the different histology that was observed in the vessels exposed to the two different lipoprotein fractions. The accumulation of lipid at the intimal surface that was seen with the native beta-lipoprotein may merely be precipitation of the protein, an aggregation that is catalyzed by surface contact with the vessel wall. The altered lipoprotein might be protected against this phenomenon because of its different molecular make-up. This hypothesis is unlikely for two reasons. First, the lipid accumulation did not have the histologic appearance of massive protein self-aggregation at the intimal surface, and in some instances penetration into the intima could be appreciated. It should be mentioned that the physical characteristics of the unmodified lipoprotein solutions within the vessel lumen remained the same throughout the experiments and showed no evidence of precipitation. Second, in experiments similar to ours Wilens demonstrated penetration of lipid through the entire intima in vessels containing plasma that were subjected to a pressure of 300 mm. Hg for 36-48 hours.[43]

The incidental finding of turbidity in the formalin solutions containing the vessels exposed to the modified lipoprotein solution, but not in the formalin

solutions containing vessels exposed to the unmodified beta-lipoprotein solution has implications in explaining the results described. It was concluded that this turbidity was due to modified lipoprotein that was physically adherent to the vessel wall and was washed off and precipitated in the formalin solution. Thus, with the vessels subjected to the modified lipoprotein solution, some of the cholesterol is accounted for by its being loosely attached to the vessel wall; and the amount of cholesterol deposited in the vessel wall by the modified protein carrier is less than table 2 indicates. In fact since the histology of vessels exposed to the modified beta-lipoprotein solution showed no lipid accumulation within the vessel wall (except in the two instances mentioned), it would be expected that there should be very little cholesterol in the vessel wall, and that the amount of cholesterol should be much greater for those vessels exposed to the native lipoprotein solution. Our permeability results did not show any real difference in the amount of cholesterol in the vessel wall deposited by the unmodified versus the modified beta-lipoprotein. However, if the amount of cholesterol that was found in the formalin solution were also subtracted from the initial quantity of cholesterol in the modified beta-lipoprotein solution, these results would very

likely show this expected difference.

It was noticed that with the unmodified beta-lipoprotein lipid deposition was quite marked around plaques. It was mentioned in the basic discussion of the connective tissue that there is evidence that sulfated mucopolysaccharides are found in increased amounts in these areas.[31] It would thus not be surprising to find intensely staining lipid material in these regions of high sulfated mucopolysaccharide concentration.

Other pieces of evidence have been mentioned which support the hypothesis that sulfated mucopolysaccharides are important in the pathogenesis of arteriosclerosis. The increased susceptibility of animals with higher intimal concentrations of mucopolysaccharides[29,30], and the increased incorporation of radioactive S^{35} into arteriosclerotic lesions induced in rabbits[32] suggest this. The protection of the female, presumably by estrogens, could be explained by the effect estrogens have been shown to have on decreasing mucopolysaccharide synthesis in the intima.[28]

The recent emphasis that has been given lipids and lipoproteins in atherogenesis has tended to divert attention from the part that the vessel wall plays in this disease. Hyperlipemic states do not necessarily cause arteriosclerosis- they may merely accelerate it. Arteriosclerosis in the light of

modern evidence probably results from an interaction between ambient plasma lipoproteins and elements in the vessel wall, an interaction which is made possible through previous vessel wall injury.

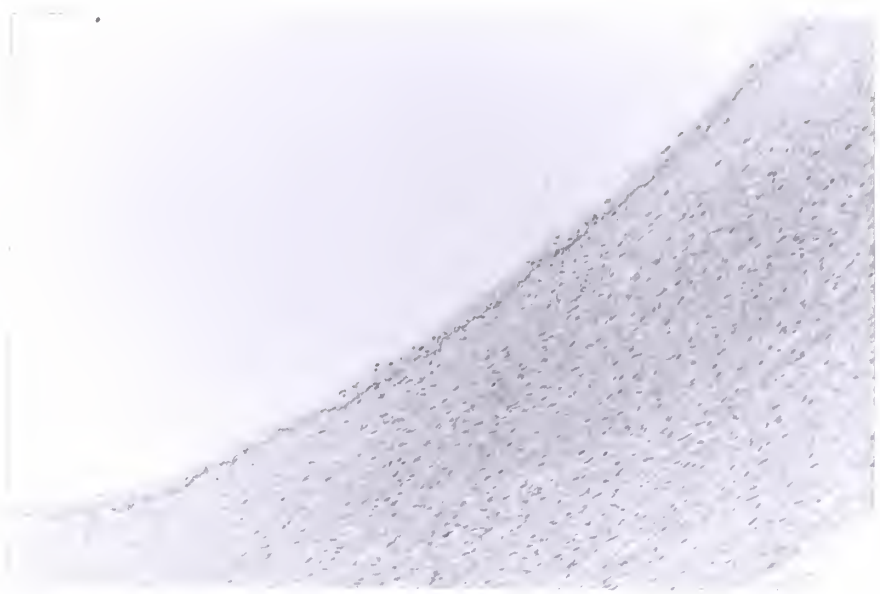
The arterial intima has remained an enigma to investigators. It is paradoxical but in order to study the intima and the cellular elements and substances that comprise it, it has been necessary to disrupt the natural environment of this organ. In doing this the actual relationships that exist in the intima and the functional sequences that occur in it are obscured. Perhaps when the structure and function of this minute vessel compartment are understood, an unequivocal pathogenesis of arteriosclerosis can be defined.

SUMMARY

This experiment has studied the permeability characteristics of native and of succinylated human plasma beta-lipoproteins through human arteries *in vitro*. In the conditions of the experiment the vessels were impermeable to both solutions. However, there was evidence that cholesterol was bound to any great extent only in the vessels exposed to the unmodified beta-lipoprotein. Furthermore, the histology of the vessels showed this lipid accumulation to be localized at the intimal surface with penetration into the first subendothelial intimal connective tissue layers.

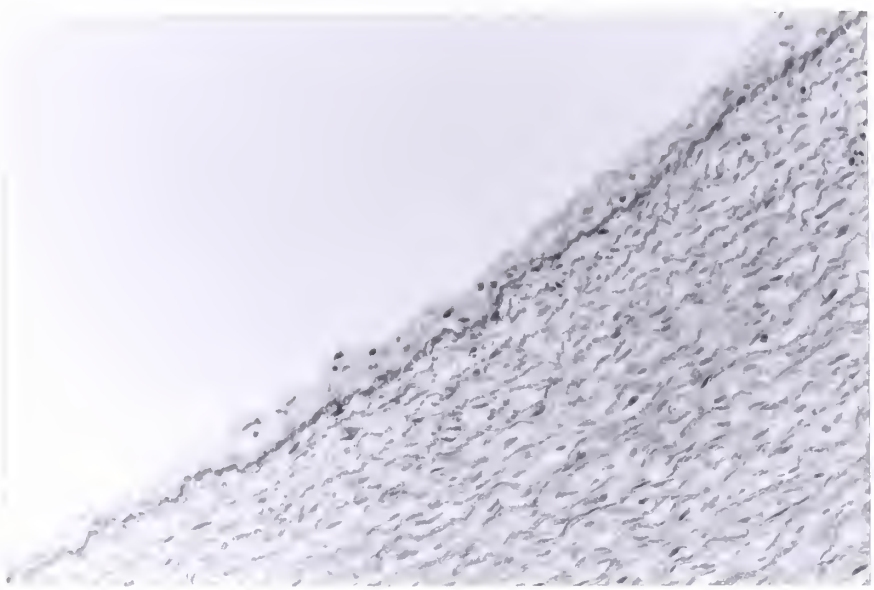
An explanation of these findings is advanced on the basis of the *in vitro* reactions of these molecules with sulfated mucopolysaccharides.

PHOTOMICROGRAPHS



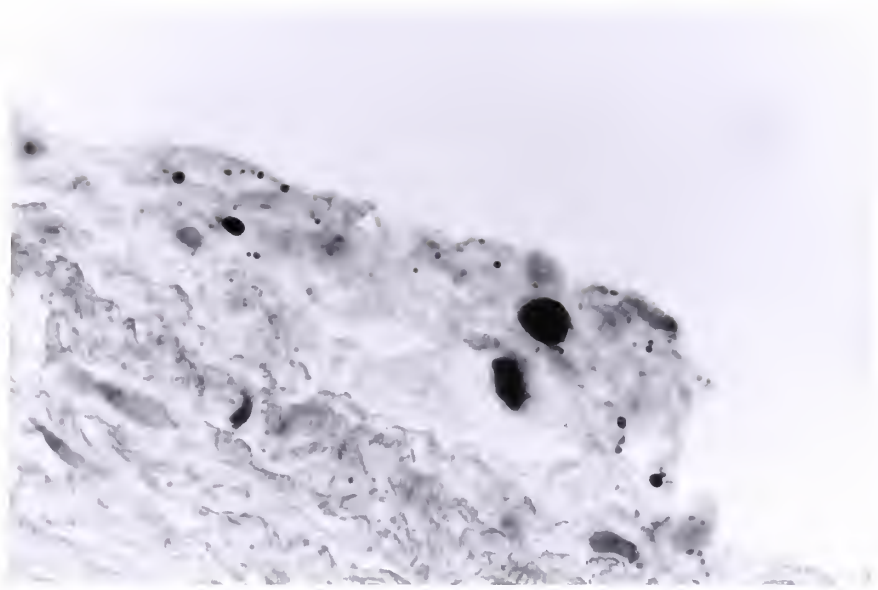
PHOTOMICROGRAPH 1. Control 13 x40 Sudan 1V

No stigmata of advanced arteriosclerosis. The intima is thin, and there are no plaques or areas of lipid accumulation except in internal elastic lamina (black line).



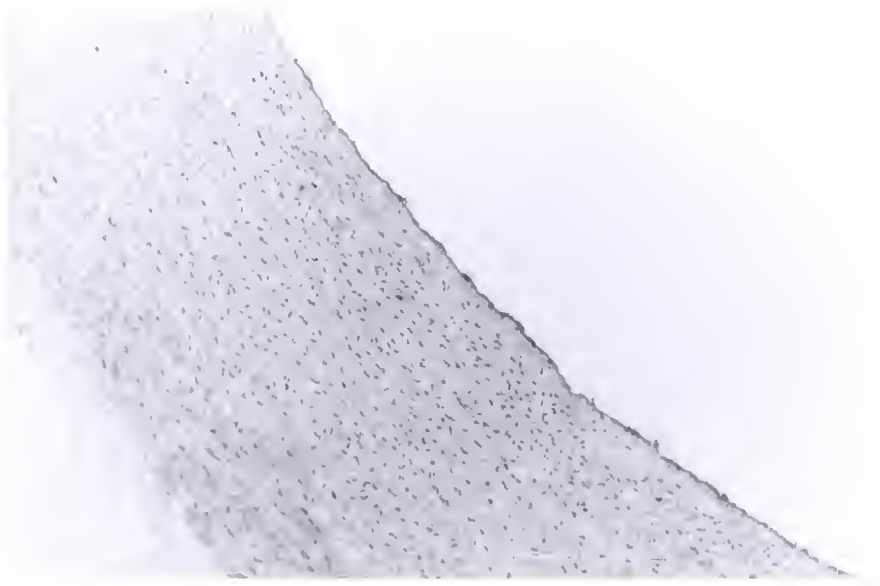
PHOTOMICROGRAPH 2. Control 13 x100 Sudan 1V

Scattered black dots in intima are probably lipid. Internal elastic lamina again clearly outlined as a black line.



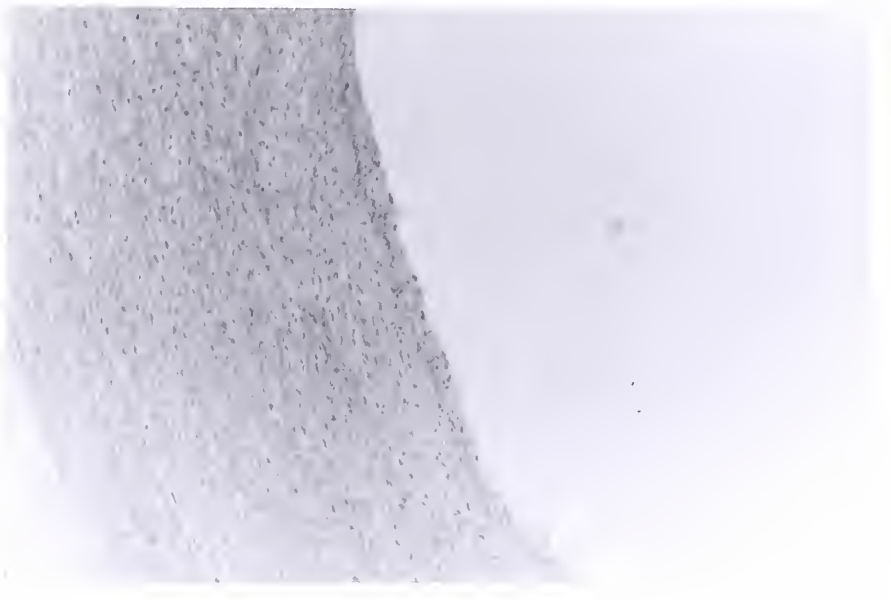
PHOTOMICROGRAPH 3. Control 13 x450 Sudan 1V

High power of intima. Large and small black dots are scattered lipid in intima.



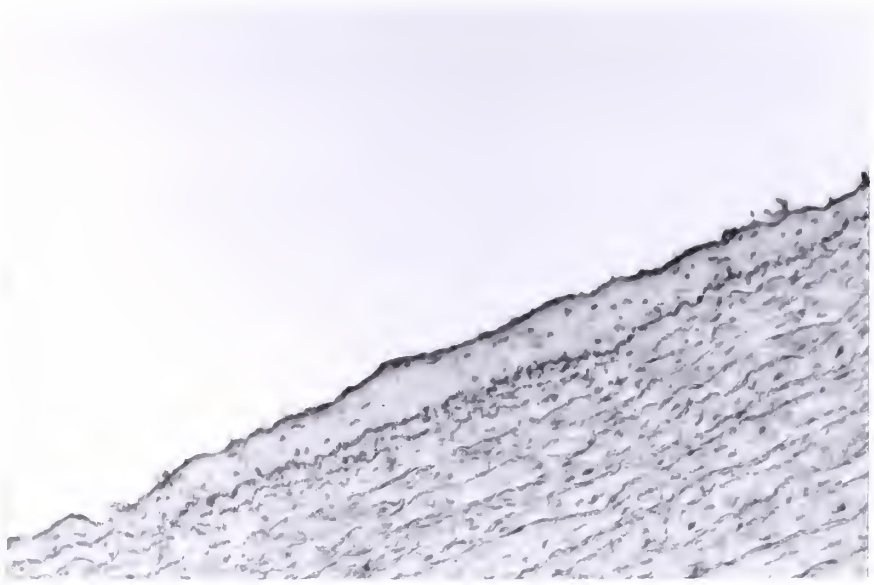
PHOTOMICROGRAPH 4. Unmodified 13 x40 Sudan 1V

Black line at intimal surface is lipid deposited from lipoprotein solution.



PHOTOMICROGRAPH 5. Modified 13 x40 Sudan 1V

Comparable to control. No lipid deposit at intimal surface.

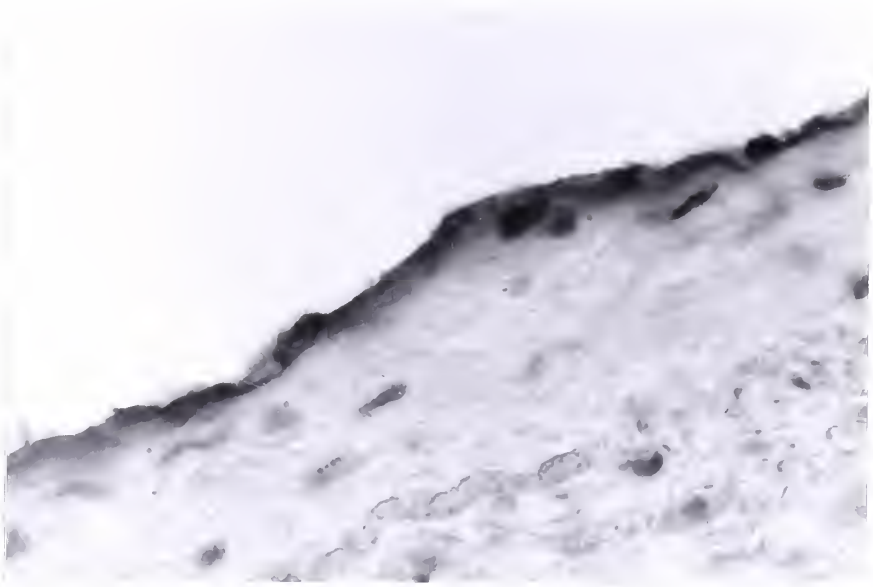


PHOTOMICROGRAPH 6. Unmodified 13 x100 Sudan IV

Lipid at intimal surface is seen with evidence of penetration into intima.

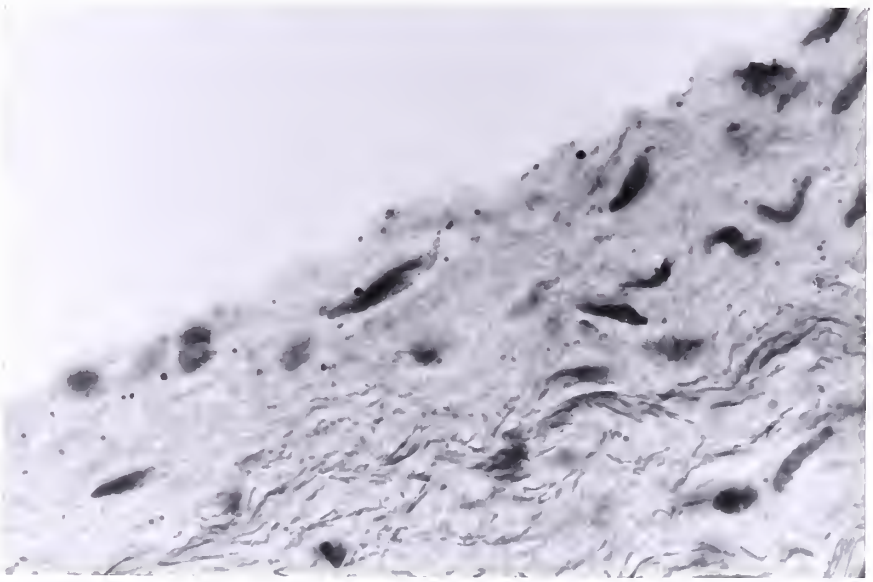
PHOTOMICROGRAPH 7. Modified 13 x100 Sudan IV

Comparable to control. Intimal surface free of lipid.



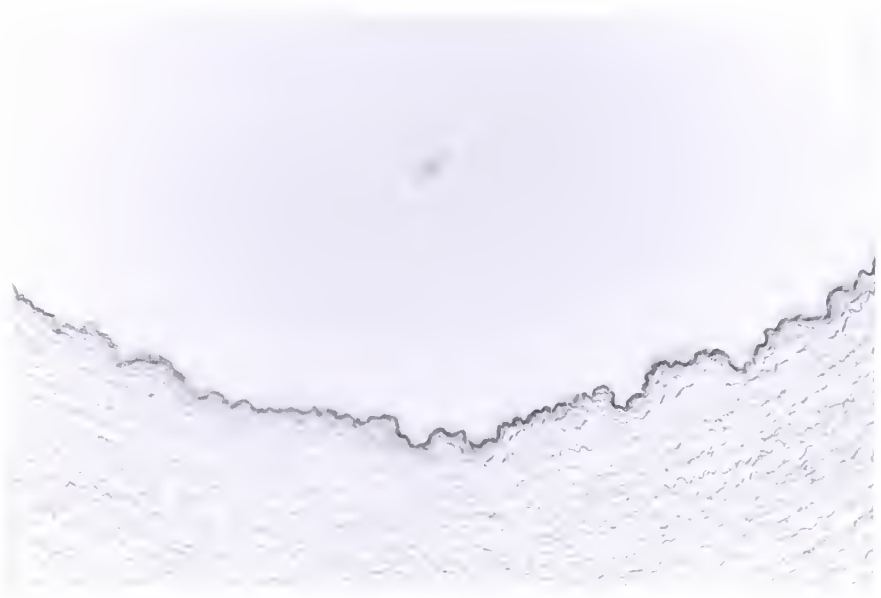
PHOTOMICROGRAPH 8. Unmodified 13 x450 Sudan IV

Sudanophilic material at intimal surface, penetrating first few layers of intima. Large black dots are cell nuclei.



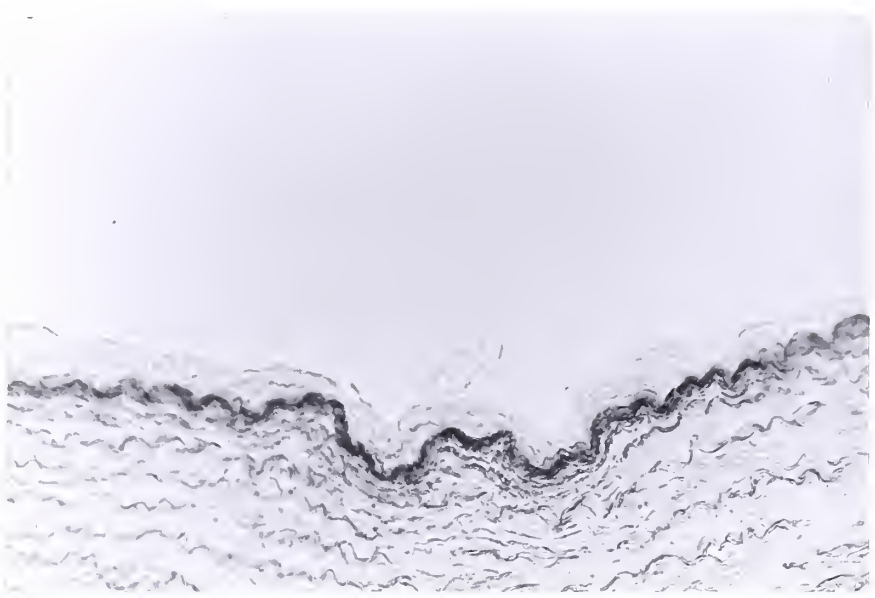
PHOTOMICROGRAPH 9. Modified 13 x450 Sudan IV

Comparable to control. No lipid at intimal surface. Surface black dots are control lipid.



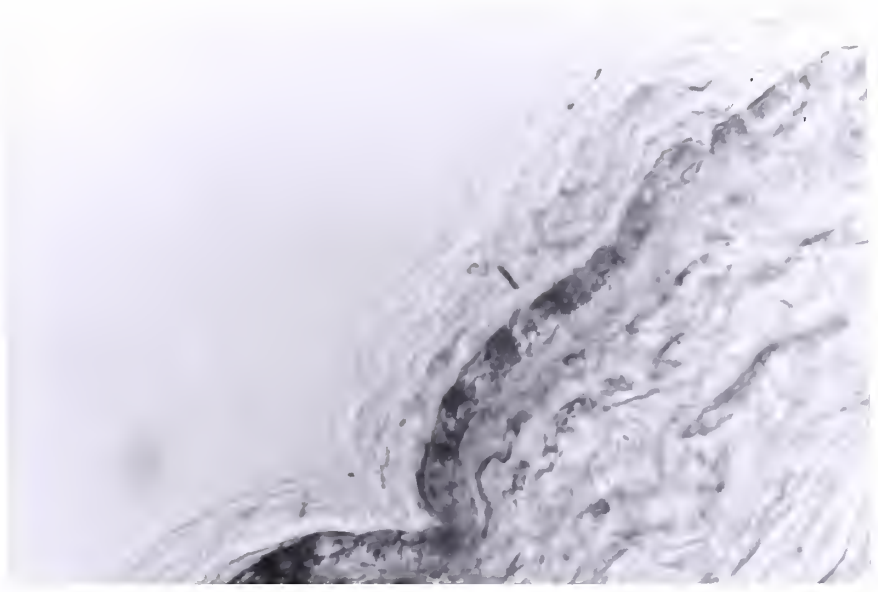
PHOTOMICROGRAPH 10. Control 8 x40 Sudan 1V

Clean vessel with thin intima. Only lipid is in internal elastic lamina (black line).



PHOTOMICROGRAPH 11. Control 8 x100 Sudan 1V

No lipid is seen except in internal elastic lamina (dark line).

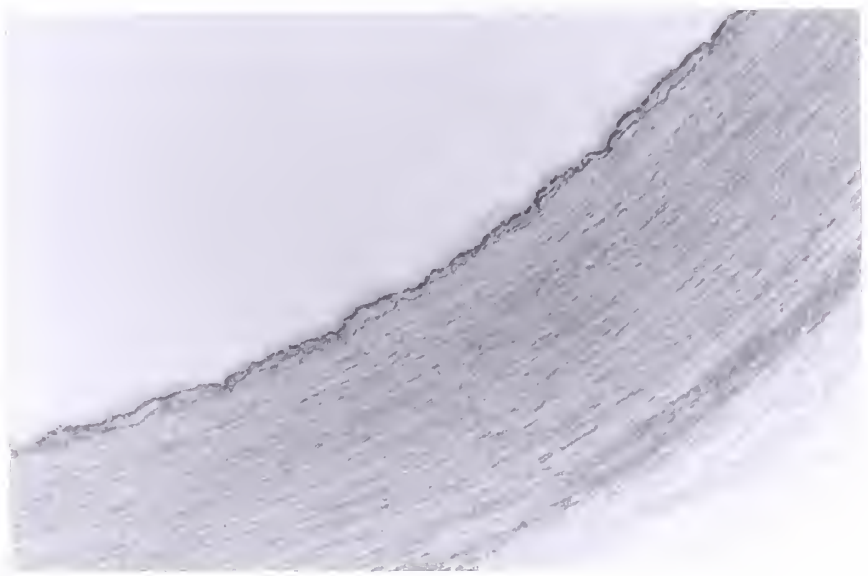


PHOTOMICROGRAPH 12, Control 8 x450 Sudan IV

High power of intima and first few layers of media. Only lipid is in internal elastic lamina (dark area).

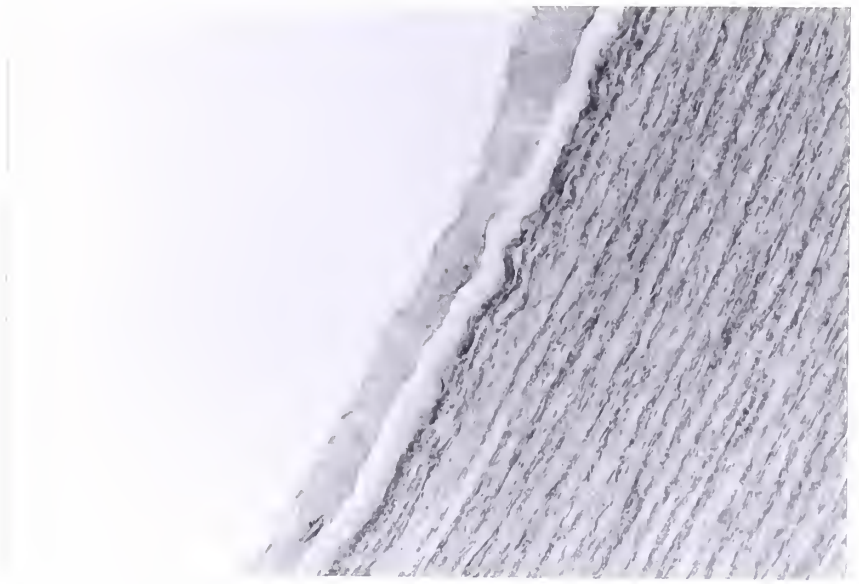
PHOTOMICROGRAPH 13. Unmodified 8 x40 Sudan IV

Lipid deposit at intimal surface after exposure to unmodified lipoprotein solution. Internal elastic lamina also stains for lipid (black).



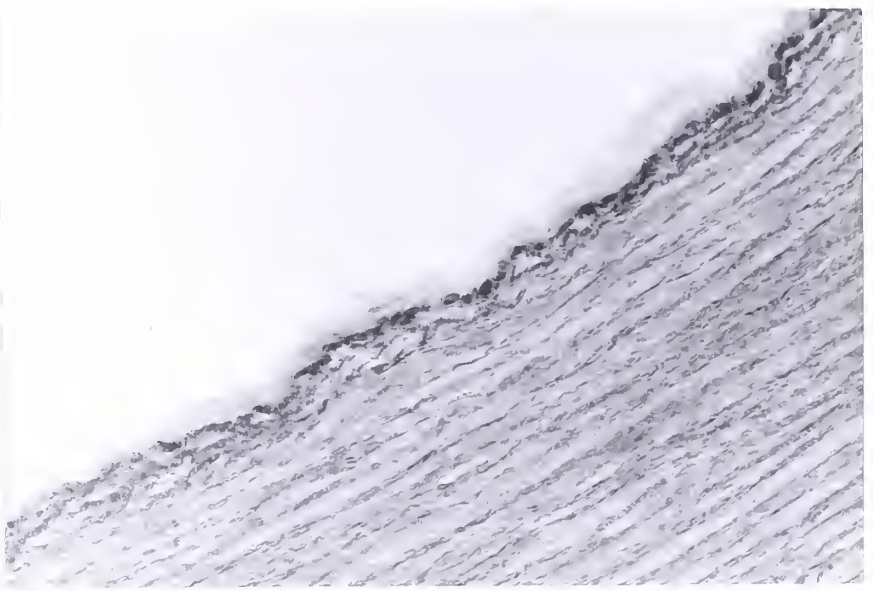
PHOTOMICROGRAPH 14. Modified 8 x40 Sudan IV

Comparable to control. Black line is control lipid in internal elastic lamina.



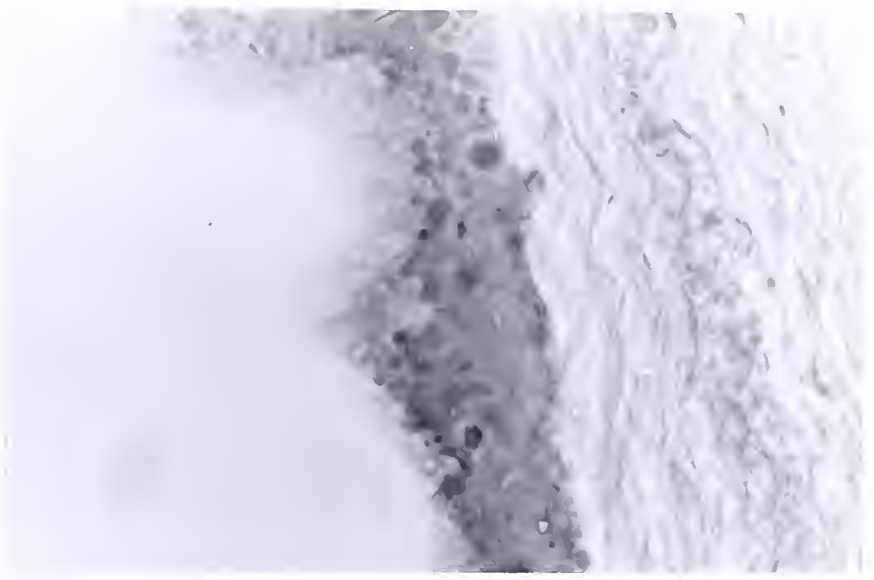
PHOTOMICROGRAPH 15. Unmodified 8 x100 Sudan IV

Thick lipid deposit at intimal surface. Internal elastic lamina is stained darker (control lipid).



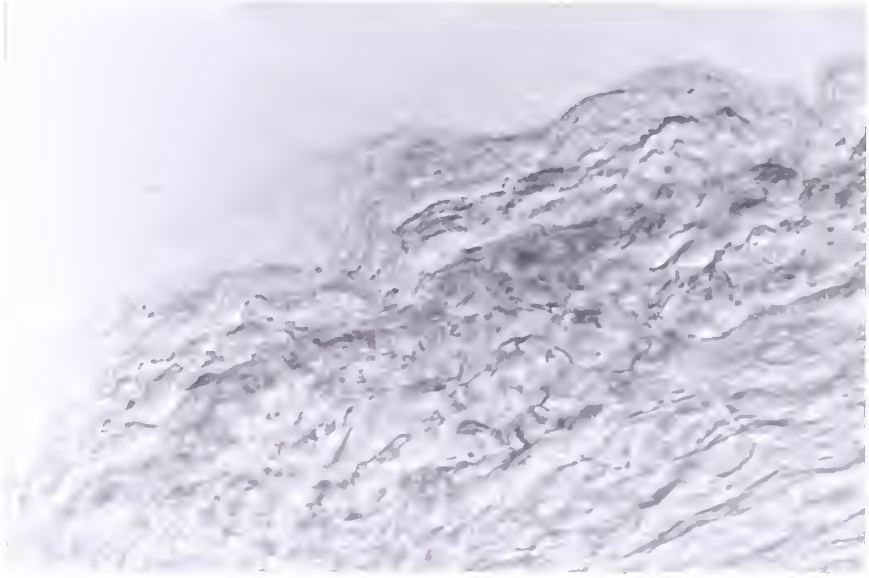
PHOTOMICROGRAPH 16. Modified 8 x100 Sudan IV

Comparable to control. Only control lipid (dark line) is present in internal elastic lamina.



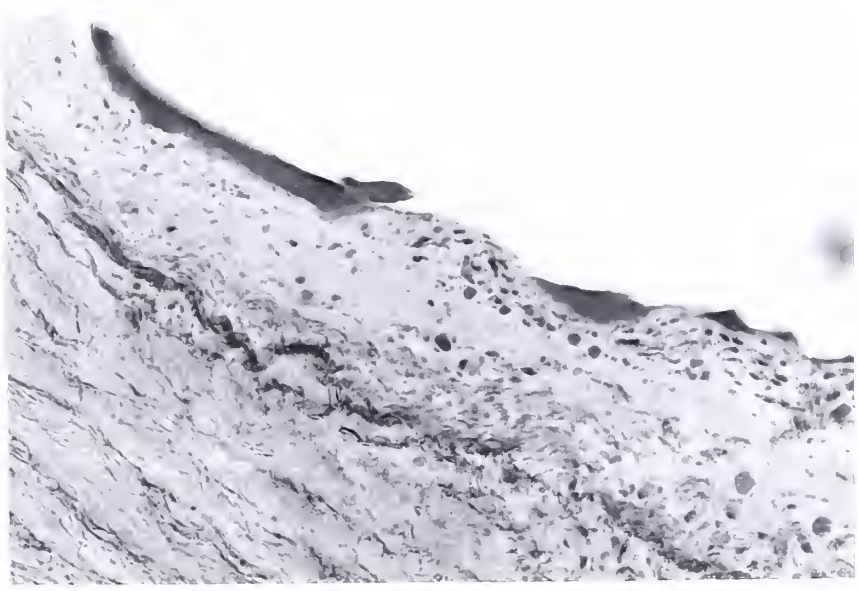
PHOTOMICROGRAPH 17. Unmodified 8 x450 Sudan 1V

Coagulated lipid at intimal surface with scattered lipid deposits.



PHOTOMICROGRAPH 18. Modified 8 x450 Sudan 1V

Comparable to control. No lipid seen at surface or in intima.



PHOTOMICROGRAPH 19. Unmodified 2 x450 Sudan 1V

Plaque with intense sudanophilic material layered on intimal surface. Lipophages and lipid droplets scattered in plaque. Slight control sudanophilia of internal elastic lamina.

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