Interactions of lipoprotein-derived cholesterol with biomaterials during short-term in vitro circulation

by

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INTRODUCTION

Biomaterials have become an increasingly more integral part of human medicine during the past two decades. They have been used in artificial organs, joints, blood vessels, and in pumps assisting defective organs, to name a few applications. Interjecting these biomaterials into a biological environment is not always a compatible match, however. The recipient's immune system may reject the alien implant, or alternately, may strive to incorporate the implant by covering it with endothelial cells, platelets, or albumens and lipoproteins.

Lipoprotein deposition—the cholesterol moiety in particular—has been a serious problem in prosthetic heart valve poppets made out of silicone rubber. Cholesterol absorption in vivo occurs to the extent where movement of the silicone poppet is impeded by the resultant swelling. In vitro trials corroborate the in vivo results: lipid deposition occurs in the same relative proportions, but in lesser quantities, as in vivo.

Since lipoprotein/cholesterol accumulation is associated with atherosclerosis in humans, this project was developed out of the premise that plaque formation is initiated by lipid infiltration into the artery wall. Other theories on atherogenesis propose mechanical or chemical damage, or endothelial and smooth muscle cell proliferation, as the primary causal agents in the disease process. The study presented in this thesis considers them as

secondary factors, and uses an in vitro circulatory system model to test the interaction of lipoprotein-derived serum cholesterol and several biomaterials at a simulated blood/arterial luminal wall boundary.

Specifically, the experiments presented here consider short-term cholesterol adsorption onto a variety of biomaterial surfaces (including silicone rubber) at several flow rates within the steady, laminar flow range. Also examined here are the effects of temperature and geometry on the in vitro circulatory model.

Although this study strives to simulate physiological conditions such as flow rates, vessel diameters, and circulating fluid (porcine serum), it does not attempt to duplicate the plaque formation process or make any implications as to parallels with human medicine. Rather, these experiments attempt to focus on the early stages of lipid deposition in situ, and to determine if a detectable quantity and/or pattern of deposition can be observed during short-term exposure.

LITERATURE REVIEW

Theories of Atherogenesis

Cardiovascular disease in general, and atherosclerosis in particular, is the major cause of death in the United States and other developed areas of the world. Atherosclerosis is a condition detected in arteries by the presence of grumous material and narrowing of the vessel lumen (stenosis). Plaque and stenoses are formed, in part, by lipoprotein infiltration through the endothelial layer of the arterial wall. In healthy individuals, this endothelial layer acts as a semi- permeable membrane. In diseased vessels, something goes awry in this system: net deposition of lipid and fibrotic material occurs subendothelially, causes local intimal thickening, and escalates after initiation.

Although the mechanism of plaque formation is fairly well understood —most experts in the field agree that plaques will not develop in the absence of mechanical or chemical injury to the luminal margin—considerable debate exists as to what the initiating factor or causal agent in the atherosclerotic sequence is. The initiation of sclerotic and thrombotic changes associated with lipid deposition and atheroma formation in arteries has been termed atherogenesis. Three major hypotheses are recognized as possible mechanisms for atherogenesis: mechanical or chemical damage to the arterial lumen, the monoclonal cell division theory, and excess lipid infiltration. In each, elements

of the other two hypotheses arise as secondary contributory factors.

The first of these theories is response to mechanical, chemical, or other types of injury. According to this theory, the endothelial layer (one cell layer thick) becomes damaged by some means, leaving the subendothelial or medial layer exposed. The arterial media is composed of elastic tissue, smooth muscle, microfibrils, and collagen. Collagen carries a net positive charge on its surface, and when exposed to the circulating bloodstream, collagen attracts platelets, which carry a net negative charge. The purpose of the endothelial layer, also holding a net negative charge, is to form a semipermeable membrane to blood elements and to repel lipoproteins and clotting factors. Also, the rate-limiting step for passage of larger macromolecules from the intraluminal blood into the arterial wall occurs at the endothelium (Weinbaum et al., 1974). Weinbaum and Caro (1976) state that the rate of macromolecule uptake by the arterial wall is dependent upon hydrodynamic interaction between vesicles and deformation of endothelial cells—this rate is sensitive to mechanical factors such as flow, pressure oscillations, sinusoidal stretch, and changes in temperature. Bjorkerud and Bondjers (1972) concur: their dye exclusion tests for cell viability show irreversible injury or cell death in areas of increased hemodynamic strain. In the aorta, the dark-staining areas are associated with vessel branching points. Injured cells release adenosine

diphosphate (ADP) and serotonin, the ADP helping local platelets to adhere to each other and the serotonin contributing to vasoconstriction. Thrombus formation is subsequently initiated and the rough surface makes an excellent trap for circulating free cholesterol, cholesterol esters, and blood proteins.

Mechanical damage can occur under high shear situations (Fry, 1968) that literally wear away at the intimal layer of the artery faster than the endothelial cells can regenerate. High shear is detected in areas of local turbulence (Reynolds number greater than 2000), such as in the aorta, or distal to obstructions in a blood vessel, such as a pre-existing stenosis or other abnormal deviation from the native vessel configuration. This latter situation manifests itself as back flow or eddy currents around the obstruction. Rotation of red blood cells due to shear produces the same effect as turbulent eddies: increased mass transport caused by molecular diffusion. Migration of particles away from solid surfaces creates a skimming layer where boundary transport is affected by surface collision and the frequency with which cells enter the skimming layer (Keller, 1971). Red blood cells stir the skimming layer at high shear and cause hemolytic wall damage, which may ultimately lead to plaque formation and clotting in the vascular system.

According to the low shear theory of atherogenesis, chemical damage can arise under normal blood lipid conditions, where cholesterol esters are

deposited in arterial tissue in similar proportions to the blood concentration (Caro et al., 1971; Smith, 1974). Low shear inhibits mass transport of cholesterol away from the arterial wall by creating a thick diffusional boundary layer. Caro believes that shear influences early atheroma development because higher shear enhances mass transport by means of a steepening effect on regional concentration gradients —the greater the gradient, the more diffusion of material away from the wall. In addition, at regions of higher shear, the diffused material is more readily swept away by circulation. The low shear stress theory is corroborated by Ku et al., 1985. Texon (1967), on the other hand, connects low lateral pressure sites with intimal thickening and endothelial proliferation, but considers atherosclerosis as being a disease with mechanical factors constituting the primary damage. Chemical damage is also promoted by viral agents in the blood that enter circulation from the lymphatic system or via leukocytes, multiplying within endothelial cells; viruses and bacteria can also attack apoproteins, freeing more cholesterol for deposition—in fact, many lipid transport disorders are related to apoprotein abnormalities rather than lipid content per se (Levy, 1981). Other examples of chemical damage include action by agents such as pharmaceuticals, bacterial endotoxins, and other substances not indigenous to the circulatory system. Transient anoxia can alter the endothelial layer by local asphyxiation—anoxia or hypoxia can be effected

by low pO_2, low hemoglobin, or transiently stagnant blood, as in the case of stroke or arterial vasospasm (Maseri et al., 1978). Nguyen (1985) positively correlates blood pressure and atherogenesis—especially in the abdominal portion of the aorta and at branch points. He suggests that high systemic pressure leads to smooth muscle cell hypertrophy and proliferation, perhaps collapsing local vasa vasorum and thus causing hypoxia in adjacent subendothelial tissue.

Constantinides (1969) tested rat artery endothelium for permeability changes with varying conditions of pH, osmolarity, anoxia and temperature, and examined the tissue under transmission electron microscope (TEM). For conditions that induced permeability changes in the tissue, his TEM studies suggested that increased permeability occurs at the interendothelial junctions due to endothelial smooth muscle contraction in response to stimuli. The open junctions lead to intra- or extracellular edema and initiate wall damage. This is thought to be a possible mechanism for free cholesterol infiltration through the semipermeable endothelial layer. Certain native compounds—such as epinephrine, dopamine and angiotensin—induce vasoactive contraction, and variations in their basal concentrations may be important in studying lipid infiltration in vivo.

Colton et al. (1974) suggest that either mechanical or chemical injury can

enhance the normal pinocytotic, diffusional, or convective movement of albumin or low density lipoproteins (LDL) into the luminal wall—which are normally reversible events, but lead to accumulation at enhanced transfer rates. It is significant to remember that cholesterol is needed within the wall for energy, membrane construction, and as a precursor in steroid synthesis, among others, but when influx is greater than efflux, lipid accumulation and damage begin. For example, arterial elastin in vivo is a protein/lipid complex with the lipid moiety bound to elastin peptide groups. In atherosclerotic lesions, lipid content (mainly cholesterol esters) of elastin increases with severity of the lesion. Elastin interacts with LDL and VLDL resulting in transfer of lipids—but not apoproteins— to elastin (Kramsch and Hollander, 1973). Again, this situation illustrates the difficulty in assigning a primary causal agent to the atherosclerotic process: along with the presence of excess lipid infiltration, the elastin in developed plaques appears to have an altered amino acid composition with an increase in polar amino acids and a decrease in cross-linking amino acids. Whether the elastin alteration occurs independently or in conjunction with cholesterol excess, or in what relative time reference, is uncertain.

The second major hypothesis on atherogenesis involves properties of the arterial wall itself—the monoclonal/cell multiplication theory. Benditt and

Benditt (1973) proposed that atherosclerotic lesions arise from single smooth muscle cells exhibiting neoplastic proliferation, smooth muscle being a constituent of the fibrous region surrounding a plaque. They found an enzyme, glucose 6-phosphate dehydrogenase, that is X-linked and produced in smooth muscle cells. Individuals who are heterozygous for the enzyme produce both isoenzymes A and B. Benditt and Benditt found plaques that were predominantly of type A or B, and a lesser proportion of mixed populations, and used these results to speculate that atherosclerotic plaques are produced by a single population of proliferating smooth muscle cells (monoclonal). If arterial damage were due to injury, a polyclonal (types A and B together) enzyme population would be seen in tissue extracts, as is the case in wound healing or new growth. They reasoned that the mechanism of monoclonal plaque generation is via mutation—mutagens may include chemical or viral agents in the blood for which a locus has an increased susceptibility. Others have disclaimed this theory based on the small percentage of monoclonal plaques, the low number of individuals sampled, and the inability to distinguish the smooth muscle cells responsible for the enzyme production within an area of gross proliferation. However, some smooth muscle cells may exhibit different sensitivities to environmental factors due to slight differences in composition. Thomas et al. (1968) showed an increase in tritiated thymidine

incorporation by cells of the aortic wall in grossly normal areas. These sites were associated with later incidence of atherosclerotic lesions and were termed preproliferative. Thomas et al. speculated that areas of damage were due to focal proliferation of endothelial smooth muscle cells concommitant with imbibition of cholesterol-rich fluid into extra- and intracellular compartments of the wall. Damage occurred when cell replacement exceeded demand and formed small masses in the intima. Intimal endothelial cells turn over significantly more slowly than either skin or intestinal endothelial cells, so any diminution of cell replacement rate in conjunction with even minor vascular trauma will alter permeability at that site. Endothelial turnover also slows with aging, whereas collagen grows with much less circulatory stress (and, therefore, does not need to be replaced as often); even in healthy arteries the hydroxyproline content (unique to collagen) in the intima increases during adulthood, and is probably a natural consequence of aging. The outcome of increased intimal collagen is stiffening of that layer, and ultimately, cracking. This allows lipid infiltration into the intima by destroying the semipermeable nature of the membrane.

The final theory is probably the one most recognizable to the general public, since it has been widely dealt with by the popular press: lipid infiltration. Lipid accumulation in the endothelial layer of arteries has been

attributed as the primary promoting factor in atherosclerosis, with fibrosis and thrombosis following the initial damage. Results of population studies such the Framingham study of the 1970's (Kannel et al., 1976), a long-term epidemiological survey of cardiovascular death versus lifestyle of a Massachusetts town, have brought diet (saturated fats and cholesterol, in particular) under close scrutiny; the Framingham study was initiated in 1951 and data tabulated over 25 years. Certainly, given a choice of surgery, drug therapy, exercise, relinquishing cigarette smoking, or changing eating habits, dietary modification is the most straight-forward approach. Although lowering blood cholesterol levels remains a major element in reducing/preventing coronary heart disease (which is directly correlated to atherosclerosis), the clinical survey identifies plasma lipid levels as one of several possible risk factors in coronary heart disease. Kannel et al. (1979) suggest that in people under the age of 50, the risk of coronary heart disease is related primarily to serum total cholesterol levels and augmented by other risk factors. The risk may be further determined by partitioning the serum cholesterol level into various lipoprotein fractions: large amounts of HDL provide a protective benefit (corroborated by Gordon et al., 1977, who noted only a weak positive correlation between LDL and coronary heart disease). VLDL and triglcerides do not exhibit a well-defined statistical trend in these

studies, varying unpredictably with sex, age, and lifestyle. Epidemiological studies, while beneficial in signaling statistical trends (Dawber et al., 1962), offer less information as to the cause/effect nature of a multiple variable problem like vascular disease. A more specific proposal has been proffered by Brown and Goldstein—the LDL receptor theory of atherogenesis (Goldstein et al., 1983; Brown and Goldstein, 1984).

Receptor-mediated endocytosis has long been recognized as a major mechanism is transmembrane exchange. Brown and Goldstein found that a certain proportion of the population has an insufficient number of LDL receptors on their endothelial cell surfaces. The receptors are responsible for removing lipids from circulation for the cell's own use (new membrane synthesis during cell division, energy, etc.). Individuals with a mutant dominant gene for the LDL receptor inherit the disease familial hypercholesterolemia (FH). Homozygotes for this gene have no normal receptors and die of heart attacks even as children; heterozygotes have one half the number of normal receptors as a healthy individual, and, therefore, two times the blood lipid concentration. When lipids, cholesterol esters in particular, are not removed from the bloodstream by receptor-mediated transport, they are removed by alternate pathways, including diffusion through and between the luminal endothelial cells. The children with homozygous FH,

who have heart attacks by the age of two, provide solid evidence for the correlation between high LDL level and initiation of atherosclerosis, for these children have none of the other risk factors associated with vascular disease in adults. Although LDLs carry a variety of lipids, cholesterol and cholesterol esters are the lipids associated with and integrated into mature plaques, and so these lipids became the primary focus of lipid infiltration studies.

Biomaterials: Lipid Interactions with Silastic®

Although polydimethyl siloxane (Silastic®) exhibits low deterioration and absence of tissue reaction in vivo, many researchers (Braley, 1970; Brash and Lyman, 1969; Homsy, 1970; Swanson and LeBeau, 1974) have discovered that medical-grade Silastic® absorbs lipids when used clinically as artificial heart valve material. These valves are configured as a ball within a cage: Silastic® is used as the ball portion and various alloys are used for the cage unit.

Cholesterol absorption in Silastic® heart valve poppets was first detected during postmortem recovery of the poppet or replacement surgery in humans fitted with artificial heart valves (Mayhan et al., 1973; Moacanin et al., 1973). Consequently, a large proportion of the literature concerning lipid/Silastic® interaction deals with case studies. The data from these investigations are varied: some groups report that the balls crack and split over time; some describe sample weight loss due to leaching out of unpolymerized polydimethyl

siloxane into circulation with time; some detect weight gain due to adsorbed/absorbed lipids; some show evidence of a tangible change in ball dimensions and shape; but all observe ball discoloration, which, when analyzed by extracting the poppet with chloroform and methanol, is due to accumulation of lipids (including cholesterol and cholesterol esters). The swelling considered above would be enough to occlude ball movement within the cage and cause wedging; the weight loss observed by others is sufficient to allow passage of the ball through the frets of the alloy cage and into circulation (where it could conceivably cause a stroke or anoxic damage). These variable observations on Silastic® interaction with the blood are most likely due to the diverse formulations of the material produced during the developmental stage of its use as a prosthetic implant. Chin et al. (1971) suggest that blood lipids are selectively absorbed by silicone rubber. Since cholesterol is carried in vivo on lipoproteins, they suggest that lipoproteins fracture at the surfaces of silicone rubber and that shearing forces generated at the occluder within the cage are sufficient to break protein/lipid bonds—forces less than those required to lyse red blood cells. Levy (1981) describes the apoprotein/lipid association in lipoproteins as noncovalent bonding, with lipids attached by H-bonding and van der Waals forces. It is important to note that all the in vivo studies range from 2 days to many years in some instances: the extreme results are generally seen

where long-term exposure to body fluids has rendered the valve unit nonfunctional.

There are several examples of in vitro systems for studying lipid deposition in the literature as well. Pierie et al. (1968) subjected Silastic® balls to continuous rubbing against a Stellite edge—to simulate cage interaction as in the in vivo state—in bovine serum at 37°C. They are the only group who makes any correlation between blood velocity and lipid deposition, stating that high velocity predisposes Silastic® to increased deposition. Starr et al. (1966) immersed balls in human plasma for 4 months and found no time correlation, after initial deposition, between lipid content and time. They reconfirmed these results by inducing mechanical damage by repeated flexion and compression of balls.

Finally, Carmen and Kahn (1968) set up a system—similar to the one utilized in this research project—to examine effects of a "lipid-containing medium" flowing past stationary poppets. They worked at the upper limits of a physiological range, considering vessels (of Pyrex glass and therefore nonabsorbing) with an inner diameter of one inch and highly turbulent Reynolds numbers of 5,000 and 10,000. Carmen and Kahn conducted their in vitro trials for 4 to 8 weeks, but their in vivo results ranged from 2 days to 2 months and yielded from 0.1-5.5% lipid pickup by weight (although

measurable quantities at the lower concentration range (0.1%) are difficult to detect using the extraction method they have employed). Their in vitro results duplicated the in vivo results, both of which demonstrate lipid absorption in the same proportions as are present in circulating plasma (Carmen and Mutha, 1972).

Carmen and Kahn were also able to enhance lipid uptake by varying degrees of post-cure: an increase of 135% was observed going from no post-cure to the maximum curing performed. Chemically, post-curing removes catalyst decomposition products from polymerized Silastic®, and since the post-curing process involves heating materials to high temperatures for long periods of time, other groups (Homsy, 1970) have employed high temperature—115°C—to simulate long-term implantation in terms of degradation chemistry.

Silastic® lipid absorption is also affected by filler content, which is generally elemental silica or silicon dioxide, by displaying decreased lipid uptake in the polymerized material as filler content increases. Dow Corning, which manufactures Silastic®, has used both post-cure and filler variation in order to ameliorate the lipid-related valve failures, but no longer uses Silastic® without combining it with other polymers or coatings such as proteins (e.g., albumin), hydrogels (e.g., 2-hydroxymethylmethacrylate), or anti-

thrombogenic coatings (e.g., heparin) (Fougnot et al., 1984). In all studies, lipid absorption is never as great in vitro as in vivo (McHenry et al., 1970, compares the two cases). Still, most types of lipids—with the exception of phospholipids and intact lipoproteins—are represented in clinically recovered poppets as well as in the in vitro trials, so Silastic® became a logical choice for a test material in the benchtop model constructed for this study. As a final note on lipid absorption, Meester and Swanson (1972) observed significantly less deposition while studying joint implants for lipid uptake, probably due to the fact that joints are not directly exposed to circulation.

MATERIALS AND METHODS

The basic concept behind these experiments was to create an in vitro circulatory sytem model to quantitate cholesterol deposition from circulating fluid onto arterial walls. This model was designed to approximate short-term events in the initiation stage of atherogenesis by considering properties of biomaterial/lipid interactions in vitro.

Figure 1 shows the actual model used to analyze lipid deposition/adsorption onto the luminal surface of arterial analogues. The model consists of a Sarns Model 3500 peristaltic roller pump to simulate the heart, various types of tubing to simulate arteries and veins, and an Erlenmeyer flask seated upon a stirplate to approximate venous capacitance. The flask is the site of fluid mixing and heating as well as sample collection; the arms of the vessel are constructed at different heights to insure that sedimented material is not pulled back into circulation (Figure 2). The entire system is modeled after the canine femoral artery with a vessel inner diameter of 1/4 inch and 1/16 inch wall thickness; steady, laminar flow at a Reynolds number (Re) of 500, which corresponds to a flow rate of 227 ml/min; and a total vessel length of 10 ft. The femoral artery was chosen as the model sytem because it is a) a large enough vessel to branch and to become sclerotic, and b) small enough to assume steady, laminar flow (which eliminates turbulence effects).

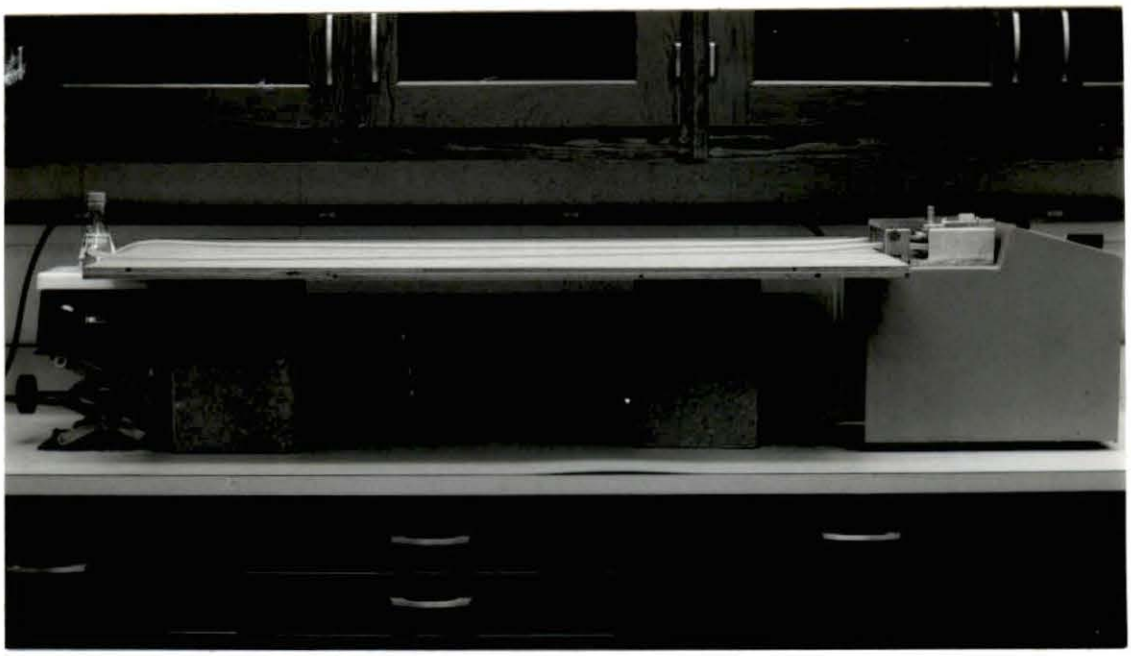

Figure 1. Actual model of the experimental system

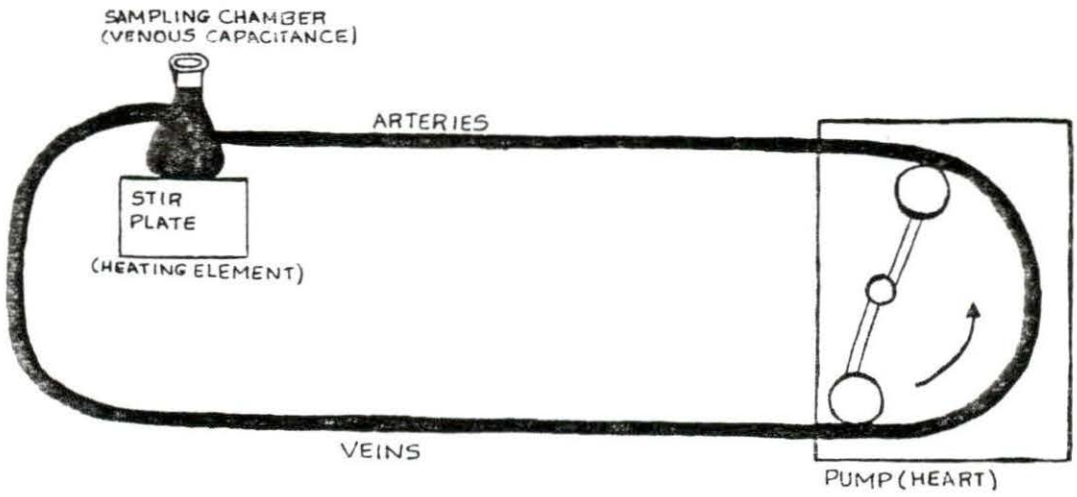

Figure 2. Schematic diagram of the experimental setup shown in Figure 1

The peristaltic pump uses polyvinyl chloride (Tygon®) tubing under its rollers; Tygon® was also chosen as the control material in the vessel simulatio because of its reported nonreactivity with proteins and lipids. Medical-grade polydimethylsiloxane (Silastic®; also known as silicon rubber) was chosen as an experimental material due to its significant lipid absorption when manufactured into artificial heart valve poppets. In vivo trials with these poppets led to material failure after excessive lipid infiltration, which either caused the material to swell and split or to swell and stop valve function. Several authors have speculated on the role of Silastic® in initiation of lipid accumulation (see Literature Review); Dow Corning—who manufactures the material—states that medical-grade Silastic® contains essentially no pores, that lipids are therefore bound covalently initially, and that lipid infiltration occurs only after long-term exposure.

As a final, additional test material, Neoprene® rubber was assayed. Carmen and Kahn (1968) measured % lipid weight gain and % volume increase of Neoprene® versus silicon rubber as a control, and showed relatively large cholesterol adsorption with Neoprene® over silicon rubber. Neoprene® was only examined as an extreme example, since it is never used as a biomaterial in vivo. Neither Neoprene® or Silastic® are compatible with the roller pump head, and therefore in trials where these materials was tested, a 2-foot section of Tygon® tubing was inserted as a spacer between the tubing distal and

proximal to the pump head. The junctions between the Tygon® tubing and the test material were sealed by containing them within tubing sleeves of larger inner diameter and bonding the two tubing types together with Silastic® Medical Adhesive Silicone Type A—this was performed to insure that the junction between spliced tubing would not leak circulating serum.

The constants in the experimental system were tubing length and diameter (and, therefore, surface area), temperature (ambient or room temperature, 22 °C) unless otherwise stated, and fluid volume within the closed system.

The cholesterol source and circulating fluid in the experiment was porcine serum. Serum was obtained from freshly slaughtered pigs, whose blood was collected into 1 L centrifuge bottles and allowed to settle at room temperature for 5 hrs. The resulting clot was then rimmed to eliminate vessel adherence and the blood centrifuged at 2500 rpm, 5°C, for 45 min. The supernatant was then decanted and recentrifuged to remove stray red blood cells. Collecting and processing 8 L of whole blood yielded 2 1/2 L clear serum that could be frozen for later use. Each experiment required 200 ml of serum—this serum was treated wilth 1 mM NaN_3 (sodium azide, an antimicrobial agent) and 1 mM PMSF (phenylmethylsulfonyl fluoride, a protease inhibitor), and allowed to circulate in the closed system for 48 hrs, sampling from the capacitance vessel at various time intervals within this period. In order to assume constant volume

as a system parameter, very small aliquots of sample (0.1 ml) were removed at each sampling time. To accomplish the goal of measuring small amounts of cholesterol leaving the circulating fluid and adhering to the vessel wall, the assay must be sensitive and accurate, and precise for small volumes. This project relied on the Sigma Diagnostics quantitative, enzymatic assay for total cholesterol in serum for data analysis.

The Sigma cholesterol assay is a colorimetric reaction, the details of which are shown in Appendix A. The assay was developed following the protocol described by Allain et al. (1974), and further substantiated by Witte et al. in the same year. The end product of this reaction is a quinoneimine dye, measured by its absorbance at 500 nm in a Beckman DB spectrophotometer on a medium slit setting. In the concentration range of 0 - 200 mg cholesterol/100 ml (the standard curve was produced with dilutions of a Sigma cholesterol aqueous standard, 200 mg/100 ml), the assay sensitivity with the above spectrophotometer was 0.65 mg cholesterol/dL per 0.001 O.D. Sensitivity was calculated as (Δ concentration) / (Δ O.D. reading), and Sigma literature states that the assay is linear to at least 600 mg cholesterol/dL. The standard curve for the conditions in these assays was linear with a correlation coefficient of 0.99966.

Samples were collected under conditions designed to avoid mechanical variables: the system was run at room temperature to lessen thermal gradients

generated between the heating plate and the pump, as well as for simplicity; temperature was examined as a possible factor in altering deposition rate in Tygon® and Neoprene®, but did not comprise a major portion of the study. The roller head and the stirplate surface were kept coplanar to eliminate the possibility of adding friction to the system as fluid moved up or down an elevation gradient. With the same logic, Silastic® and Tygon® were both tested at Re = 0 (static flow) to assess the contribution of fluid mechanics on deposition pattern and rate. The static flow study was performed with tubing clamped to insure zero flow conditions. A set of experiments was also conducted on Silastic® inserting a Y-connector into the arterial (distal to the pump) portion of the tubing, keeping the total tubing surface area a constant. The mechanical effect being tested here was the contribution, if any, that shear at bifurcations has on lipid deposition, since plaque formation is often seen at branch points in vivo.

As a final note on assay procedures, the initial cholesterol extraction and quantitation were done with an older, less reliable technique. Cholesterol was first extracted with a chloroform-methanol mixture and washed with a 0.88% KCl solution. This is a classical cholesterol chemistry technique that acts via methanol disrupting the H-bonding or electrostatic forces between lipids and proteins in the lipoproteins (alcohol also inactivates degradative enzymes); chloroform solubilizes cholesterol but not most other complex lipids; the KCl

wash aids in separating the fractions more completely. The remaining cholesterol in chloroform is evaporated rapidly and reconstituted/assayed with the Liebermann-Burchard reaction. The reaction was used with reliable results clinically for years before enzymatic assays were developed and involves reacting cholesterol in chloroform with an acetic anhydride/sulfuric acid mixture to obtain a blue-green colorimetric reaction that is read spectrophotometrically at 620 nm. The assay proved inappropriate for this study due to the large number of samples being processed at once: it became too difficult to control time, temperature and chloroform evaporation rate. The early results using these techniques were therefore statistically too variable to consider and the Sigma assay was adopted as an alternative. When the enzymatic assay was chosen, the circulating fluid/lipid source was changed from citrated plasma to serum to avoid citrate interference with the reaction products. Lastly, a cholesterol decolorizing reagent marketed by Sigma consisting of 0.5 mole/L sodium ascorbate in saline was added to all samples to compensate for any turbidity detected in the serum samples—this acted to normalize any changes seen in a set of samples collected at room temperature between 0 to 48 hrs (the serum tended to darken with time).

RESULTS

Raw data for the experiments in this project are contained in Appendix B. These data were collected at constant conditions and include 1) Tygon® samples at room temperature and 40°C, 2) Silastic® samples at room temperature, straight and branched sections, 3) Neoprene® samples at room temperature and 40°C, and 4) Silastic® and Tygon® tested at Re = 0 (static flow). All the samples collected in these trials were analyzed for total cholesterol content and the results expressed as mg cholesterol/100 ml serum. An important point to consider in this data is that the actual measurements are the change in serum cholesterol concentration with time—the assay is therefore an indirect measurement of cholesterol adsorption/absorption onto biomaterials ascertained by its removal from circulation.

Figure 3 shows summary data for Tygon® sampled at 22°C with regression slopes drawn in. Figure 4 is a plot of Tygon® at 40°C alone. All of these data are somewhat variable and the curves lie in the linear correlation coefficient range of $P>0.01$ to $P<0.05$, which is marginally significant given the small sample populations. Also, some of the slopes are positive, a situation that cannot occur in a closed system with no exogenous cholesterol source (a positive slope would imply that cholesterol concentrations were increasing with time). Qualitatively, Figure 4 represents a realistic control situation: the slopes

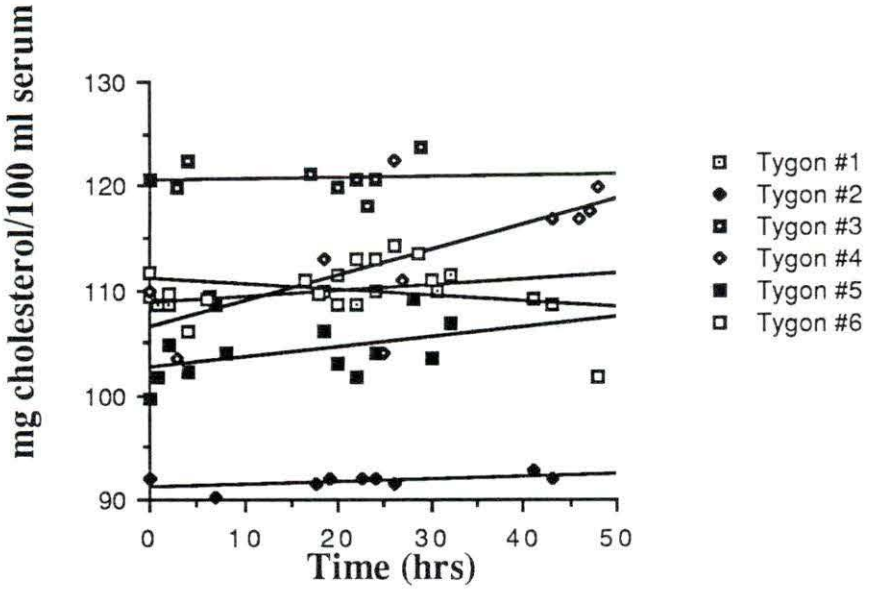

Figure 3. Cholesterol concentration of circulating serum vs time on Tygon® held at room temperature. Linear correlation coefficients: r1=0.67063, r2=0.51621, r3=0.056579, r4=0.67059, r5=0.40614, and r6=0.23669

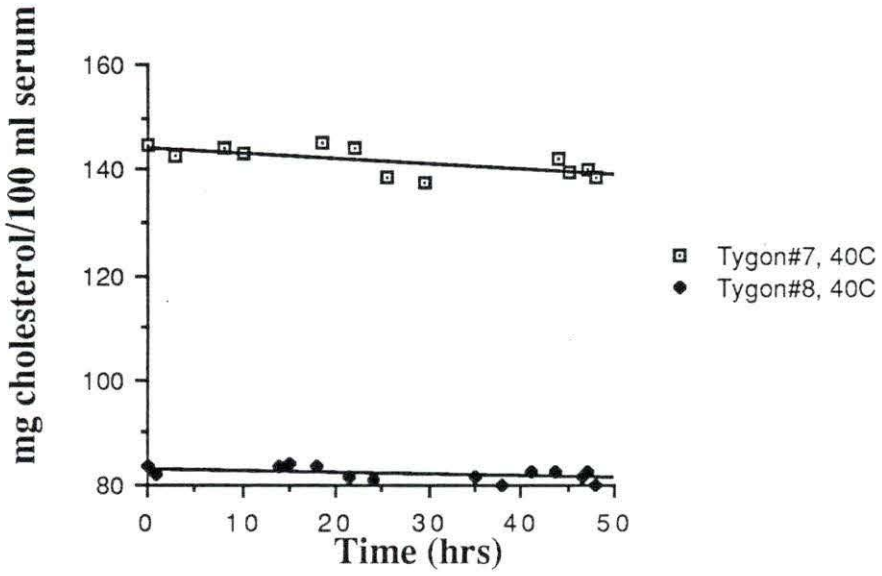

Figure 4. Cholesterol concentration of circulating serum vs time on Tygon® held at 40°C. Linear correlation coefficients: r7=0.67363, and r8=0.47430

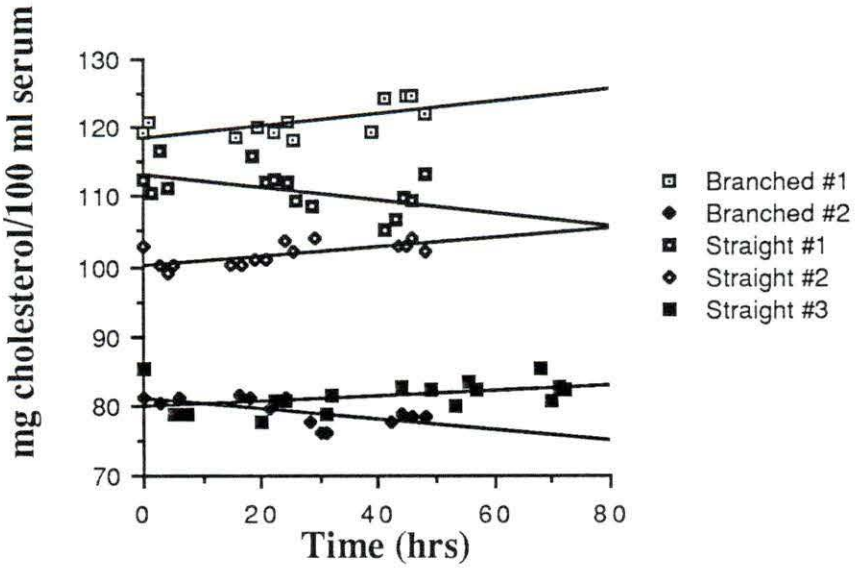

Figure 5. Cholesterol concentration of circulating serum vs time on all Silastic® trials, straight and branched distal segments, for qualitative comparison

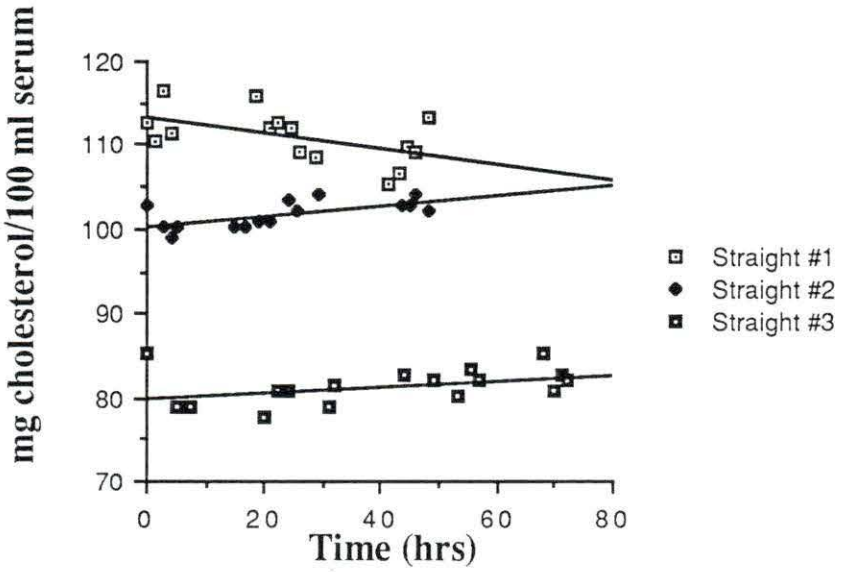

Figure 6. Cholesterol concentration of circulating serum vs time on straight Silastic® tubing. Linear correlation coefficients: r1=0.52667, r2=0.64302, and r3=0.38830

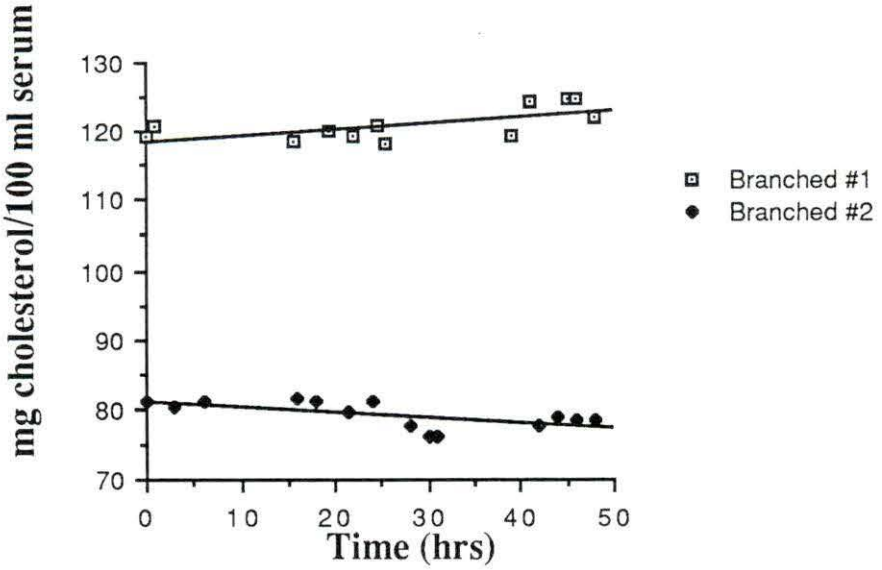

Figure 7. Cholesterol concentration of circulating serum vs time on Silastic® containing a distally (to the pump head) branching section. Total luminal surface area was held constant with the other experiments. Linear correlation coefficients: r1=0.64474, and r2=0.65150

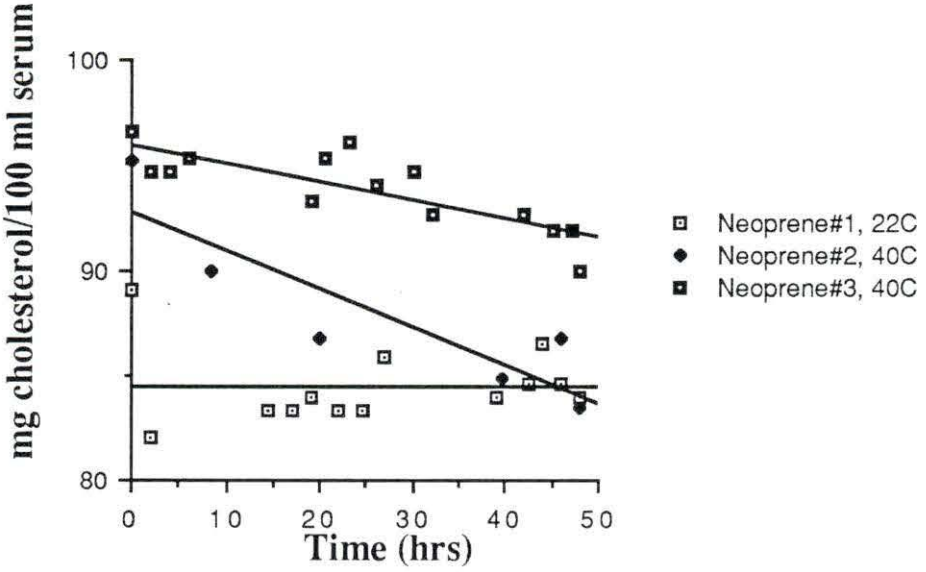

Figure 8. Cholesterol concentration of circulating serum vs time on Neoprene®, experiments conducted either at room temperature or at 40°C. Linear correlation coefficients: r1=0.0097829, r2=0.88551, and r3=0.81632

are small and slightly negative.

Figure 5 shows the summary regression slopes for all Silastic® trials, straight and branched. The correlation coefficients are in the same confidence range as the Tygon® data, but are qualitatively no different than the control data. The graph is broken down into its component parts, straight and branched, respectively, in Figures 6 and 7.

Figure 8 is qualitatively and quantitatively the most significant data in the set. Neoprene® at 22°C exhibits no correlation between time and cholesterol concentration due to scatter in the data—the regression slope drawn through these points is very slightly negative and approximates a control situation. The data obtained by circulating serum through Neoprene® at 40°C for 48 hrs, however, represents a strong linear correlation between time and cholesterol removal from circulation (negative slope), and is a promising candidate for an experimental material. Table 1 summarizes the regression slopes for the above data.

Table 1. Regression slopes for raw data (mg cholesterol/100 ml serum/hr)

Tygon, 22°C	Tygon, 40°C	Silastic, straight	Silastic, branched	Neoprene	Static Flow
5.3749e-2	-1.0247e-1	-9.4278e-2	9.1713e-2	(22°C)	(Silastic)
2.6124e-2	-3.5672e-2	6.1847e-2	-7.4134e-2	-1.0891e-3	-4.3031e-3
8.5711e-3		3.5132e-2		(40°C)	(Tygon)
2.4100e-1				-1.8225e-1	-6.7727e-2
9.6324e-2				-8.8616e-2	
-5.0732e-2					

In order to assess the relationship between control data and various experimental setups, the slopes of the regression curves were compared using the t distribution for unpaired data. These results are summarized in Table 2.

Table 2. Comparison of regression slopes and t-test calculations between tubing types

Comparison	n	d.f.	t statistic	Probability
Tygon vs all Silastic	11	9	1.0375	P>0.200
Tygon vs straight Silastic	9	7	0.91058	P>0.200
Tygon vs branched Silastic	8	6	0.63755	P>0.500
Tygon, 22°C vs 40°C	8	6	1.7249	P>0.100
Tygon vs all Neoprene	9	7	2.2214	P>0.050
Tygon vs Neoprene, 40°C	8	6	2.5432	P<0.050
Silastic, straight vs branched	5	3	-0.089941	P>0.500

Tygon® vs branched Silastic®, and straight Silastic® vs branched Silastic® are both strongly unrelated statistically (P>0.500). It is tempting to speculate from these numbers that branched Silastic® is the cause of this relationship—however, there is an ambiguous correlation between Tygon® and straight Silastic® and therefore it can only be concluded that these protocol have different effects on cholesterol concentrations over time. There is a strong positive relationship between Tygon® and Neoprene®, 40°C (P<0.050). This statistic only exists in the absolute because the two sets of slopes have opposite signs that are not taken into account in the t distribution. The rest of the data compared in Table 2 lie outside of the 95% confidence interval but are not so strongly unrelated that a definitive statement can be made about their

role. More experiments are needed to form any conclusions; nevertheless, several plausible explanations and suggestions for further studies can be derived from these data.

DISCUSSION AND CONCLUSIONS

Lipoprotein deposition and infiltration into artery walls is one of the key processes in atherogenesis, if not the initiating factor. This study uses biomaterials to observe the short-term behavior of cholesterol in vitro. Since cholesterol interactions in vivo are linked with a number of biological variables such as immune system stimulation by points of cellular damage, red blood cell accumulation, fibroblast ingrowth, and collagen exposure, the actual mechanical contribution of cholesterol is difficult to isolate. Certainly by the time that atherogenesis is clinically detectable, the above factors are too intermeshed to isolate primary from secondary factors.

Post-mortem and clinical studies of mechanical failure in heart valve prostheses fabricated out of Silastic® implicated cholesterol (and other lipids) as the causal agent. The lipids reputedly infiltrated the biomaterial, causing swelling, cracking, and splitting in some cases and leaching out of plasticizers by circulating blood in others. Researchers implicated cholesterol, but these studies were of long duration relative to this project, where deposition was allowed to take place over 48 hrs. Indeed, the success of an in vivo implant (if not rejected) relies on other biological processes; in particular, the formation of a pseudoendothelial layer to prevent blood clotting or emboli formation on the artificial surface is essential. Note that lipids in these studies refer to fatty

acids, neutral fats, steroids, steroid esters, and phospholipids—cholesterol is readily detectable biochemically since it comprises the largest percentage of a developing plaque. One of the questions that arose in this project was whether deposition occurred at short exposure time, and if it appeared not to, was cholesterol present in significant or nonmeasurable quantities.

To answer that question, the mass of a monlayer of cholesterol on the inner surface of the system's tubing was calculated (Table 3). The sensitivity of the enzymatic assay coupled with the particular spectrophotometer used was 0.65 mg cholesterol/dL for every 0.001 O.D. increment read. If the mass of a monolayer is 0.1 mg, 6 1/2 layers of cholesterol would have to be laid down to cause a 0.001 O.D. deflection on the instrument. But the concentrations where most of the data fell could be read to 0.001, and corresponding fluctuations in concentrations were large enough to be readily detected and significant.

Table 3. Calculation of monolayer quantity of cholesterol in tubing lumen

- Unstressed molecular area of cholesterol = $38 Å^2$
- Molecular weight of cholesterol = 386.67 g/mole
- Tubing surface area = πDL
  $= \pi(0.635 \text{ cm})(304.8 \text{ cm})$
  $= 608.0 \text{ cm}^2$
- Cholesterol monolayer mass:

$3.80 \times 10^{-15} \text{ cm}^2/\text{molecule}(6.023 \times 10^{23} \text{ molecule/mole}) = 2.29 \times 10^9 \text{ cm}^2/\text{mole}$

$386.67 \text{ g/mole}(1/2.29 \times 10^9 \text{ mole/cm}^2) = 1.69 \times 10^{-7} \text{g/cm}^2$

$1.69 \times 10^{-7} \text{g/cm}^2(608.0 \text{ cm}^2) =$ 0.10 mg

These calculations assume that cholesterol was deposited as a monolayer on the lumen surface, providing a linear relationship between time and concentration. Because there are no interactive biological variables in a benchtop model such as the underlying medial and adventitial layers of an artery in vivo or blood components associated with a living organism, this assumption is better than it would be were the tubing implanted; however, a serious drawback in assuming linearity lies in the wide range of initial cholesterol concentrations seen in serum obtained from different pigs. The whole blood was harvested from the same source, but there was no control over age, weight, sex, or species variation in the pigs slaughtered—initial concentrations ranged from 80.9 to 144.7 mg/dL serum with a mean of 101.3 ± 16.7. Linearity of concentration with time may fall anywhere in or above that range.

The original premise of these experiments was that Tygon® would serve as a control material and Silastic® would be the test material. Actual results le to the conclusion that, although there was not enough data available to be certain, Tygon® could still be considered for a control—the statistical data was outside of the 95% confidence interval, but not greatly. Silastic® showed no discernible deposition pattern and appeared to be nonreactive with cholesterol. If this were true, Silastic® results would tend to refute the sheer mechanical interaction of Silastic® and lipid proposed in the literature.

The static tests, whose results are listed in Appendix B and Table 1, were designed to isolate cholesterol/biomaterial interactions to an even greater extent by removing the fluid mechanics variables of flow and shear stress. Mass transport is also influenced by low and high shear rates. Both static Tygon® and static Silastic® trials held surface area at a constant value, and sampling time was kept at 48 hrs. The regression data for these tests showed poor linear correlation. It is interesting to note that when second-order polynomial regression was applied to these data, the curves generated showed strong positive correlation (Figures 9 and 10).

The possibility then exists that the ambiguous correlation seen in the experimental data was due to nonlinearity of deposition, at least in the concentration range considered. In humans, serum cholesterol levels are "pathological" when greater than 250 mg/dL. The porcine serum concentrations used here are far below that value. Considering that the data may have been nonlinearly related, all of the data presented in this study were also analyzed for the relationship between time and the natural logarithm of cholesterol concentration. These calculations were virtually identical to the nonlogarithmic results due to the small slope values.

Plaque formation in human has been associated with high systemic blood pressure. Hydrostatic systolic pressure readings for the in vitro model—measured by tapping into the tubing—were 4.78 mm Hg in the arterial portion

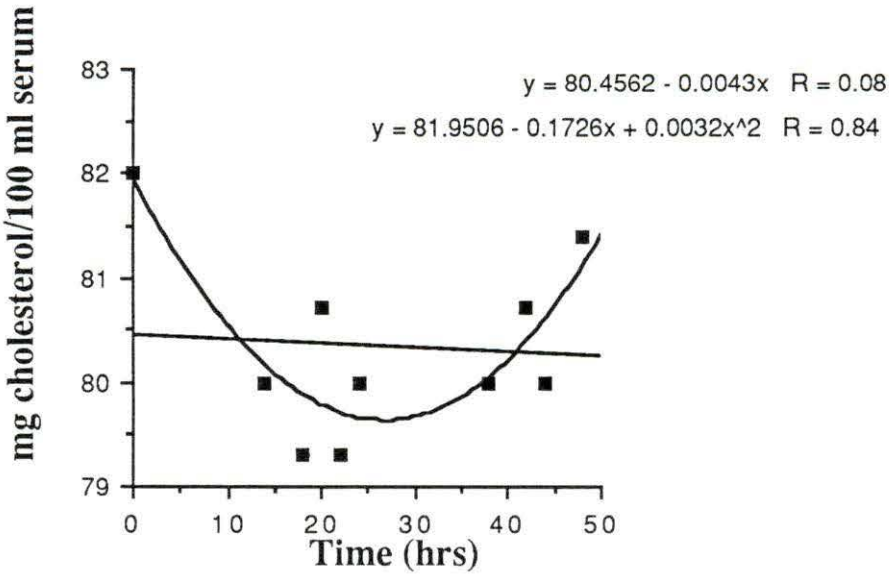

Figure 9. Cholesterol concentration of noncirculating serum vs time on static Silastic®, Re = 0. Linear and second-order polynomial regression are shown

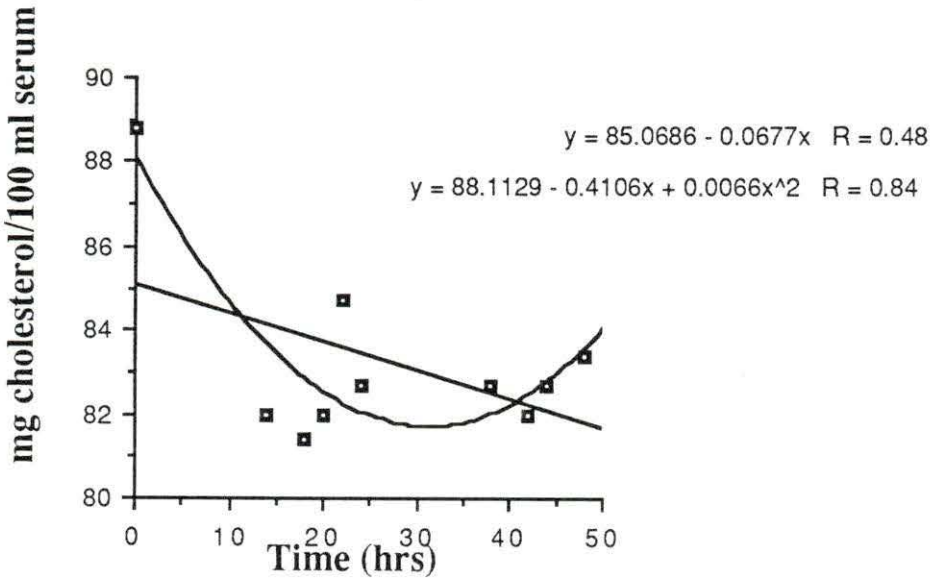

Figure 10. Cholesterol concentration of noncirculating serum vs time on static Tygon®, Re = 0. Linear and second-order polynomial regression are shown

and 3.75 mm Hg in the venous portion.

The results of this project do not definitively predict a specific pattern of cholesterol deposition onto vessel lumens. More experiments are needed to delineate particular patterns. From these preliminary results, Tygon® at 40°C could serve as a control and Neoprene® at 40°C could be an effective test material.

Some additional experiments to consider are testing the effects of temperature on concentration, increasing the Reynolds number to the turbulent range (Re>2000) to test shear effects, and creating a stenotic environment by either choosing a narrower vessel lumen (modeling the coronary artery, for example) or inserting a physical impedance to flow. These factors would obviously only be tried after static studies showed a clear distinction between control and test material.

Once significant deposition was detected, the question of adsorption vs absorption could be addressed. Decreases in circulating cholesterol concentrations may be caused by precipitation (and, therefore, nonadherence to the wall), luminal surface adsorption, or infiltration into the biomaterial. Scanning electron microscopy or light microscopy using a cholesterol-binding dye such as Oil Red 0 would answer these questions.

In conclusion, short-term cholesterol adsorption was not detected in this project. Deposition is possibly a long-term phenomenon—in vivo, cholesterol

deposition may be so entangled with all the other factors involved in atherogenesis that it cannot be isolated as a primary causal agent. Sheer mechancial wall interaction between circulating cholesterol and the endothelium may be an insignificant factor in a highly complicated disease.

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APPENDIX A

Sigma Diagnostics Enzymatic, Quantitative Assay for Total Cholesterol in Serum

Manual procedures

1. To cuvet labeled BLANK, add 0.02 ml deionized water.
   To cuvet labeled STANDARD, add 0.02 ml Cholesterol Aqueous Standard (200 mg cholesterol/dL)
   To cuvet labeled TEST, add 0.02 ml serum.

2. To each cuvet, add 1.0 ml Cholesterol Assay Solution. Cover cuvets with Parafilm® and invert several times to mix.

3. Incubate cuvets at 37°C for 15 min.

4. Read and record absorbance of STANDARD and TEST vs BLANK as reference at 500 ± 15 nm. Complete readings within 30 minutes.

5. Calculate serum total cholesterol as follows:

$$\text{Serum Total Cholesterol [mg/dL]} = \frac{A_{TEST}}{A_{STANDARD}} \times 200 \text{ mg/dL}$$

Biochemistry

$$\text{Cholesterol Esters} \xrightarrow[\text{Esterase}]{\text{Cholesterol}} \text{Cholesterol + Fatty Acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow[\text{Oxidase}]{\text{Cholesterol}} \text{Cholest-4-en-3-one} + H_2O_2$$

$$2\ H_2O_2 + \text{4-Aminoantipyrine} + \text{p-Hydroxybenzenesulfonate} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine Dye} + 4H_2O$$

APPENDIX B

Tygon® Raw Data

Re = 500, tubing inner diameter = 1/4 inch, wall thickness = 1/16 inch
Q = 227 ml/min, system volume = 200 ml

	Time (hrs)	Cholesterol Concentration (mg/100 ml)
Tygon #1	0.0	109.3
	1.0	108.7
T = 22°C	2.0	108.7
	6.5	109.3
	18.5	110.0
	20.0	111.3
	22.0	108.7
	24.0	110.0
	30.5	110.0
	32.0	111.3
Tygon #2	0.0	92.1
	7.0	90.2
T = 22°C	17.5	91.5
	19.0	92.1
	22.5	92.1
	24.0	92.1
	26.0	91.5
	41.0	92.7
	43.0	92.1
Tygon #3	0.0	120.5
	3.0	119.9
T = 22°C	4.0	122.4
	17.0	121.1
	20.0	119.9
	22.0	120.5
	23.0	118.0
	24.0	120.5
	29.0	123.7

	Time (hrs)	Cholesterol Concentration (mg/100 ml)
Tygon #4	0.0	109.8
	3.0	103.5
T = 22°C	18.5	112.9
	25.0	104.1
	26.0	122.4
	27.0	111.0
	43.0	116.7
	46.0	116.7
	47.0	117.4
	48.0	119.9
Tygon #5	0.0	99.7
	1.0	101.6
T = 22°C	2.0	104.8
	4.0	102.2
	7.0	108.6
	8.0	104.1
	18.5	106.0
	20.0	102.9
	22.0	101.6
	24.0	104.1
	28.0	109.2
	30.0	103.5
	32.0	106.7
Tygon #6	0.0	111.6
	2.0	109.7
T = 22°C	4.0	106.0
	6.0	109.1
	16.5	111.0
	18.0	109.7
	20.0	108.5
	22.0	112.9
	24.0	112.9
	26.0	114.1
	28.5	113.5
	30.0	111.0

	Time (hrs)	Cholesterol Concentration (mg/100 ml)
Tygon #6 (continued)	41.0	109.1
	43.0	108.5
	48.0	101.6
Tygon #7	0.0	144.7
	3.0	142.8
T = 40°C	8.0	144.1
	10.0	143.4
	18.5	145.4
	22.0	144.1
	25.5	138.8
	29.5	137.5
	44.0	142.1
	45.0	139.5
	47.0	140.1
	48.0	138.8
Tygon #8	0.0	83.4
	1.0	82.1
T = 40°C	14.0	83.4
	15.0	84.0
	18.0	83.4
	21.5	81.4
	24.0	80.8
	35.0	81.4
	38.0	80.1
	41.0	82.7
	43.5	82.7
	46.5	81.4
	47.0	82.7
	48.0	80.1

Silastic® Raw Data
Re = 500, tubing inner diameter = 1/4 inch, wall thickness = 1/16 inch
Q = 227 ml/min, system volume = 200 ml

	Time (hrs)	Cholesterol Concentration (mg/100 ml)
Silastic #1	0.0	112.6
(straight section)	1.5	110.6
	3.0	116.6
T = 22°C	4.0	111.3
	18.5	115.9
	21.0	111.9
	22.0	112.6
	24.5	111.9
	26.0	109.3
	28.5	108.6
	41.0	105.3
	43.0	106.6
	44.5	109.9
	46.0	109.3
	48.0	113.2
Silastic #2	0.0	102.9
(straight section)	3.0	100.3
	4.0	99.0
T = 22°C	5.0	100.3
	15.0	100.3
	16.5	100.3
	19.0	101.0
	21.0	101.0
	24.0	103.5
	25.5	102.2
	29.0	104.1
	43.5	102.9
	45.0	102.9
	46.0	104.1
	48.0	102.2

	Time (hrs)	Cholesterol Concentration (mg/100 ml)
Silastic #3	0.0	85.3
(straight section)	5.0	78.8
	7.5	78.8
T = 22°C	20.0	77.5
	22.0	80.8
	24.0	80.8
	31.0	78.8
	32.0	81.4
	44.0	82.7
	49.0	82.1
	53.0	80.1
	55.5	83.4
	57.0	82.1
	68.0	85.3
	70.0	80.8
	71.0	82.7
	72.0	82.1
Silastic #4	0.0	119.3
(branched section)	1.0	120.7
	15.5	118.6
T = 22°C	19.5	120.0
	22.0	119.3
	24.5	120.7
	25.5	118.0
	39.0	119.3
	41.0	124.1
	45.0	124.7
	46.0	124.7
	48.0	122.0
Silastic #5	0.0	80.9
(branched section)	3.0	80.3
	6.0	80.9
T = 22°C	16.0	81.6
	18.0	80.9
	21.5	79.6
	24.0	80.9

	Time (hrs)	Cholesterol Concentration (mg/100 ml)
Silastic #5 (continued)	28.0	77.6
	30.0	76.3
	31.0	76.3
	42.0	77.6
	44.0	78.9
	46.0	78.3
	48.0	78.3

Neoprene® Raw Data
Re = 500, tubing inner diameter = 1/4 inch, wall thickness = 1/16 inch
Q = 227 ml/min, system volume = 200 ml

	Time (hrs)	Cholesterol Concentration (mg/100 ml)
Neoprene #1	0.0	89.1
	2.0	82.1
T = 22°C	14.5	83.3
	17.0	83.3
	19.0	84.0
	22.0	83.3
	24.5	83.3
	27.0	85.9
	39.0	84.0
	42.5	84.6
	44.0	86.5
	46.0	84.6
	48.0	84.0
Neoprene #2	0.0	95.1
	8.5	90.0
T = 40°C	20.0	86.7
	39.5	84.8
	46.0	86.7
	48.0	83.5
Neoprene #3	0.0	96.6
	2.0	94.6
T = 40°C	4.0	94.6
	6.0	95.3
	19.0	93.3
	20.5	95.3
	23.0	96.0
	26.0	94.0
	30.0	94.6
	32.0	92.6

	Time (hrs)	Cholesterol Concentration (mg/100 ml)
Neoprene #3	42.0	92.6
(continued)	45.0	91.9
	47.0	91.9
T = 40°C	48.0	89.9

Raw Data for Static Tygon® and Silastic® Experiments
Re = 0, tubing inner diameter = 1/4 inch, wall thickness = 1/16 inch
Q = 0 ml/min

	Time (hrs)	Cholesterol Concentration (mg/100 ml)
Static Silastic	0.0	82.0
	14.0	80.0
T = 22°C	18.0	79.3
	20.0	80.7
	22.0	79.3
	24.0	80.0
	38.0	80.0
	42.0	80.7
	44.0	80.0
	48.0	81.4
Static Tygon	0.0	88.8
	14.0	82.0
T = 22°C	18.0	81.4
	20.0	82.0
	22.0	84.7
	24.0	82.7
	38.0	82.7
	42.0	82.0
	44.0	82.7
	48.0	83.4