CROSS-FLOW FILTRATION, TRANSMISSION ELECTRON MICROGRAPHIC ANALYSIS AND BLOOD COMPATIBILITY TESTING OF COLLAGEN COMPOSITE MATERIALS FOR USE AS VASCULAR PROSTHESES

by

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B. Eng., McGill University (1977)

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Department of Mechanical Engineering, May 9, 1980

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Thesis Supervisor

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Chairman, Departmental Graduate Committee
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Submitted to the Department of Mechanical Engineering on May 9, 1980 in partial fulfillment of the requirements for the Degrees of Master of Science in Mechanical Engineering and Mechanical Engineer

ABSTRACT

The Medical Profession has a great deal of interest in the development of vascular prostheses suitable for replacing small arteries or veins. The applications for such prostheses include varicose vein replacement and bypass graft operations of the femoropopliteal (behind knee), renal (kidney) and coronary (heart) arteries.

Collagen is one of the major constituent materials of natural blood vessels and is mechanically very strong, but it is thrombogenic (induces blood clotting) in its native state. A device for making a non-thrombogenic material from rat tail tendon was discovered by Yannas (1975), leading to the desire to make artificial blood vessels from collagen. Bovine hide collagen was chosen as a raw material for the vascular prostheses, since, unlike rat tail tendon collagen, it is readily available in commercial quantities.

The procedure for processing this type of collagen was studied in great detail. The intermediate materials produced by the manufacturing process were examined under the transmission electron microscope and tested by platelet aggregation experiments. An examination of the relative thrombogenicity of the intermediate materials determined that three steps are required to eliminate platelet aggregation by collagen. These steps are: swelling in pH 3 acetic acid, treatment with glutaraldehyde and air-drying or dehydrothermal treatment. It was found that the order of the glutaraldehyde treatment and air-drying steps could be interchanged.

The correlation of the transmission electron micrographic appearance of the samples with the platelet aggregation experiments revealed that some nonbanded bovine hide collagen specimens did cause platelet aggregation. Nonbanded, swollen collagen fibrils are, therefore, not a sufficient requirement for non-thrombogenicity but may be a necessary requirement.
The design and construction of an apparatus for manufacturing seamless tubes from bovine hide collagen for use as vascular prostheses paralleled the development of the non-thrombogenic material. The manufacturing principle involves the deposition of suspended collagen particles on a cylindrical filter membrane through which the dispersion flows. This procedure called cross-flow filtration, was chosen because of the unsuitability of other manufacturing methods for processing collagen. It was found that seamless tubes could be manufactured with sufficient strength to be easily suturable and to withstand the transmural pressures required of blood vessels.

Thesis Supervisor: Dr. Ioannis V. Yannas

Title: Professor of Polymer Science and Engineering
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During the course of this thesis, my life was touched by many people to whom I owe a great debt of gratitude.

To Professor Ioannis Yannas, my mentor and friend, I owe the success of this project. He was always available with advice and encouragement.

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CHAPTER I

Introduction

The thrust of the research described within this thesis is to develop a vascular prosthesis suitable for replacing small arteries and veins. Generally good results are obtained with the current vascular prostheses designed for the replacement of large blood vessels, in spite of the fact that the grafts are not ideal in all respects. Materials are also available for use in ex vivo applications, such as kidney dialysis machines, which are suitable for the short term blood contact required. However, currently available artificial grafts are completely unsatisfactory for use as venous replacements (Faulkner 1979) and are less than satisfactory for use as small arterial replacements.

The interest in small vessel grafts is driven by the need for femoropopliteal (behind knee), renal (kidney) and coronary (heart) vascular replacement grafts. The grafts are used to replace sections of blood vessels which are narrowed or occluded, threatening the survival of the tissue serviced by the defective blood vessel. Of particular interest is the coronary arterial bypass graft operation. This operation is performed to relieve the symptoms of angina and stave off the possibility of a myocardial infarct (heart attack), a major cause of death for middle-aged North Americans. The operation has proved to be very successful (Wysham 1979),

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using the saphenous vein as a bypass graft. Unfortunately, a suitable autogenous saphenous vein is not always available with which to perform this life saving operation.

This work is organized to provide some background in the physics and physiology of the natural circulatory system, so that design specifications for vascular prostheses can be derived. Following this, is a description of the candidate collagen materials which were examined by transmission electron microscopy and platelet aggregation tests to determine their suitability for vascular prostheses. Chapter VIII describes the apparatus which was designed to fabricate seamless tubes for use as vascular prostheses. And finally, the mechanical testing and evaluation of the material for use as vascular prostheses are discussed in the last chapters.
CHAPTER II

Physiology and Physics of the Circulatory System

In order to understand the demands that the body makes upon the prosthesis, and the demands that the prosthesis makes on the body, an understanding of the natural blood vessels is necessary. A development of the salient physiological points will lead to design recommendations for the vascular prosthesis.

The vascular system of any mammal is very complex and composed of many components, but the schematic representation of Figure 2.1 shows the major elements. The major purpose of the circulatory system is to transport oxygen and nutrients to the parts of the body where they are required, and to remove the waste materials. The transfer of nutrients and wastes takes place almost exclusively at the capillaries. The rest of the circulatory system is simply there to transport the nutrients between the various capillary sites. The flow of the blood is from the high pressure (arterial) side to the low pressure (venous) side, at rates and pressures controlled by the heart. The blood is distributed among the various organs by controlling the resistance to the flow at key points, which are marked by X's in Figure 2.1. At these points, the muscular control of the diameter of the arterioles permits the distribution of the blood to the organs with the greatest need. During a typical day, the 5 liters of blood in the circulatory system are recirculated through the heart.
FIGURE 2.1 ROUTES OF BLOOD FLOW FROM THE AORTA TO THE VENA CAVA. RA = RIGHT ATRIUM. RV = RIGHT VENTRICLE. LA = LEFT ATRIUM. LV = LEFT VENTRICLE. (BURTON 1972, P. 52)
Table 2.1, based on the painstaking work of the German histologist, F. Mall, shows the complexity of the circulatory system. The system consists of billions of vessels entangled in an incredible mesh. The vessel size varies over four orders of magnitude, from the vena cava, which is the size of your thumb to the capillaries, which cannot be seen with the naked eye. This chart also shows that 80% of the total volume of the blood is found in the venous system. This is an indication that the vessels of the venous and arterial systems have very different physical characteristics. Indeed, the blood flow rates, pressure-time dependence, pressure magnitude, physical construction and basic characteristics are all different.

The relative velocities and cross-sectional areas are shown in Figure 2.2. Although the diameter of each type of blood vessel decreases as the circulatory system branches from the main arteries to capillaries, the total cross-sectional area increases due to the large numbers of smaller vessels. The cross-sectional area of the capillaries is about 800 times that of the aorta. By the equation of continuity, there must be a proportional drop in velocity. The graph in Figure 2.2 illustrates this drop, from 28 cm/sec (10 ft/11 sec) in the aorta to 0.05 cm/sec (10 ft/1hr 42 minutes) in the capillaries.

The venous system generally has a lower velocity than the arterial system for a given degree of branching. The
<table>
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<th>Type of Vessel</th>
<th>Diameter (mm)</th>
<th>Number</th>
<th>Total Cross-Sectional Area (cm²)</th>
<th>Length cm</th>
<th>Total Volume (cm³)</th>
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<td>Aorta</td>
<td>10</td>
<td>1</td>
<td>0.8</td>
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<td>30</td>
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<tr>
<td>Large Arteries</td>
<td>3</td>
<td>40</td>
<td>3.0</td>
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<td>60</td>
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<tr>
<td>Main Artery branches</td>
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<td>600</td>
<td>5.0</td>
<td>10</td>
<td>50</td>
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<tr>
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<td>1,800</td>
<td>5.0</td>
<td>1</td>
<td>25</td>
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<td>Arterioles</td>
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<td>125</td>
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<td>25</td>
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<td>Capillaries</td>
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<td>0.1</td>
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<td>570</td>
<td>0.2</td>
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<td>1</td>
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<td>11</td>
<td>20</td>
<td>220</td>
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<td>Vena Cava</td>
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<td>1</td>
<td>1.2</td>
<td>40</td>
<td>50</td>
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*Data of F. Mall, 1888 in Burton, 1972.
FIGURE 2.2. SCHEMATIC GRAPH SHOWING THE CHANGES IN RELATIVE TOTAL CROSS-SECTIONAL AREA OF THE VASCULAR BED (BROKEN LINE), AND THE MEAN VELOCITY IN THE DIFFERENT TYPES OF VESSELS. (BURTON 1972, P. 54)

mean velocity of blood in the vena cava (20 cm/sec) is only two-thirds that found in the aorta. In exercise the mean velocities increase up to four times the resting values given above, so that the velocity in the aorta may be over one meter per second. Also, the actual velocity is pulsatile, rising to a peak value of three times the mean value in systole, and much less in diastole.

A comparison of the distensibility of the major blood vessels (Figure 2.3) illustrates the macroscopic differences between the arterial and venous systems. The veins are sensitive to relatively small changes in pressure, whereas relatively large pressures are required to affect the arteries. Both curves illustrate a kink that is due to their internal structure. A loosely strung outer layer of collagen exerts little influence upon the distensibility of the vessel until after the kink in the curve is reached; then it becomes the dominant element. Since large veins are normally collapsed to a dumbbell shape, this also adds to their ability to act as reservoirs. Another feature of veins is that even if the internal pressure is less than the external pressure, the blood can flow fairly easily through the tunnels formed on either side of the collapsed vein. The cross-section of the vessels in Figure 2.3 show the typical round shape of the arteries and collapsed shape of the veins.

Each type of blood vessel is formed from four types of tissue, the endothelial lining, elastin fibers, vascular smooth
muscle and collagen fibers. The relative proportions of the fibers is different for each type of blood vessel and are illustrated in Figure 2.4. Large arteries are usually known as elastic vessels, due to the predominance of elastin. Smaller arteries are often called muscular vessels since the vascular smooth muscle dominates the vessels' structure. Veins usually have much thinner walls than arteries for a given size of lumen. Figure 2.5 shows the difference in wall thickness for similar sized veins and arteries.

The walls of the vessels are usually divided into three layers or tunicae: the tunica intima (inner layer), consisting of the endothelial cells, subendothelial connective cells and elastic tissue; the tunica media (middle layer), consisting of vascular smooth muscle, and variable amounts of collagen and elastin; and the tunica adventitia (outer layer), which is primarily collagenous tissue.

In each type of blood vessel a single layer of endothelial pavement cells forms the inner lining. In Figure 2.6, a few of these cells can be seen on the inner surface of an arteriole. The purpose of these cells is to provide a smooth wall that offers selective permeability to water, electrolytes, sugars and other substances. Oxygen and carbon dioxide are also exchanged through the walls of all vessels to a certain extent. The presence of the endothelial cells plays little importance in the total elasticity of the vessel, since very little force is required to deform them. The intercellular material
FIGURE 2.4 SIZES, THICKNESS AND TISSUE COMPOSITION OF DIFFERENT BLOOD VESSELS. THE NUMBERS UNDER THE VESSEL NAME ARE THE LUMEN DIAMETER AND WALL THICKNESS. END.= ENDOTHELIAL LINING CELLS. ELA.= ELASTIN FIBERS. MUS.= SMOOTH MUSCLE. FIB.= COLLAGEN FIBERS (BURTON 1972, P.64)
FIGURE 2.5 SMALL ARTERY AND VEIN OF SIMILAR SIZE. NOTE THAT THE VEIN HAS A MUCH THINNER WALL
**ARTERIOLE**

**Red blood cells**: Abnormally clumped together in the lumen of the arteriole as a result of the fixation of the tissue.

**Internal elastic lamina**: Corrugated band due to the contraction of the circular smooth muscle. Prominent diagnostic structure found between intima and media.

**Smooth muscle**: Circularly arranged in the media. Note the presence of delicate elastic fibers in this layer. Although not shown, collagenous and reticular connective tissue fibers are also found in this layer.

**Collagen**: In the thick adventitia. Note the thin layer of slightly corrugated elastic fibers between the adventitia and media.

**FIGURE 2.6** (BERGMAN AND AFIFI, 1974)
of this layer is composed largely of mucopolysaccharides, which, by themselves, have been shown to have no adverse effect upon the blood (Yannas, Silver and Salzman 1976). The lining should not be considered rigid, fixed and unchanging, since it is felt that the endothelial cells migrate freely along the vessel walls, and reproduce by mitosis (Burton 1972, p.64).

The elastin fibers often form a layer or layers just behind the lining cells and are also scattered through the media and adventitia. Generally, the elastin, adjacent to the endothelial cells forms a membrane. that is, the elastin fibers form tubular sheets of material instead of just individual fibers, as shown in Figure 2.6. These fibers are very easily stretched (about ten times more easily than rubber; see Table 2.2) and can be extended many times their unstretched length before reaching their elastic limit. In the aorta the elastic membranes are often found in multiple concentric sheets near the intima. These elastic membranes are particularly important in large arteries, as they absorb the pressure shock of the pulse.

The collagen fibers form networks through the media and adventitia. Collagen resists stretching much more than elastin. However, these fibers are loosely strung around the blood vessels (see Figure 2.6) so that their full resistance to stretching is not felt until the elastin has already stretched to a great degree. This jacket of fibers is responsible for
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<th>Elongation at break (%)</th>
<th>Source of Information</th>
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<td>210</td>
<td>4,400</td>
<td>750</td>
<td>Hand book of Industrial Materials, p.298</td>
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<td>Aluminum Alloys</td>
<td>10,000,000</td>
<td>20,000</td>
<td>4</td>
<td>Baumeister 1978, pp.5-3, 5-5</td>
</tr>
<tr>
<td>Cast Carbon Steel</td>
<td>30,100,000</td>
<td>63,000</td>
<td>30</td>
<td>Lynch 1975, p.39</td>
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<tr>
<td>Endothelium</td>
<td>Negligible</td>
<td>?</td>
<td>Very great</td>
<td>Burton 1972, p.66 (Micro-manipulation studies)</td>
</tr>
<tr>
<td>Muscle (Rectus abdominis)</td>
<td>3 to 100</td>
<td>21</td>
<td>65</td>
<td>Yamada 1970, pp.93,95</td>
</tr>
<tr>
<td>Elastin Fibers</td>
<td>43.5</td>
<td>145</td>
<td>&gt;100</td>
<td>Burton 1972, p.66</td>
</tr>
<tr>
<td>Collagen (Calcaneal Tendon)</td>
<td>28,000 to 140,000</td>
<td>8,000</td>
<td>10</td>
<td>Yamada 1970, p.100</td>
</tr>
</tbody>
</table>
the kink in the distensibility curve of Figure 2.3. This loose array of collagen can be compared to that found in tendons, (see Figure 2.7) which is very oriented and results in high a Young's Modulus and tensile strength in the longitudinal direction. In Figure 2.6, the collagen fibers can be seen to have a slackness about them unlike the collagen fibers in tendons. The collagen and elastin fibers are oriented circumferentially as can be seen in the longitudinal section of Figure 2.8.

The collagen and elastin fibers maintain the vessel walls in equilibrium by offering passive resistance to the transmural pressure. The vascular smooth muscle, on the other hand, is the active component in the system. It is only through the combination of the elastin and the vascular smooth muscle, that graded vasomotor control is possible. If only muscle were present, the blood vessel would be either wide open or completely closed with no stable intermediate position (Burton 1972, pp. 70-71). The muscle cells are oriented diagonally between the elastin membranes (see Figure 2.9) in order to intimately connect the active and passive components. The greatest control over the blood flow is found in the arterioles, where the smaller lumen can be virtually closed, if necessary. In this way, the distribution of blood to the various parts of the body can be controlled. The vascular smooth muscle is quite apparent in the distributing arteries, but here its physiological function is not clearly understood.
Collagenous Connective Tissue
Tendo calcaneus (Tendon of Achilles)
Longitudinal section

Collagen: Fibers oriented in one direction and in dense aggregates and bundles separated by a small amount of areolar connective tissue containing vessels and nerves. Collagen fibrils are flexible, have high tensile strength and are inelastic. This large tendon links the gastrocnemius and soleus muscles with the calcaneus bone at the rear of the foot.

Fibrocyte nuclei: The predominant cell in this type of connective tissue (dense collagenous type) is oriented primarily in the longitudinal axis, in rows between the bundles of collagenous fibers.

Figure 2.7 (Bergman and Afifi, 1974)
This is a longitudinal section of an arteriole. The stain used in this preparation provides good differentiation between collagenous connective tissue, which stains blue, and muscular elements in the wall of the arteriole, which stain reddish brown. Note the centrally placed nuclei in the smooth muscle cells which are seen in cross-section. The lumen of the artery is filled with blood cells.

FIGURE 2.8 (BERGMAN AND AFIFI, 1974)
AORTA, VERHOFF'S STAIN, 850 x

20

FIGURE 2.9 ARTERIAL WALL, SHOWING THE INTER/connection OF THE ELASTIN MEMBRANES AND VASCULAR SMOOTH MUSCLE.
The mean pressure in veins and arteries differs substantially as indicated in Table 2.3. This table shows typical mean pressures at various points in the circulatory system for a person at rest. The pressure is highest (~100mm Hg), at the distributing arteries, which are nearest the heart, and decreases due to the flow resistance as the arteries branch further from the heart. The pressure remains fairly high (60mm Hg), even at vessels as small as arterioles. As the venous system is entered, the pressure is only a small percentage of the original value, and finally, in the vena cava the pressure is only 10% of that found on the output side of the heart.

The different pressures result in different average tensions in the blood vessels. The Law of Laplace, 
\[ \text{Tension} = \text{Pressure} \times \text{Radius} \] 
(T=P·R), can be used to relate the pressure to the tension. If the internal pressure were to become too great, the vessel would "blow out" causing a local aneurism. Normal, healthy arteries can withstand pressures of about 1000 mm Hg, although the maximum peak pressure value found in a healthy individual is only about 300 mm Hg.

The true pressure in the vessels is not constant, but is pulsatile, varying between the systolic (maximum) and diastolic (minimum) pressures with each heart beat as shown in Figure 2.10. The blood pressure, as measured by a physician, is usually given as the ratio of systolic to diastolic pressures in mmHg/mmHg. For example, 120/80 is considered to be a normal
Table 2.3  **Circumferential Tension in The Walls of Blood Vessels***

<table>
<thead>
<tr>
<th>Type of Vessel</th>
<th>Mean Pressure (mmHg) (psi)</th>
<th>Radius</th>
<th>Tension (lb/in)</th>
<th>Amount of Elastic Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aorta and large arteries</td>
<td>100 1.93</td>
<td>1.3 cm or less</td>
<td>0.97</td>
<td>Very elastic, two coats</td>
</tr>
<tr>
<td>Small distributing arteries</td>
<td>90 1.74</td>
<td>0.5 cm</td>
<td>0.33</td>
<td>Much elastic tissue but more muscular</td>
</tr>
<tr>
<td>Arterioles</td>
<td>60 1.16</td>
<td>0.15 mm to 62 um</td>
<td>0.0069 to 0.0028</td>
<td>Thin inner elastic lamina only</td>
</tr>
<tr>
<td>Capillaries</td>
<td>30 0.58</td>
<td>4 um</td>
<td>0.00009</td>
<td>None</td>
</tr>
<tr>
<td>Venules</td>
<td>20 0.39</td>
<td>10 um</td>
<td>0.00015</td>
<td>None except in the largest venules</td>
</tr>
<tr>
<td>Veins</td>
<td>15 0.29</td>
<td>200 um</td>
<td>0.0023</td>
<td>Elastin fibers present</td>
</tr>
<tr>
<td>Vena cava</td>
<td>10 0.19</td>
<td>1.6 cm</td>
<td>0.12</td>
<td>Very elastic, with large fibers</td>
</tr>
</tbody>
</table>

*Burton 1972, p.70.
FIGURE 2.10 CYCLIC PRESSURE FOUND IN ARTERIES. (BURTON 1972, P.88)

FIGURE 2.11 EFFECTS OF POSTURE ON LEVEL OF ARTERIAL AND VENOUS PRESSURES. THE GRADIENT DOWN ARTERIES AND VEINS DUE TO FLOW (FLOW x RESISTANCE) IS INCLUDED IN THE DIAGRAM. THE FIGURES ARE ESTIMATED LEVELS OF THE PRESSURES, IN MILLIMETERS OF MERCURY, REFERRED TO THE LEVEL OF THE RIGHT ATRIUM AS DATUM LEVEL. (BURTON 1972, P.99)
resting blood pressure, but pressures as high as 240/160 are
typical in cases of essential hypertension. The mean pressure
can often be approximated by the formula: \( \frac{1}{3} \times (\text{systolic}
\text{ pressure} + 2 \times \text{diastolic pressure}) \). The rate at which this
cyclic pressure change occurs is about 70 cycles per minute,
for an individual at rest. The body, of course, is not always
resting. During fairly heavy exercise, the pressure may in-
crease to 180/90 and the pulse rate will rise to well over
150 beats per minute. As the pulse pressure wave travels
from the larger arteries to the smaller distributing branches,
the basic shape remains the same, but the excursion from the
mean value decreases to virtually nil at the capillaries.

The physician measures the blood pressure on the arm,
at the same height as the heart to eliminate any hydrostatic
effects. The hydrostatic effects are very real, though, and
have a marked effect upon the local transmural blood pressures
throughout the body (see Figure 2.11). The difference in
pressure, for example, between the feet while in a horizontal
position, and while in a vertical position is approximately
88 mm Hg.

The venous system appears to have less stringent demands
made upon it, which perhaps accounts for the thinner walls
as compared to arterial system. The mean venous pressure is
only about 15 mm Hg and is also pulsatile. The shape of the
pulse wave is not well defined and varies from vein to vein,
but generally the peaks are not as sharp and pressure gradients
are not as large as those found in the arteries (see Figure 2.12).
It is interesting to note the effect that posture has upon the mean venous pressure. In the vertical position, the pressure in the foot may be as high as 90 mm Hg, however, the pressure in the upper extremities may drop to -40 mm Hg. The veins in the neck or a raised arm, therefore, will collapse under this negative transmural pressure. As noted earlier in the report, the collapsed vein will still allow the flow of blood through the tunnels formed on either side. This effect is not observed within the cranium, as the tissue pressure is adjusted to maintain a positive transmural pressure.

Another effect of the increased pressure in the lower extremities, is that valves are necessary in the veins to maintain the flow of blood toward the heart. The valves allow the flow of blood to be facilitated by the contraction of muscles. The importance of these valves is apparent when they malfunction, as in the case of varicose veins. When the veins varicose, the only non-surgical solution is to raise the veins often enough to allow the blood to flow to the heart, and prevent venous pooling in the lower extremities.

The foregoing has been a brief summary of some of the pertinent physiological characteristics of the circulatory system. Additional information may be found in a variety of books, including: Guyton, 1976; Berne and Levy, 1977; Burton, 1972; and Middleman, 1972.
CHAPTER III

Hemostasis

The body has many defense mechanisms which it implements when something goes wrong. Hemostasis, the prevention of blood loss, is one of these defense mechanisms. It is a very complex process which is designed in such a way that it can handle both the very small day-to-day leaks, as well as major wounds.

Initially, the vascular smooth muscle contracts in the wall of the damaged blood vessel. If the lumen is small enough, the endothelial cells on opposite sides of the lumen stick together in a further effort to prevent the loss of blood. Platelets enter into the response by clumping to form a plug and releasing chemicals to aid in the formation of a fibrin clot. Finally, the clot retracts to complete the mechanism and, hopefully, stop the loss of blood.

Platelets are intimately involved in the coagulation process; there are links at several points in the chain which the platelets must fill before the reaction can continue. Platelets are small (~1 μm dia.) anucleate cells which are present in large numbers in the blood (~250,000,000 per ml). The lifetime of the platelets in the blood stream is about seven days, if they are not involved in a clot. They have a propensity to adhere to practically everything, other than the endothelial lining cells and the mucopoly saccharide intercellular material between the endothelial cells.

Platelets initiate the coagulation process by adhering
to a foreign surface. The foreign surface may be the collagen within the wall of the blood vessel which is exposed during trauma or, perhaps, a glass vial containing some extracted blood. Following the adhesion step, the platelets undergo a shape change and extend pseudopods. The change of shape is also accompanied by the release of the contents of the granules in the platelets, commonly called the release reaction. The chemicals released from the granules include ADP, which makes the surface of the platelets sticky, encouraging the clumping of more platelets on the platelets which have adhered to the foreign surface. During this positive feedback process, epinephrine and serotonin, which cause vasoconstriction, are also released. The chemical contents of the granules and perhaps the ADP-affected platelet surface are required to allow the coagulation cascade to continue. The coagulation cascade is the name given to the very complex series of chemical reactions which eventually result in the formation of a fibrin clot, whether initiated by the intrinsic pathway, or the extrinsic pathway.

A variety of references which discuss the role of platelets in hemostasis are listed in the bibliography. The chapter by Deykin in Reich (1978) offers a more complete, but not unduly complicated description of the hemostasis. The article by Mustard (1976) concentrates on the role of platelets in the hemostatic mechanism. Lindon (1974) illustrates the changing morphology of platelets during hemostasis with a series of very beautiful scanning electron micrographs.
Chapters 128 to 141 in Williams (1977) give very detailed descriptions of many aspects of hemostasis and include extensive reference lists.
CHAPTER IV
The Design of Vascular Prostheses

Design Recommendations

The design of vascular prostheses involves meeting biological, chemical and mechanical design criteria, simultaneously. When a prosthesis is implanted, the body makes many demands upon the prosthesis, but also, merely by its presence in the body, the prosthesis makes demands upon the body. It is the double action nature of the design problem which increases the difficulty of reaching a solution. A piece of glass tubing, for example, would probably meet most of the mechanical design requirements, but the presence of the glass causes many strange reactions in the blood.

Several basic requirements for a vascular analog have been isolated. The material and geometry of the prosthesis must not upset the body's natural defenses. This includes both the immunological response to foreign bodies and the hemostatic response to vascular damage. For example, an adverse allergic reaction should not be elicited by the body. Nor should the material alter or destroy any of the cells or proteins in the blood. The material should not be toxic to the blood or surrounding tissue. Also a malignant reaction to the material is also an undesirable occurrence. The material, on the other hand, must withstand the attack of the various chemicals and enzymes found in the body.
Two major specifications are made on the strength of the material. From the surgeon's point of view, the prosthesis must be easy to suture in place, without being too fragile to handle. The vascular prosthesis must also be able to perform its major function of transporting blood, while withstanding the transmural pressure and fatigue caused by the pulse. Of course, anything which is implanted must be sterile or capable of being sterilized.

The most important decision for a vascular prosthesis, is the choice of material. This is because the detailed mechanisms of the immunological and hemostatic responses are not fully understood. A material can be tested to see if it is blood compatible, or if the body will reject it as a foreign body, but the reason for the variation of compatibilities among materials is still open to discussion. Therefore, control of materials to improve their compatibility characteristics is still difficult and uncertain. A discussion of some of the available materials follows this section.

The geometry of the prosthesis is also important. Platelets can also react with rough surfaces, initiating the clotting process (Turitto 1977). For this reason, designs which involve seams should be avoided. The simple rolling of a flat sheet to a tube with an overlapping seam is not a suitable process for forming a vascular analogue (Yannas 1975).

Making a material sutureable, means that the material must be soft enough to put a needle through, but tough enough that
it will not rip when some force is applied to the stitch. The implications of this requirement are that the material must have strength in many directions. For example, a material oriented like Christmas wrap ribbon would not be suitable. It has strength in the longitudinal direction, but if it were sewn to something, the fibers would simply separate. A strength of 100 psi for a material 4 mils thick has been determined by an experienced surgeon, Dr. R. Collins, as a minimum value for suturability (Yannas 1977). This specification must be used with care, as it tacitly assumes an isotropic material.

The most obvious mechanical requirement of vascular prosthesis is for it to withstand the transmural pressure. The determination of that pressure, however, is slightly more involved than it appears at first glance. The mean blood pressure of a resting person is about 100 mmHg—at the level of his heart. When standing, the pressure at his feet is actually 88 mmHg higher. The flow is also pulsatile, 120 mmHg/80 mmHg typical resting value. While exercising the pressure can easily jump to 180/90, which implies a peak pressure of about 270 mmHg in the distributing arteries of the feet. The actual peak pressure may be some 30 mmHg higher, depending upon the individual. The pressure may also be 10-30 mmHg lower than this value, depending upon weather the vessel in question is a distributing artery or a vessel as small as an arteriole. Therefore 300 mmHg can be considered a maximum peak pressure for normal healthy individuals.
An all too common disease, in the North American Society, is varying degrees of essential hypertension. The nominal resting pressures of 240/160 which are typical of this disease, would correspond to a peak exercise pressure of 300 mmHg. This implies a maximum peak pressure of 388 mmHg in the feet, due to the hydrostatic pressure. It is suggested that for the design of artificial arteries, 388 mmHg should be used as a minimum design to encompass persons with relatively high blood pressure. Two points should be noted. The pressure of 240 mmHg is not the maximum value which can occur, in essential hypertension. Resting pressures of 300 mmHg have been recorded. Such individuals, however, are not usually capable of exercising to any great degree, and would not be capable of raising their blood pressure much above the 300 mmHg value. This, again, corresponds to a maximum value of 388 mmHg in the lower extremities, due to the hydrostatic pressure. If the vascular prosthesis is to be implanted at the level of the heart, then 300 mmHg would be an adequate design pressure and 388 mmHg would cover even the most extreme cases.

This design pressure does not contain a safety factor. This value should be considered as a peak pressure which could actually be experienced by the prosthesis. Therefore, exceeding this specification results in additional safety; insufficient strength to meet this specification should be regarded as possible failure. The pressure that healthy arteries can withstand is in excess of 1000 mmHg, implying that nature's
safety factor is greater than 2 1/2.

The internal pressurization of a tube causes a tangential tension, in accordance with the Law of Laplace: \( T = P \cdot R \). This formula shows that for a given pressure, the tension increases with the size of the vessel. Therefore, the radius and wall thickness must be considered when calculating the tangential stress. Since the wall thickness, \( t \), of a vascular prosthesis is small compared to the radius, the tangential stress, \( \sigma \), may be considered uniform as calculated by \( \sigma = T/t \). Table 4.1 shows values for the tangential stress as calculated by this formula and assuming the design pressure of 388 mmHg. It is evident from this chart, that depending upon the wall thickness and radius, large variations are found in the permissible tangential stress. Three radii of vascular prostheses are of particular interest, 0.262 cm (0.206 in. dia.) 0.176 cm (3.52 mm dia.) and 0.15 cm (3 mm dia). The first is the size of present prototype vascular prostheses the second is the size at which tubing is required for use in Dr. Baumgartener's experiments, and the third is the size required for Dr. Salzman's experiments with sheep. At a 4 mil wall thickness, the expected maximum stresses are 193 psi, 130 psi, and 111 psi for these radii. These are the stresses which the material of the vascular prosthesis should be able to withstand to prevent a local aneurism.

The maximum stresses given above are peak stresses in a cyclic process involving more than 100,000 cycles per day.
Table 4.1: Tangential Stress In Pressurized Tubes (PSI)

<table>
<thead>
<tr>
<th>Radius, R (cm)</th>
<th>Tension, T (lb/in)</th>
<th>Wall Thickness of the Tube, t (mils)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.3</td>
<td>3.84</td>
<td>1920 1280 980 768 512 384 192</td>
</tr>
<tr>
<td>1.0</td>
<td>2.95</td>
<td>1477 985 738 591 394 295 148</td>
</tr>
<tr>
<td>0.5</td>
<td>1.48</td>
<td>738 492 369 295 197 148 74</td>
</tr>
<tr>
<td>0.318</td>
<td>0.938</td>
<td>469 313 235 188 125 94 47</td>
</tr>
<tr>
<td>0.262</td>
<td>0.774</td>
<td>387 258 193 155 103 77 39</td>
</tr>
<tr>
<td>0.2</td>
<td>0.591</td>
<td>295 197 148 118 79 59 30</td>
</tr>
<tr>
<td>0.176</td>
<td>0.520</td>
<td>260 173 130 104 69 52 26</td>
</tr>
<tr>
<td>0.15</td>
<td>0.443</td>
<td>222 148 111 89 59 44 22</td>
</tr>
<tr>
<td>0.1</td>
<td>0.295</td>
<td>148 98 74 59 39 30 15</td>
</tr>
</tbody>
</table>

Pressure assumed to be 388 mmHg = 7.5 psi
Since the process is cyclic, fatigue effects are present. The effect of fatigue, is to cause the material to break after many cycles of a stress, which may be well below the ultimate tensile strength. Usually the number cycles required to fatigue a specimen to breakage increases as ratio of the applied stress to the ultimate tensile strength decreases. In many common materials there is a hold stress, above which fatigue will eventually cause breakage, and below which there is no limit to the number of cycles a specimen can undergo. The material presently used for vascular prostheses does not have well characterized fatigue properties, particularly when formed by the method of cross-flow filtration. A rule of thumb often used, is to limit the applied stress to 1/2 to 2/3 the ultimate tensile strength, that is, a safety factor of 1 1/2 to 2 times. Therefore, the expected maximum stresses, found in Table 4 should be multiplied by this safety factor to reduce the fatigue effects.

The results are, that for a material of 4 mils thickness, the ultimate tensile strength of the material should be between 290 to 386 psi for 0.206 in. diameter tubing, 195 to 260 psi for 3.52 mm diameter tubing, and 167 to 222 psi for 3 mm diameter tubing. If these specifications are met by an isotropic material, there should be no problem with the suturability of the material.

The above design criteria are directed toward the production of vascular prostheses for use as arteries. It would
seem that any tubing which meets these specifications, would also meet the requirements for an artificial vein. While it is true that the venous system is at a much lower pressure, and, therefore, has less stringent demands made upon the walls to resist the transmural pressure, there are several additional criteria to obey. For instance, when the transmural pressure becomes negative, as can happen when you raise your arm, the collapsed venous analogue must still permit the flow of blood. In the natural vein, this is achieved by maintaining a certain minimum radius so that a tunnel formed on either side of the collapsed vein. This same technique could be applied to a vascular prosthesis, but other suitable methods could be found. The basic implication of this requirement is that the venous analogue must have some structural rigidity, which is not necessary in an arterial analogue.

Another problem, which is present to a lesser extent in arterial analogues, is that the distensibility of the implant should be similar to the natural vessel on either side of it. If the distensibilities are not closely matched, there will be undue stress on the sutured joint. Also, this difference could result in reflected pressure waves, which can cause many problems, including interference of the closing of the heart valves. The distensibility characteristics of the veins are quite important, as the veins are required to act as reservoirs of blood during changing pressure situations.
A further problem, unique to veins, is the presence of valves to maintain the flow of blood in the correct direction. They are usually found in the larger veins directly after a smaller branch joins the main vein. These valves are particularly important in the lower limbs, and indeed a probable application for venous analogues is in the replacement of varicose veins.

There are many secondary problems which may or may not prove to be of importance in practice. One of these, which would probably become evident if large numbers of vessels were replaced, is the fact that blood vessels are not impermeable cylinders. The walls of blood vessels permit carbon dioxide, oxygen, electrolytes and sugars to pass through. It is therefore conceivable that the permeability of the vascular analogue could be important.

A problem which is not of immediate importance, is the fabrication of bifurcated tubes. The branches of arteries are not simply tubes joined together, but have a special form so that a portion of the blood from the fast flowing center of the main artery is diverted to the branch. (See Figure 4.1).

FIGURE 4.1 ILLUSTRATION OF AN ARTERIAL CUSHION WHERE A SIDE ARTERY LEAVES ITS PARENT ARTERY (BURTON 1972).
This discussion has included what are envisioned as the major problems to overcome in the design of a vascular prosthesis. Berger and Salzman, 1978; Kolff, 1976; Myers and Parsonnet, 1969; and Mason 1972; discuss many of the problems encountered in designing blood contacting devices to a greater degree. Once the given design criteria are met, the completion of a suitable vascular analogue should be a matter of solving the fine tuning problems.

Materials for Vascular Prostheses

Dacron has been used for many years, chiefly to repair the aorta, in spite of the fact that this material is dec- cided by thrombogenic. It is usually implanted as a knitted or woven material which is preclotted to prevent leakage. With such a porous structure the platelets and fibroblasts are encouraged to react with the material forming a neointima over a period of months. The relative success of this graft is due to the rapid embolization of thrombus by the rapidly flowing blood stream. The lumen of the graft remains open, but the emboli may cause minor infarcts throughout the body, in spite of the efficiency of the reticulo-endothelial system. Two further disadvantages of this graft are its propensity to calcify and its tendancy to develop aneurysms, (Wright 1979). Green (1979) notes that dacron grafts were often occluded by hyperplasia of the neointima. The hyper- plastic or cancerous reaction of the body to plastics is
common, particularly if large unbroken sheets of plastic are present. The dacron grafts have not been successful for use as small arteries (Mason, 1972) and would not be suitable for use as veins.

A variety of materials containing heparin have been used for prostheses which must contact blood. Heparin is normally used as an anticoagulant. Its presence improves the blood compatibility of many materials, but the heparin can leach out with time. This results in a material which is no longer as non-thrombogenic as the original material. The use of heparinized materials for use as small veins or arteries has, however, not been very promising (Mason, 1972).

Heparin or other anticoagulating agents are often administered to patients for short or long terms, following surgery involving vascular grafts. This therapy does help to reduce the risk of thromboembolic complications. However, it is difficult to monitor this drug's level over the long term and the patients are also subject to the risk of hemorrhage. A truly bland graft would not need this therapy.

Excluding the autogenous saphenous vein, polytetraflouroethylene (PTFE or "Gore-Tex") grafts currently are the only grafts which are close to acceptable for small artery replacement. One year patency rates of 40% and as high as 75% are reported (Haimov, 1979) for femoropopliteal and femorotibial artery replacements. However, Faulkner (1979) reports a 100%
failure rate when the PTFE grafts are used as venous re-
placements.

The autogenous saphenous vein is still the vascular
graft of choice (Haimov 1979). It is not without problems, however. The vein is often much larger than the artery
to which it must be sutured. This causes surgical dif-
ficulty in anastomosing the graft, as well as flow problems, which can result in stagnant regions and clotting. The vein is not designed to withstand the much higher arterial pres-
sures, and often develops intimal thickening and stenosis. The saphenous vein is not always available to the surgeon. The patients which require a bypass vascular graft operation have a vascular disease. This may prevent the harvesting of a suitable saphenous vein due to the systematic vascular disease or the vein may have already been used in a previous operation. Not the least of the problems associated with this graft are the additional pain and trauma to the patient caused by the harvesting operation.

Umbilical cord veins are occasionally used as vascular
grafts when the saphenous vein is not available. The vein is removed from the umbilical cord, crosslinked in glutaralde-
hyde and is sheathed in a polyester mesh to prevent aneurysms. These grafts have shown promise for use as vascular prostheses but have some complications (Dardik 1976). The collagen which composes the graft has not been treated to eliminate its thrombogenic nature, and hence, thrombus formation is a major
complication; as with the dacron grafts. The availability of these grafts and lack of surgical trauma to the patient make these grafts attractive. The fact that they are made from crosslinked collagen assures longevity (Carpentier 1969) of the graft without a hyperplastic response common with some plastics. An interesting article by Sauvage (1979) evaluates the relative thromboresistance of the umbilical vein graft in comparison with several other types of materials.

The vascular prostheses prepared in this thesis were developed from a desire to make vascular prostheses from the non-thrombogenic collagen reported by Silver (1977). A prosthesis made from non-thrombogenic collagen could be made even more useful than the umbilical vein grafts. Bovine hide collagen was chosen as the source of the collagen, since it is inexpensive and readily available in the large quantities which would be necessary for the commercial production of vascular prostheses. This is an advantage of collagen over most proteins which are available only in small quantities suitable for laboratory work. Collagen is also a very strong protein, making it a desirable engineering material. Collagen was long thought to be immunologically inert but does actually possess some antigenic activity which is of no clinical importance (Ramanandran 1967). Also, it does not show any evidence of eliciting a hyperplastic response from the body.
The glutaraldehyde crosslinking stabilizes the collagen against the degrading effect of the body's chemicals. A bonus derived from using a reconstituted material is that it can be made any size or shape including tapered or bifurcated tubes.
CHAPTER V

Preparation of the Collagen Composite Samples

Introduction

The purpose of these samples is to show the transmission electron micrographic structure of the materials, and to determine roughly which steps in the material preparation are responsible for the nonthrombogenic behavior of the tested materials. The materials were tested by platelet aggregation and viewed under the transmission electron microscope at various stages in the procedure. The sample numbers on Figure 5.1 indicate the stage at which the materials were tested.

The material which is used for the preparation of the collagen specimens is bovine hide collagen. It was prepared by the method described by Komanowsky et al (1974) and donated by H.I. Sinnamon of the U.S. Department of Agriculture. The purified collagen is refrigerated in large freeze-dried chunks which are Wiley milled (Arthur H. Thomas, Philadelphia, Pennsylvania) to a powder before use.

Collagen is a large protein composed of three amino acid chains. It is found in skin and tendons and comprises about 30% of the body's protein. Evenly spaced glycine residues constitute one third of the amino acids in each chain. The chains are arranged in an intertwining triple helix which is referred to as a tertiary structure.
FIGURE 5.1: SAMPLE PREPARATION FLOW CHART

- Disperse in physiological saline
- Blend for 15 minutes
- Wait for 1 hour
- Add 0.5% glutaraldehyde
- Dry and mill
- Form membrane
- Crosslink (0.5% glutaraldehyde)
- Dry and mill
- Form membrane
- Crosslink (0.5% glutaraldehyde)
- Dry and mill
- Form membrane
- Crosslink (0.5% glutaraldehyde)
- Dry and mill
- Form membrane
- Crosslink (0.5% glutaraldehyde)
- Dry and mill
- Form membrane
- Crosslink (0.5% glutaraldehyde)
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- Dry and mill
- Form membrane
- Crosslink (0.5% glutaraldehyde)
- Dry and mill
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The rigid, rod-like molecules are approximately 3000 Å long and 15 Å in diameter, and have a molecular weight of 300,000. These molecules are arranged in a quarter-staggered quaternary structure to form fibrils, which gives rise to the banding pattern observed under the transmission electron microscope. A more detailed discussion of collagen can be found in Silver (1977), Ramachandran and Reddi (1976), Gould (1968) and Ramachandran (1967).

The preparation of the experimental materials begins by Wiley milling bovine hide collagen (BHC) to a 60 mesh particle size. These particles are then dispersed in distilled water, physiological saline, or acetic acid. Most samples were dispersed in acetic acid by blending for one hour, and allowing to swell for three days in the refrigerator, before adding chondroitin-6-sulfate or filtering on a Buechner funnel, or both. Transmission electron micrographic specimens were made from the dispersions. The filtered membranes were sometimes crosslinked in glutaraldehyde before Wiley milling and mixing in saline. This final dispersion was then used in the aggregation tests. In some acid dispersions, glutaraldehyde was added to the dispersion to determine the effect of gluteraldehyde without airdrying. The reason for the three-day swelling time was based on the preliminary transmission electron microscopic data of Paul Denny, which said that three days of swelling were required for the destruction of the native banding in the collagen
fibrils. It was assumed that the destruction of this banding was necessary for the collagen to exhibit a non-thrombogenic behavior.

The sample numbers, D.BS:X, are partially indicative of the sample's history. The first digit, D, indicates the initial dispersing medium of the sample. Physiological saline (0.15 M NaCl, pH 7.0) is indicated by a "1". Distilled water alone, with a pH of approximately 5.8, is indicated by a "3". The usual dispersing medium, 0.05 M acetic acid, is indicated by a "4" for the first digit. The second digit, B, indicates the amount of blending. Hand shaking in a vial or 2 minutes of blending is indicated by a "1". 15, 20, 30, 60 and 120 minutes of blending are indicated by "2","3" "4","5","6" and "7". The final number after the colon is simply a number to denote samples which have more complex procedures than simply blending and swelling. Generally, more complex procedures have a higher value for X. A prime after the number indicates that the sample was retested after 9 or 10 months of refrigeration. Since the sample number only indicates the history, the exact preparation procedure for each sample is detailed below.

Samples 1.10:0 and 1.10:0'

Bovine hide collagen was Wiley milled to a 60 mesh particle size. These particles were dispersed in physiological saline (PS) by simply shaking the vial. The composition of physiological saline is 0.15 M NaCl or 8.77 gm NaCl/per
liter distilled water with pH adjusted to pH 7 by adding dilute NaOH. The concentration was not measured, but estimated to be 50 mg BHC/ml PS. The particles in this sample were very fluffy (like cotton balls) making the sample look similar to a dispersion, although the pH was 7.0. Unlike a dispersion, the particles would eventually settle out. This sample was prepared approximately 3 hours before use in Runs 8 and 9 of the Series A platelet aggregation experiments. This sample was allowed to equilibrate for 292 days (42 weeks), and relabeled 1.10:0' before use in Runs 1B and 1R of Series C experiments.

Sample 2.20:0

0.55 gm of 60 mesh sized particles of bovine hide collagen were dispersed in 200 ml of distilled water. The bovine hide collagen used for this work has been shown to contain approximately 10% (v/v) water. Therefore, 0.55 gm of collagen, wet weight, is approximately 0.50 gm of collagen, dry weight.

The pH of the water had previously been adjusted to 7.0 by adding dilute sodium hydroxide. The dispersion was blended for 15 minutes in an ice-jacketed Eberbach container assembly (model 8590). The blender was powered by a Waring two-speed blender power unit (model 8420) set to high, but with an auto-transformer reducing the line voltage to 50% of 120 volts. This dispersion showed many large clumps of collagen particles.
which would quickly settle out if the dispersion were allowed to stand. A small portion of this sample was used to make transmission electron microscopic specimens while the remainder of the dispersion was used to make sample 2.10:1.

Sample 2.60:0

0.1375 gm of 60 mesh sized particles of bovine hide collagen were dispersed in 50 ml of distilled water adjusted to pH 7.0 by adding dilute sodium hydroxide. After 2 hours of blending at the highest setting on a portable overhead Greiner blender, the dispersion was used to make transmission electron microscopic specimens.

Sample 2.20:1

The pH 7.0 collagen dispersion described in Sample 2.10:0 was used to make this sample. The dispersion was filtered on a Buechner funnel using Schleicher and Schuell filter paper. The majority of the fluid had passed through the filter paper after about 15 minutes using only gravity as a driving force. After thorough air drying, the membrane was crosslinked for 24 hours in a 0.5% (v/v) glutaraldehyde bath. The bath was made by adding 20 ml of 25% glutaraldehyde to 1 liter of distilled water and subsequently adjusting the pH to 7.0 with dilute sodium hydroxide. The crosslinking bath was maintained at room temperature, 24 to 25°C. Following the crosslinking, the membrane was rinsed, several times, with deionized water to remove any free glutaraldehyde.
Once the membrane had air dried, it was Wiley milled to a 60 mesh particle size and dispersed in physiological saline (2.79 mg BHC ml/PS) approximately 3 hours before use in runs 3B, 3R, 4B and 4R of the Series G experiments.

**Samples 3.20:1 and 3.20:1′**

0.55 gm of 60 mesh Wiley milled BHC was blended on a Waring one-speed power unit, model 8400, (50% of 120 V) for 15 minutes in 200 ml of Waugh water, pH = 5.8. The dispersion was immediately filtered in a 150 mm Buechner funnel on Schleicher and Schuell filter paper for about 15 minutes, then air-dried overnight (≈16 hours). The dried membrane was Wiley milled to a 60 mesh particle size and dispersed in physiological saline. A concentration of 100 mg collagen to 10 ml of physiological saline was used in Runs 1 and 17, and 10 mg/10 ml was used for Run 2 of the Series A experiments. The 100 mg/10 ml sample was re-used 9 days later for Run 1 of Series B. This sample was relabelled 3.10:1' and used again for Runs 5B and 5R of Series D 293 days (42 weeks) after it was made. This sample had particles which were much more dense than those in Sample 1.10:0, and settled more quickly to the bottom. The particles also appeared to be whiter in color, perhaps due to their greater density. The particles settled out to approximately 1/8 the total volume of the sample.
Sample 3.20:1a

This sample is the same material as that described in Sample 3.10:1 except that the particles were added, dry, to the platelet-rich plasm (PRP) during the aggregation run, rather than dispersed in physiological saline. This sample was used for Run 13 of Series A.

Sample 4.10:0

A dispersion was made from 0.55 gm of 60 mesh sized particles of bovine hide collagen and 200 ml of 0.05 M acetic acid. A dispersion made in this ratio is known as a 1/4% (w/w) dispersion. The dispersion was blended in an ice-jacketed Eberbach blender on a two-speed Waring power unit set to high, and the line voltage reduced to 50% of 120 volts. At the two minute mark a dropperful of dispersion was used to make transmission electron micrographic specimens. The procedure of staining and blotting dry each grid was completed within 1.5 minutes to assure faithful preservation of the specimens. As the blending of the dispersion continued, this dispersion was used to form some of the specimens for Samples 4.20:0, 4.40:0 and 4.50:0.

Sample 4.20:0

The dispersion of sample 4.10:0 was used to make this sample. After 15 minutes of blending the bovine hide collagen in acetic acid, a dropperful of the dispersion was used to make the transmission electron micrographic specimen.
As before, the staining and blotting procedure was completed within 1 1/2 minutes.

**Sample 4.20:1**

A dispersion was made from 0.55 gm of bovine hide collagen milled to a 60 mesh particle size and 200 ml of 0.05 M acetic acid. The dispersion was blended (50% of 120 V, high) for 15 minutes in an ice-jacketed blender before filtering in a Buechner funnel. The filtering was virtually completed after 1 1/2 hours. Half of this membrane was used for Sample 4.20:2. The membrane was air-dried, overnight, before Wiley-milling to a 60 mesh particle size and dispersing in physiological saline at a concentration of 64.6 mg BHC/2 ml PS. This sample was used for Runs 5B and 5R of Series G.

**Sample 4.20:2**

As noted above, one half of the membrane made by the procedure of Sample 4.20:1 was used for this sample. After air-drying, the membrane was crosslinked in a 0.5% (v/v) glutaraldehyde bath adjusted to pH 7.0 with dilute sodium hydroxide. Following 24 hours of crosslinking at room temperature, 24 to 26°C, the membrane was washed with distilled water to remove the free glutaraldehyde and air-dried. The dried membrane was Wiley-milled to a 60 mesh particle size and dispersed in physiological saline (16.4 mg BHC/0.5 ml PS) for use in Runs 1B, 1R, 2B and 2R of Series G.
Samples 4.20:3 and 4.20:3'

The preparation of these samples involved adding 3.3 gm of 60 mesh Wiley-milled bovine hide collagen to 320 ml of 0.05 M acetic acid. The collagen dispersion was blended (50% of 120 V) for 15 minutes. Without allowing the collagen any time to swell, 0.396 gm of chondroitin-6-sulfate, dissolved in 80 ml of 0.05 M acetic acid, was added over the next 6 minutes while the blender was operated at 100% of 120 v. The chondroitin-6-sulfate used in the preparation of dispersions has also been shown to have a moisture content of about 10% (w/w). Therefore, a dispersion made with 3.3 gm of collagen plus 0.396 gm of chondroitin-6-sulfate contains a 12% dry weight ratio of chondroitin-6-sulfate to collagen. The blending continued at this high rate for an additional 9 minutes. This resulted in a 12% (w/w) chondroitin-6-sulfate. 3/4% (w/v) collagen dispersion which was refrigerated until it was used in Run 14 of Series A, 16 days later. The sample was relabelled 4.20:3' after equilibrating for 302 days (43 weeks), when it was used to make a transmission electron micrographic specimen. One week later, the reactivity of platelets to this specimen was tested by performing Runs 3B and 3R of Series D. Samples 4.20:3 and 4.30:1 were very similar in appearance, a homogenous thick fluid, which appeared to be whiter than collagen-acid dispersions with no chondroitin-6-sulfate.
Samples 4.30:1 and 4.30:1'

The history of these samples is quite similar to those described above. 3.3 gm of 60 mesh Wiley-milled bovine hide collagen were blended (50% of 120 V) for 20 minutes in 320 ml of 0.05 M acetic acid. With no swelling time allowed, 0.264 gm of chondroitin-6-sulfate, dissolved in 80 ml of 0.05 M acetic acid was added over 7 minutes while blending at a setting of 100% of 120 volts. 8 minutes of additional blending was allowed. This 8% (w/w) chondroitin-6-sulfate, 3/4% collagen dispersion was refrigerated for 28 days before it was used in Run 15 of Series A. This sample was relabelled 4.30:1' after 314 days of equilibriating. A transmission electron microscopic specimen was made at this time. One week later this sample was used in Runs 2B and 2R of Series D.

Sample 4.40:0

This sample was prepared from the same dispersion that was used to make sample 4.10:0. A dispersion was made from 0.55 gm of collagen blended at 50% of 120 volts in 200 ml of acetic acid. After 30 minutes of blending, a dropperful of dispersion was used to make transmission electron micrographic specimens. The staining and blotting dry procedure was completed within 1 1/2 minutes after the time the dropper was filled.

Sample 4.50:0

Several different dispersions were made, each with the same history. 20 ml of 0.05 M acetic acid and 0.55 gm of 60 mesh sized particles of bovine hide collagen were blended at 50% of 120 volts on the high setting for 60 minutes. A drop
of the dispersion was then used to make a transmission electron micrographic specimen.

Sample 4.51:0

The method for making a 1/4% collagen dispersion, as described above, was used as a starting for all samples numbered with 4.5S:X. Sample 4.51:0 was simply made by storing a 1/4% collagen dispersion, which had been blended for one hour, in the refrigerator for 12 hours. At the end of that time, a transmission electron micrographic specimen was made. This sample was also stored to provide one of the dispersions described as Sample 4.53:0.

Sample 4.52:0

A 1/4% bovine hide collagen dispersion was blended for one hour, then refrigerated for 24 hours. A transmission electron micrographic specimen was prepared, then this dispersion was returned to the refrigerator to continue swelling, to be used for Sample 4.54:0.

Sample 4.53:0

A number of the following samples used the procedure described for this sample as a starting point. 0.55 gm of bovine hide collagen, Wiley-milled to a 60 mesh particle size, was blended in 200 ml of 0.05 M acetic acid for 60 minutes. The blending was performed in an ice-jacketed Eberbach blender, on a two-speed Waring power unit set to high, but with the line voltage reduced to 50% of 120 volts. Following the blending,
the dispersion was placed in a tightly closed glass jar and allowed to swell for 72 hours in a refrigerator (−8°C). At this point, transmission electron micrographic specimens were made from many dispersions, and most were saved to provide the dispersions required for the other 4.53:X samples.

**Samples 4.53:1 and 4.53:1'**

These samples were made from one of the dispersions used to make Sample 4.53:0. The procedure was to take 0.55 gm of 60 mesh Wiley-milled bovine hide collagen particles and blend (50% of 120 V) for 60 minutes. After 73 hours of swelling, the dispersion was filtered for 17 hours, then allowed to air-dry for several days. The dried membrane was Wiley-milled to a 60 mesh particle size. The particles were dispersed in physiological saline at a concentration of 100 mg/10 ml for use in Runs 2, 3 and 10 of Series B. After an additional 284 days (41 weeks) of exposure to the physiological saline, the sample was relabelled 4.53:1' and used in Runs 1B, 1R, 2B, 2R, 3B, 3R, 4B and 4R of Series D. This sample had small dense particles as described in Sample 3.20:1, except that these were less white.

**Samples 4.53:2 and 4.53:2'**

The major portion of the dispersion used to make Sample 5.53:3 was used in the preparation of these samples. The preparation for these samples was identical to that used in Sample 4.53:1 up to the filtering step. Following the filtration
the membrane was placed in a pH 3.0, 0.125% glutaraldehyde crosslinking bath for 9 1/2 hours, then removed and allowed to air dry for several days. The glutaraldehyde bath was formed by putting 2.5 ml of 15% glutaraldehyde in 500 ml of 0.05 M acetic acid. The membrane was Wiley-milled to a 60 mesh particle size and subsequently dispersed in physiological saline at a concentration of 100 mg/ml. This sample contained very small dense particles which were orange in color. The particles were more dense than those in Samples 3.10:1 and 4.53:1, as the particles took up only about 1/14 of the volume when settled. The orange color seemed to intensify with the age of the sample. This sample was used for Runs 4 and 11 of Series B. This sample was left in contact with the physiological saline for 283 days (41 weeks) to simulate long-term exposure of the material to physiological conditions. The sample was then relabelled 4.53:3' and used in Runs 5B, 5R, 6B, 6R, 7B, 7R, 8b, and 8R of Series C. The next day, Runs 7B and 7R of Series D were performed with this sample, and 8 days later this sample was used for Runs 1B, 1R, 2B, and 2R of Series E.

Sample 4.53:3

0.55 gm of 60 mesh Wiley-milled bovine hide particles were blended (50% of 120 V) for 60 minutes in 200 ml of 0.05 M AcOH. The dispersion was allowed to swell under refrigerated conditions for 71 1/2 hours, when 5 ml of this dispersion was placed in a 3 liter dialysis bath. The remainder of this dis-
persion was used to prepare Sample 4.53:2. Samples 4.53:3 and 4.53:8 were dialyzed in the same bath for the same length of time. 5 ml of each dispersion were placed in dialysis bags, which had previously been boiled in sodium carbonate and rinsed with dilute acetic acid and deionized water. The filled bags were placed in physiological saline (0.15 M NaCl), adjusted to pH 7.5 by adding dilute sodium hydroxide. The dialysis was carried out for a period of 44 hours. During the dialysis, the pH of the bath was readjusted to 7.5 every few hours. During the last 12 hours, the pH changed from 7.5 to 7.0. Therefore, a pH of 7.0 was assumed to be the value of the samples. The samples were removed from the dialysis bath and used in Run 10 of Series A, approximately 5 hours later. This sample was stringy and was inserted as a lump, rather than pipetted, but the lump dispersed rapidly when it contacted the platelet rich plasma used in the platelet aggregation experiments.

The transmission electron micrographic specimens were prepared from a second dispersion prepared in a similar manner to the first. A 1/4% bovine hide collagen dispersion, prepared as outlined in Sample 4.53:0, was swollen for 79 hour (3 days). Ten ml of this dispersion were dialyzed, at room temperature, against 4 liters of pH 7 distilled water. The pH was adjusted twice per day over the 5-day dialysis, and the dialysys bath was changed after the second day. The final pH reading was 6.8. At the end of this dialysis, transmission electron micrographic specimens were prepared.

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Sample 4.53:4

Ten ml of a 1/4% bovine hide collagen dispersion, swollen for 78 hours, prepared as described in Sample 4.53:0, was dialyzed to pH 3.0. The dialysis bath was prepared from 4 liters of 0.05 M acetic acid, the same dispersing medium as was used to make the dispersion. The bath was changed after two days and the dialysis was continued for 5 days. At the end of this time, transmission electron micrographic specimens were prepared.

Samples 4.53:5 and 4.53:5'

0.55 gm of 60-mesh Wiley-milled bovine hide collagen were dispersed in 200 ml of 0.05 M acetic acid by blending at 50% of 120 volts for 65 minutes. 74 hours later, 0.66 gm of chondroitin-6-sulfate, dissolved in 40 ml of 0.05 M AcOH, were added, while blending (50% of 120V) over a period of 3 minutes. The blending was continued for a total of 8 minutes. Ten ml of this dispersion were refrigerated until used in Run 12 of Series A, approximately 49 hours after the addition of the chondroitin-6-sulfate. The remainder of this 12% (w/w) chondroitin-6-sulfate 1/4% (w/v) collagen dispersion was used for the preparation of Sample 4.53:6. The same preparation procedure outlined above was used to make a new sample which was used in Runs 2B and 2R of Series J. This sample looked very much like Sample 4.53:0, that is, a white homogeneous thick fluid.
Samples 4.53:6 and 4.53:6'

The bulk of the dispersion used to form Sample 4.53:5 was used to make this sample. 0.55 gm of 60 mesh bovine hide collagen particles were blended (50% of 120 V) for 65 minutes in 200 ml of 0.05 M acetic acid. The dispersion was placed in a sealed jar and refrigerated, allowing the collagen to swell for 73 hours. At this time 0.066 gm of chondroitin-6-sulfate, dissolved in 40 ml of acetic acid was added dropwise to the dispersion over 3 minutes while blended at 50% of 120 volts. After 5 minutes of additional blending and a wait of 27 minutes, this 12% (w/w) chondroitin-6-sulfate dispersion was filtered for 1 1/2 hours in a Buechner funnel using an aspirator for suction. The membrane was allowed to air dry for approximately 43 hours, then it was Wiley-milled to a 60 mesh particle size and dispersed in physiological saline at a concentration of 100 mg/10 ml. This sample was used for Runs 3, 5, 6 and 16 of series A, and Run 5, 6, 7, 8 and 9 of Series B. The relabelled sample, 4.53:6', was used for Runs 6B and 6R of Series D, 293 days (42 weeks) after its preparation.

Sample 4.53:6 was very similar in appearance to Samples 3.10:1 and 4:53:1. The particles were very dense, white and granular, again, taking up only about 1/8 of the volume when settled.

Samples 4.53:7 and 4.53:7'

The bulk of the dispersion used to form Sample 4.53:8 was used to make this sample. The quantities and times for
each step in the preparation of this sample are identical to those given for Sample 4.53:5 up to the filtering step. After 1 1/2 hours of filtering, the membrane was air dried for 2 1/2 hours. This membrane was placed in a pH 3.0, 0.125% glutaraldehyde crosslinking bath identical to that described in Sample 4.53:2. After 24 hours of crosslinking, the membrane was rinsed with deionized water and allowed to dry for approximately 16 hours. The membrane was then Wiley-milled to a 60 mesh particle size and dispersed (by hand shaking) in physiological saline at a concentration of 100 mg/10 ml.

This sample was used for Run 7 of Series A and Run 12 of Series B. Runs 4B and 4R of Series D retested this sample 293 days (42 weeks) after its preparation, at which time it was relabelled as 4.53:6'. The appearance of this sample was similar to Sample 4.53:2. Its particles were dense, taking up only ≈1/14 of the total volume when settled, but were less orange than those in Sample 4.53:2.

Sample 4.53:8

0.55 gm of 60 mesh Wiley-milled bovine hide collagen were blended (50% of 120 V) for 65 minutes in 200 ml of 0.05 M AcOH. A refrigerated swelling time of 72 hours was permitted. Afterwards, 12% (w/w) chondroitin-6-sulfate (0.066 gm chondroitin-6-sulfate in 40 ml 0.05 M AcOH) was added during 3 minutes of blending (50% of 120V) followed by an additional 5 minutes of blending. Five ml of this dispersion were dialyzed according to
the schedule described under Sample 4.53:3. The remaining portion of the dispersion was used in the formation of Sample 4.53:6. The dialyzed product, Sample 4.53:8, was used for Run 11 of Series A. This sample was very similar, in appearance, to Sample 4.53:3, as it was stringy and had to be inserted in the platelet rich plasma as a lump, rather than pipetted. The lump broke up into many smaller pieces upon contacting the platelet rich plasma.

Sample 4.53:10

In this sample glutaraldehyde was added to the collagen while it was in the dispersed state. The standard dispersion preparation outlined in Sample 4.53:0 was followed to make two 1/4% (w/v) collagen dispersions in acetic acid. After the 1 hour blending, the dispersions were swollen for 78 hours in the refrigerator. At this point 7 ml of 25% glutaraldehyde was added to the 350 ml of dispersion, making the final concentration equal to 0.5% v/v of glutaraldehyde. The remaining 50 ml of the dispersions were stored in the refrigerator for use in samples 4.53:8 and 4.53:9. The glutaraldehyde was added while the combined dispersions were blended for 1 hour in a Hamilton Beach overhead blender set to low, with the line voltage reduced to 60% of 120 volts. The transmission electron micrographic specimens were made from this dispersion 24 hours after the addition of the glutaraldehyde.
Sample 4.53:11

Half of the dispersion made for sample 4.53:10 was used to make both this sample and Sample 4.53:12. After adding the glutaraldehyde, the dispersion was filtered on Schleicher and Schuell filter paper in a Buechner funnel to form a membrane. The membrane was allowed to air dry before half of it was Wiley-milled to 60 mesh sized particles and dispersed in physiological saline. The remaining half was used to make Sample 4.53:12. This sample was similar in appearance to other samples made from membranes, such as Sample 4.53:2. Shortly after dispersion in the physiological saline, this sample was used in Runs 7B and 7R of Series J.

Sample 4.53:12

The half of the membrane remaining from Sample 4.53:11 was crosslinked for 24 hours in a 5% v/v glutaraldehyde pH 3.0 bath at room temperature. The crosslinking bath was composed of 800 ml of 0.05 M acetic acid plus 200 ml of 25% glutaraldehyde. The membrane was then thoroughly washed with several rinses of deionized water to remove the residual glutaraldehyde. The membrane was then air-dried, Wiley-milled to a 60 mesh particle size and dispersed in physiological saline so that it could be used in Runs 5B and 5R of Series J.

Sample 4.53:13

Ten ml of the dispersion made for Sample 4.53:10 were used for this sample. This 1/4% collagen-acetic acid dis-
persion with glutaraldehyde was dialyzed to pH 7 against a 4 liter bath. The dialysis bath was formed from 4 liters of distilled water and the pH was adjusted to pH 7.0 by adding dilute sodium hydroxide. The dialysis bath was change after 2 days, but the dialysis procedure was continued for 5 days, adjusting the pH as often as necessary. The final pH of the bath was 6.8, which was stable over at least the last 12 hours. After removal from the dialysis bath this sample was used to make transmission electron micrographic specimens. Three days later, this specimen was used in Run 9 of Series I.

Sample 4.53:14

Ten ml of the dispersion made for Sample 4.53:10 were also used for this sample. This sample, however, was dialyzed against 4 liters of 0.05 M acetic acid. The dialysis was carried out at room temperature as in Sample 4.53:13. The bath was changed after 2 days, and the dialysis continued for 5 days in total. After completion of the dialysis, transmission electron micrographic specimens were made. Three days later, this sample was used in Run 6 of Series I.

Sample 4.53:15

The other half of the 1/4% collagen-acetic acid-glutaraldehyde dispersion made for Sample 4.53:10 was used in Samples 4.53:15, 4.53:16, 4.53:17, 4.53:18 and 4.53.19. The next step after the addition of the glutaraldehyde, was to add
chondroitin-6-sulfate. Ten ml of 0.05 M acetic acid were used to dissolve 0.0578 gm of chondroitin-6-sulfate. This solution was added, dropwise, to 175 ml of the glutaraldehyde-treated dispersion, 1 1/2 hours after the glutaraldehyde was added. The dispersion was blended in a Hamilton Beach overhead blender set to low, with the line voltage reduced to 60% of 120 volts for 5 minutes while the chondroitin-6-sulfate was added. Transmission electron micrographic specimens were prepared from this dispersion before it was relegated to the following samples.

Sample 4.53:16

The glutaraldehyde-treated collagen-chondroitin-6-sulfate dispersion made for Sample 4.53:15 was shared between this sample and Sample 4.53:17. Shortly after the addition of the chondroitin-6-sulfate, the dispersion was filtered in a Buechner funnel. This filtering step was completed in only 20 minutes, and then air dried. Half of this membrane was reserved for Sample 4.53:17. The remaining half was Wiley-milled to a 60 mesh particle size and dispersed in physiological saline to form Sample 4.53:16. This sample was used for Runs 8B and 8R of Series J.

Sample 4.53:17

The remaining half of the air-dried membrane of Sample 4.53:16 was crosslinked for 24 hours in a 5% glutaraldehyde bath at room temperature. The crosslinking bath was made
from 800 ml of 0.05 M acetic acid plus 200 ml of 25% glutaraldehyde. The crosslinked membrane was thoroughly rinsed with deionized water and allowed to air dry. The membrane was then ground to 60 mesh-sized particles in the Wiley mill and dispersed in physiological saline. This sample was used for Runs 6B and 6R of Series J.

**Sample 4.53:18**

Ten ml of the dispersion made for Sample 4.53:15 were used for this sample. The procedure was to dialyze this sample against 4 liters of distilled water at room temperature with the pH adjusted to 7.0 by dilute sodium hydroxide. The bath was changed after 2 days and continued for 5 days before this sample was used to make transmission electron micrographic specimens. Three days later this sample was used in Run 8 of Series I.

**Sample 4.53:19**

This sample was made from 10 ml of Sample 4.53:15. The 1/4% collagen-glutaraldehyde treated dispersion with 12% chondroitin-6-sulfate was dialyzed to pH 3. The dialysis medium was 4 liters of 0.05 M acetic acid, the same solvent as the initial dispersing medium. The dialysis was performed at room temperature for 5 days, with a complete change of the dialysis bath on the second day. Upon completion of the dialysis, electron micrographic specimens were made.
The remainder was stored in the refrigerator for 3 days until it was used for Run 7 of Series I.

Sample 4.54:0

The 1/4% bovine hide collagen dispersion used to make Sample 4.52:0 was also used for this sample. At the end of 5 days of swelling, after 1 hour of blending, this dispersion was used to make transmission electron micrographic specimens. A dispersion with the identical history was also used in Run 4 of Series A. The dispersion used for these platelet aggregation tests was saved to become Sample 4.57:0. A new 5 day swollen dispersion was also prepared for testing in Runs 1B, 1R and 9 of Series J.

Sample 4.55:1

This sample was created by blending 1.10 gm of bovine hide collagen in 400 ml of 0.05 M acetic acid for 1 hour (50% of 12 V, high) in two blenders. The dispersion was refrigerated for 14 days, when 0.132 gm of chondroitin-6-sulfate, dissolved in 20 ml of 0.05 M acetic acid was added. The chondroitin-6-sulfate was added, dropwise, while blending, over 2 3/4 minutes. The ice-jacketed blending was continued for a total of 8 1/2 minutes, with the blender set to high, and the line voltage reduced to 50% of 120 volts. The next day, this dispersion was put into the cross-flow filtration apparatus and a tube (tube #8) was made. This tube was crosslinked for 25 1/4 hours in 0.5% (v/v) glutarald-
ethylene in distilled water. After thorough washing in deionized water, the tube was placed in a solution of 70% ethanol plus 30% water. The sample was refrigerated for 8 weeks, then air-dried and Wiley-milled to a 60 mesh particle size and dispersed in physiological saline. This sample was then used for Runs 1B, 1R, 2B and 2R of Series F.

**Sample 4.56:0**

This sample was prepared by blending (50% of 120 V, high) 0.55 gm of 60 mesh bovine hide collagen in 200 ml of acetic acid for 1 hour. This ice-jacketed blending was followed by refrigerated swelling for 65 days (9 weeks). Transmission electron micrographic specimens were then made and the remainder of the dispersion was used for some of the following samples.

**Samples 4.56:1**

Chondroitin-6-sulfate in acetic acid (0.0132 gm/1 ml) was added to 36 1/2 ml of the dispersion of Sample 4.56:0 to make a chondroitin-6-sulfate to collagen ratio of 12% (w/w). The sample was blended for 5 minutes with the Greiner overhead blender. Transmission electron micrographic specimens were prepared 5 days after the addition of the chondroitin-6-sulfate.

**Samples 4.56:2**

After swelling the 1/4% collagen-acetic acid dispersion of Sample 4.56:0 for 70 days, 25% (w/w) chondroitin-6-sulfate
was added. 36 1/2 ml of the dispersion plus 0.0264 gm of chondroitin-6-sulfate dissolved in 1 ml of 0.05 M acetic acid were blended for 5 minutes with the Greiner overhead blender at full speed, approximately 5000 rpm. Shortly after the addition of the chondroitin-6-sulfate, transmission electron micrographic specimens of this sample were prepared. Another set of specimens were prepared 36 days later.

Sample 4.56:3

The dispersion of Sample 4.56:0 was swollen for 77 days after the 1/4% collagen-acetic acid, 1 hour-blended (50% of 120 V, high) dispersion was initially made. After the swelling, 0.73 ml of 25% (v/v) glutaraldehyde was added to the 36 1/2 ml of the dispersion to bring the concentration of glutaraldehyde in the dispersion to 0.5%. Transmission electron micrographic specimens were made from this sample 17 and 36 days after the addition of the glutaraldehyde.

Sample 4.56:4

A dispersion was made by blending (50% of 120 V, high) 1.10 gm of 60 mesh bovine hide collagen in 400 ml of 0.05 M acetic acid in two blenders for 1 hour. Twelve refrigerated weeks of swelling were permitted after the ice-jacketed blending. Then, 7.3 ml of 25% glutaraldehyde were added to 365 ml of the dispersion. The dispersion was blended for 10 minutes while the glutaraldehyde was added.

This dispersion provided the material for most of the samples remaining in this chapter. To form Sample 4.56:4,
one-half of this dispersion was filtered on a Buechner funnel, 11 hours later. Half of this membrane was air-dried, then Wiley-milled to a 60 mesh particle size and dispersed in physiological saline. This sample was then used in Runs 4B and 4R of Series H.

Sample 4.56:5

The other half of the membrane prepared for Sample 4.56:4 was crosslinked in a 5% glutaraldehyde bath after air-drying. This bath was made from 200 ml of 25% glutaraldehyde plus 800 ml of acetic acid. The crosslinking was performed at room temperature for 12 hours. Due to the high concentration of the glutaraldehyde bath, it was important to wash the membrane many times with deionized water before air-drying. The air-dried membrane was Wiley-milled to 60 mesh particle size, and was then dispersed in physiological saline. This sample was used for Runs 3B and 3R of Series J.

Sample 4.56:6

A 10 ml portion of the dispersion of Sample 4.56:3 was dialyzed to pH 7.0. The dialysis bath was made by adding dilute sodium hydroxide to 4 liters of distilled water and changed once during the 2-day dialysis procedure. Following the room temperature dialysis of the glutaraldehyde-treated dispersion, the sample was stored in the refrigerator for several hours before use in Runs 1B and 1R of Series H.
Sample 4.56:7

Two dispersions with similar histories comprise this sample. The first dispersion was made from 25 ml of the glutaraldehyde-treated dispersion of Sample 4.56:3. Thirty-six days after the addition of the glutaraldehyde, 0.0084 gm of chondroitin-6-sulfate in 1 ml of 0.05 M acetic acid was added. The dispersion was blended for 5 minutes during the addition of the dissolve chondroitin-t-sulfate. Transmission electron micrographic specimens were prepared several hours later. The second dispersion was made from half of the dispersion described in Sample 4.56:4. A thin 1/4% collagen dispersion was treated with glutaraldehyde after 12 weeks of swelling. Twelve hours later, 0.066 gm of chondroitin-6-sulfate were added to 200 ml of the dispersion. Transmission electron micrographic specimens were formed from a few drops of this dispersion, while the remainder was used to make samples 4.56:8, 4.56:9 and 4.56:10.

Sample 4.56:8

The second dispersion of the previous sample was filtered in a Buechner funnel. Half of this membrane was used for the next sample, while half was air-dried, Wiley-milled to 60 mesh sized particles and dispersed in physiological saline. This sample was used for Runs 5B and 5R of Series H.
Sample 4.56:9

The remaining half of the membrane for the previous sample was crosslinked in a 5% glutaraldehyde bath. The bath was formed largely of 0.05 M acetic acid (800 ml) plus 200 ml of 25% glutaraldehyde. The crosslinking was performed at room temperature for 12 hours. At the end of the crosslinking, the membrane was thoroughly washed with deionized water and ground up in the Wiley mill. The 6-mesh sized particles of the membrane were then dispersed in physiological saline and used in Runs 3B and 3R of Series H, and Runs 4B and 4R of Series J.

Sample 4.56:10

A small portion of the second dispersion of Sample 4.56:7 was used to make this sample. Ten ml of the dispersion were dialyzed against 4 liters of distilled water, which was adjusted to pH 7.0 by the addition of dilute sodium hydroxide. This bath was changed once during the 2 days of dialysis which were performed at room temperature. The sample was removed from the dialysis bath and used in Runs 2B and 2R of Series H, several hours later.

Sample 4.57:0

This sample was prepared by blending 0.55 gm of 60-mesh sized particles of bovine hide collagen in 0.05 gm M acetic acid for 1 hour, and allowing it to swell for 308 days (44 weeks). At the end of this time it was used in Runs 7B and 7R of
Series E. It should be noted that this is the same sample which was used for the platelet aggregation tests of Sample 4.54:0. Two days later, this sample was used to prepare transmission electron micrographic specimens.
CHAPTER VI

The Transmission Electron Micrographic Appearance of Collagen Composite Materials

Introduction

Collagen has long been known to be a potent platelet aggregating agent. Shortly after the advent of the transmission electron microscope, collagen was found to display a banded structure with a 640 Å periodicity which relates to the quaternary structure of the fibrils (Schmitt & Gross 1948). The effect which chondroitin-6-sulfate has upon the ultrastructure of collagen was described in a series of papers by Wood (1960). Pease and Bouteille (1969 & 1971) investigated the fine structure of collagen in more detail. The quaternary structure was also analyzed by X-ray diffraction techniques (Ellis 1970). Bruns and Gross (1974) developed a very complete theory concerning the reasons for the banded structure and its correspondence to the actual amino acid sequences. In the same year, Jaffe and Deykin (1974) and Brass and Bensusan (1974) offered evidence that it was this quaternary structure which was responsible for causing platelets to aggregate in the presence of collagen. Zucker and Mason (1976) studied the ultrastructural aspects of the platelet-collagen interactions in more detail. Muggli (1978) continued this work and claimed that the quaternary structure was not necessary in order for
collagen to cause platelet aggregation. The controversy surrounding this relationship of the quarternary structure to platelet aggregation is not yet resolved.

The transmission electron microscope is viewed as a tool to help analyze the characteristics of non-thrombogenic collagen materials. The development of the collagen materials described in Figure 5.1 was guided by an effort to eliminate the quaternary structure of collagen. In this way it was hoped to produce a non-thrombogenic material based on the claims of Jaffé and Deykin (1974) and Brass and Bensusan (1974). The analysis of the lack of banded structure in potential collagen-based vascular prostheses would therefore be a useful check on the quality control of the non-thrombogenicity of the vascular prostheses.

The methods used to make the transmission electron micrographs of the collagen materials are described in the next sections. These are followed by a series of micrographs of the collagen specimens so that the effect on the ultrastructure by the material processing can be analyzed. The micrographs of each sample follow a description of the microscopic appearance. The fibril widths and banding are quantified by the histograms grouped near the end of the chapter. A table summarizing these histograms is found at the end of the chapter. The ultrastructural appearance can also be related to the platelet response to the material, and is discussed more fully in the final chapter.
Preparation of the Transmission Electron Microscope Grids

Copper grids, 3.05 mm in diameter, were used as the supporting medium for samples which were viewed in the transmission electron microscope. Usually, the grids were coated with a parlodian film followed by a thin carbon coating, before a sample was placed on the grid. The grids are available in sizes from 75 to 400 mesh. Many of the first samples were made on 75 mesh grids, but it was quickly realized that the smaller holes of a 400 mesh grid made for a more stable sample in the electron beam. Also, the parlodian film was less likely to break during the carbon coating procedure (or other handling) if a 400 mesh grid was used. The copper grid supports the sample and conducts the heat assuring the thermal stability of the sample and preventing charging. The carbon coating also helps to conduct the electrons from the sample, and to further stabilize the parlodian film so that it will not deteriorate with time. Since the grids are quite thin and fragile, they are handled with a pair of No. 5 Watchmaker's balance forceps.

The parlodian, a solid, was dissolved in a nonpolar solvent, such as amyl acetate, to form a stock solution from which films were formed. Several hours were required to completely dissolve the 0.075 gms of parlodian in the 24 ml of amyl acetate. This stock solution was stored at room temperature and seemed to keep indefinitely. Parlodian films were formed by allowing the amyl acetate to evaporate from
a thin layer of the stock solution. This was done by mixing approximately 1/4 ml of the stock solution with 300 ml of distilled water in a 500 ml beaker. The parlodian solution would rapidly rise to the surface and form a film. Some experimentation was necessary to obtain a good film. Often, if a film did not form within a few minutes, too much parlodian was present, and part of the beaker's contents was poured into another beaker.

Once a film had been formed, the grids were placed on the film. The grids have two sides. The "shiny" side appears either light or dark in colour depending upon the reflection of the light. The "dull" side always appears light in colour, since the roughness of this surface diffuses the light. Approximately 40 grids were placed on the parlodian film with the dull side down. The rougher surface of the dull side resulted in a better bond between the grid and the parlodian. In order to remove the grids, a piece of cellulose filter paper was placed over the grids and allowed to become wet. When the filter paper was removed, the parlodian film would adhere to the filter paper, sandwiching the grids between the film and filter paper. The filter paper was then allowed to dry for several hours, or overnight, before the carbon coating was performed.

The carbon coating was performed in an evacuated chamber, in which current could be passed through a carbon junction.
The carbon junction was formed by two 1/4 inch diameter pure carbon rods, one of which had been pointed by a special "pencil sharpener" and the other which had been made flat by sanding with emery paper. The grids were placed in the chamber, on the filter paper, after being turned over, to put the parlodian coated side down. The carbon appeared to adhere better to the shiny side, and the parlodian film seemed less likely to break during the procedure. A bolt or some other raised piece was also placed on the filter paper, so that the original colour of the filter paper could be contrasted with the carbon coated areas. A vacuum of at least $2 \times 10^{-5}$ torr was required for the carbon coating procedure. A voltage of approximately 150 to 180 volts, passed through the carbon junction for 10 to 20 seconds, was sufficient to coat the grids with the evaporated carbon. A very bright white light was created by the junction. To prevent eye damage, this light was not looked upon directly. When the current was stopped, the filter paper was a light buff colour, not very different from the original colour of the filter paper. The grids were then removed from the chamber and stored until ready to use. The grids were usually used as soon as possible, but correctly prepared grids could be stored at least a couple of weeks in advance of the specimen preparation.

Hayat (1970a, 1970b, 1970c, 1974 & 1975), Pease (1964) and Meek (1976) were useful references for many of the
microscopic techniques used to prepare and view specimens.

**Preparation of Transmission Electron Micrographic Specimens**

The transmission electron micrographic (TEM) specimens were prepared from samples of small particles of bovine hide collagen dispersed in a solvent. The basic procedure is to place a drop of the collagen dispersion onto a parlodian and carbon coated copper grid, blot off the excess dispersion, stain with phosphotungstic acid and allow to dry.

Often, the concentration of the collagen dispersions to be viewed (usually 0.5% w/v) was so high that simply placing a drop of the dispersion on the grid and allowing it to dry resulted in a grid too densely covered with fibrils to view properly under the transmission electron microscope. A well-prepared grid had no particles visible on the surface and only a few fibrils visible under a light microscope. Adding 10 times the volume of the solvent to the dispersion before dropping the sample on the grid is one method of reducing the density of fibrils on the grid. An easier method, which resulted in good samples, was to place a drop of the concentrated dispersion on the grid, but immediately wash it off of the grid with a stream of distilled water from a wash bottle. This method worked well with collagen dispersions at pH 3. Collagen dispersions at pH 3 precipitated with chondroitin-6-sulfate required less vigorous washing. Dispersions at pH 7 required little or no
Collagen was found to be relatively electronlucent, offering little contrast between the internal structures. The specimens were, therefore, stained with electron dense materials to give more satisfactory transmission electron micrographs. Both of the major types of staining, positive staining and negative staining, were used. Positive staining involves the combining of the stain with the collagen so that the bound stain is not removed by washing with distilled water. The negative staining process allows the solvent of the electron-dense material to evaporate, so that the electron-dense material collects in the depressions and around the edges of the fibrils. Although both methods showed the banding pattern of the collagen fibrils, the positive staining method generally made the finer structures more visible. Figure 6.1 illustrates both staining methods. In both cases phosphotungstic acid was used. The positively stained specimen was stained with 0.5% (w/v) phosphotungstic acid for 60 seconds, then washed with distilled water and allowed to dry. The negatively stained specimen was also stained with 0.5% (w/v) phosphotungstic acid, but it was allowed to dry without washing the stain off. This specimen may be partially positively stained as well.

The procedure for staining is outlined as follows. A stock solution of 0.5% w/v phosphotungstic acid was made by adding 0.15 gms to 30 ml of distilled water, and was
stored at 4°C between uses. The pH of this solution was varied from the pH 2.5 which results from this concentration of phosphotungstic acid by adding HCl or NaOH. It was found that a pH of 1.5 seemed to give the best staining characteristics. A stock solution of 0.002 M uranyl acetate, made by adding 0.025 gm to 30 ml of distilled water, was also stored at 4°C. Within a few minutes after dropping the dispersion on the grids, the grids were each floated on a drop of the stock staining solution on parafilm. Dispersions at pH 3 were generally stained for 30 to 60 seconds with 0.5% w/v phosphotungstic acid, and washed with distilled water. Sometimes this was followed by a counterstain with uranyl acetate for 10 minutes, and a wash with distilled water. Dispersions at pH 7 and dispersions at pH 3 with chondroitin-6-sulfate were stained in a similar manner. Dispersions to which glutaraldehyde had been added, required much longer staining times. Typically, specimens were made with a 5 minute stain with 0.5% w/v phosphotungstic acid, with or without a distilled water wash. These specimens were also prepared with a 10 minute stain of 0.5% w/v phosphotungstic acid, followed by a distilled water wash. A counter-stain of 15 minutes of 0.002 M uranyl acetate and distilled water wash occasionally followed the 10 minute phosphotungstic acid stain. After staining, the grids were blotted dry from the side to remove residual moisture, and allowed to dry. The grids were generally
Figure 6.1: The effect which (a) positive staining and (b) negative staining has upon the appearance of a collagen fibril.
dried at least overnight, otherwise the specimen would deform when placed in the high vacuum of the transmission electron microscope.

**Transmission Electron Microscopic Technique**

All transmission electron microscopy was performed on a Philips EM 300 microscope. The microscope was carefully aligned and corrected for condenser astigmatism before each session. Accelerating voltages of 80 and 100 kV were used, with most micrographs taken at the lower voltage. The 20 μm and 30 μm objective apertures were primarily used, but the 50 μm aperture was used at low magnification (1200x to 1500x onto the plate) to prevent vignetting. Initially, electron micrographs were taken at arbitrary magnification, but later the plate magnifications were standardized at 5 convenient levels to permit easier comparison. The magnification settings chosen were 2, 8, 11, 15 and 21, corresponding to magnifications onto the photographic plate of 1500x, 4770x, 10255x, 26335x and 96053x at an accelerating voltage of 80 kV. Since the magnification of the microscope had recently been calibrated, these calibrated magnifications were used for the micrographs.

The 8.1 cm by 10 cm Dupont Graphic Arts film was exposed at 50 units on the exposure meter using the small screen. The exposure was halved when only very swollen fibrils were present, to decrease the density of the negative and make printing easier. The photographic plates were developed for 5 minutes, washed for 1 1/2 minutes in water and fixed for 5
to 10 minutes. Following thorough drying, the negatives were printed with an Omega 4 x 5 enlarger with a 135 mm lens. The Kodabromide paper was typically exposed for 20 seconds at f/8, developed for 90 seconds in Dektol (1:2 dilution), stopped in an acetic acid stop bath and fixed for 5 to 10 minutes. Three minutes in Kodak Hypoclearing agent preceded a half-hour water wash and drying. All grades of paper were used; grade 1 for high contrast, intensely banded fibrils; and grade 5 for low contrast, swollen fibrils. To improve the quality of reproduction for this thesis, the final prints were rephotographed by a PMT (Photomechanical transfer) process using an 85 lines per inch half tone screen.

Analysis of the Transmission Electron Micrographs

Several pieces of information could be gleaned from the transmission electron micrographs. The first thing that was noted was whether or not the fibrils were arranged in clumps. Generally, materials which had many clumps of fibrils were not suitable for viewing under the transmission electron microscope, since even accelerating voltages of 100 kV could not penetrate clumps of fibers. Often, though, individual fibrils which protruded from the clump could be examined.

The next most apparent observation of fibers is their appearance, whether banded, unbanded, swollen or narrow. The banding period was measured, usually by assuming that all bands were of an equal period, measuring several periods and dividing by the number of periods measured. In order to quantify the
appearance of the fibrils, a scale of banding intensity from 0 to 4 was made. The standard magnifications of the transmission electron micrographs, 1500x, 4770x and 26335x onto the plate assisted in differentiating between the intensities.

An intensity of "0" means that no banding was apparent in the transmission electron micrographs. This could apply to either fibrils which were very swollen or which were quite narrow, but in either case, neither type of fibril exhibited banding perpendicular to its longitudinal axis.

An intensity of "1" is used to indicate fibrils which had any hint of banding at all. Generally, these fibrils were felt to exhibit banding only upon a very close examination. In wide, swollen fibers, the bands were found only in the very central region. In narrow fibrils the banding was simply very weak, or, in other words, there was limited contrast between adjacent light and dark bands. The fibrils which were rated at this intensity usually only exhibited a banded structure upon examination of plates made at a magnification setting of 15.

An intensity of "2" was used to denote fibers which had banding which was visible, but not overwhelming. Wide, swollen fibrils generally had banding which extended over approximately one-third of the width, and was more distinct than the banding of intensity of "1". Narrower fibrils also showed a banding pattern which was more visible even at a lower magnification. Fibrils with this intensity could
be seen at a magnification setting of 8 and were quite visible at a setting of 15. An intensity of "3" was used to denote fibrils which had clearly visible banding. The banding pattern consisted only of light and dark bands, but had a great deal of contrast between the adjacent light and dark bands. These fibrils were quite visible at a magnification setting of 8 and often readily visible at a magnification setting of 2. Wide, swollen fibers seldom exhibited banding clearly defined enough to enter this intensity category. In swollen fibrils, the banding would have to be nearly all of the way across the fibril and have a great deal of contrast between adjacent light and dark bands.

An intensity of "4" was reserved for fibrils which not only displayed a strong major periodicity but also many of the intraperiod bands. These fibrils were invariably quite narrow. The intraperiod bands were occasionally visible at a magnification setting of 8, but generally an intensity of "4" could only be given to fibrils which were examined at a magnification setting of 15 or greater. The major periodicity could easily be seen at a magnification of 8 and usually at a magnification of 2. Due to the fact that occasionally a higher magnification plate of a fiber was not available, a few fibrils which are labelled intensity "3" may actually be intensity "4".

The previous intensity scale did not take into account the fibril widths. The fibril widths were measured on the
transmission electron micrographs either with Mitutoyo model #505-637 dial guage calipers on the photographic prints, or with an SKS 7X optical comparator on the negatives, to accuracies of ±0.0025 inches or ±0.00125 inches.

A true histogram, which is simply the measurement versus the frequency of each measurement, is only valid for multiple measurements of the same object. Since, in this case, many measurements of different widths must be made, a histogram is unlikely to give the true relative percentages of each width. Therefore, a different measurement system was devised. The lengths of all fibrils of a given width on a transmission electron micrograph were measured with a Tacro inchmeter (a device which uses a wheel to measure linear distances along a curved line). The ratio of the length of fibrils with this width divided by the total length of all fibrils measured, then gives an accurate percentage of the fibrils with this width. If the width of the fibril changed along its length, then the fibril was divided into sections of equal widths and the length of each section was individually measured. The data which resulted from these measurements was plotted as a percentage of length versus the width in nanometers. The resulting graphs, which show the widths in blocks of 50 nm, have been grouped at the end of this chapter to permit easy comparison between samples. A summary chart of these graphs is located at the end of the discussion of this chapter.
Additionally, weighted means and standard deviations were calculated for some samples with single peaks in the fibril-width distribution graphs. These were calculated from each specific fibril width, using the percentage of the total length of measured fibrils as a weighting factor (Bevington 1969).

Representative transmission electron micrographs of each sample follow a description of the microscopic appearance of each sample. The prints have been made at 4000x, 15000x and 75000x to show the overall appearance of the sample, as well as the detailed appearance of individual fibrils. The sample descriptions which follow have been grouped to examine the effect of swelling collagen in acetic acid, the effect of chondroitin-6-sulfate on swollen collagen, the effect of glutaraldehyde on collagen after a short period of swelling and the effect of glutaraldehyde after a long period of swelling.

The Transmission Electron Micrographs

Samples 2.20:0 and 2.60:0

The natively banded collagen of these samples were prepared by dispersing Wiley-milled bovine hide collagen in water at pH 7. Dispersions made in this way are quite fibrous, since the clumps of collagen fibrils do not easily break apart simply under the action of blending. If allowed to stand, the clumps in the dispersion would rapidly settle out. No statistical measurement was made of clump sizes,
but the clump in Figure 6.2 is approximately 32 µm by 6 µm, probably a little on the small side. Nearly all of the fibrils in this sample were arranged in electron-dense clumps with few fibrils emanating from these clumps, making the electrom microscopy difficult. A smaller clump, exhibited in Figure 6.3, was found in the sample which was blended for 2 hours. The fibrils separated from this clump are shown at a higher magnification in Figure 6.4. The most distinctive feature of these fibrils is the regular banding pattern which contains many intraperiod bands within each main period. A very high magnification view of these fibrils is presented in Figure 6.5, which also shows the banding notation originally suggested by Schmidt and Gross (1948), and the new designation suggested in 1974 by Bruns and Gross. The true period of this banding pattern is generally accepted to be 66.8 nm, based on an amino acid analysis. The measured banding periods, however, were 60 to 62 nm, indicating a slight error in the microscope's calibration. The calibrated magnifications of the microscope were used, though, so that the relative differences between the swollen and narrow fibrils of the following samples could still be detected.

The blending time at pH 7 does not appreciably affect the fibril widths, therefore, the data for the 15 minute blended sample, 2.20:0, and the 2 hour blended sample, 2.60:0, are combined in Figure 6.76. The weighted average fibril width
in this sample is 70.7 nm with a standard deviation of 23 nm. The widths of the bovine hide collagen fibrils at pH 7 (unlike rat-tail tendon fibrils, for example), have a fairly narrow distribution and are the smallest widths encountered in all of the samples.
Figure 6.2: Transmission electron micrograph (Plate #17232, Sample 2.20:0) of bovine hide collagen blended for 15 minutes in pH 7 distilled water, 4000 X. Stain: 0.5% PTA (phosphotungstic acid) (pH 1.5) for 1 minute, then rinsed with distilled water.
Figure 6.3: Transmission electron micrograph (Plate #17012, Sample 2.60:0) of bovine hide collagen blended for 2 hours in pH 7 distilled water, 15,000 x. Stain: 0.5% PTA (pH 1.5) for 1 minute, then rinsed with distilled water.
Figure 6.4: Transmission electron micrographs (Plates #17014, 17015 and 17013, Sample 2.60:0) of bovine hide collagen blended for 2 hours in pH 7 distilled water, 75,000 X. Stain: 0.5% PTA (pH 1.5) for 1 minute, then rinsed with distilled water.
Figure 6.5: Transmission electron micrograph (Plate #17013, Sample 2.60:0) of bovine hide collagen in pH 7 distilled water 450,000 X. The photograph on the left, an optically averaged image of the fibril on the right, emphasizes the banding pattern. Stain: 0.5% PTA (pH 1.5) for 1 minute, then rinsed with distilled water.
Sample 3.20:1

This sample is bovine hide collagen which has soaked in physiological saline for 9\(\frac{1}{2}\) months. Figure 6.6 shows that this sample is very strongly banded and that the fibrils are quite uniform in their widths. The weighted mean fibril width is 71.8 nm with a weighted standard deviation of 7.3 nm. This is not significantly different from the fibril widths found in the previous sample. Figure 6.77 shows that all fibrils maintained the native banding, with an average period of 60.3 nm, and usually displayed the intraperiod bands as well. This indicates that the presence of salt at pH 7 does not seem to greatly affect the microscopic appearance of bovine hide collagen.
Figure 6.6: Transmission electron micrograph (Plate #16843, Sample 3.20:1) of bovine hide collagen dispersed in physiological saline for 10 months, 75,000 ×. Stain: 0.5% PTA, no rinse.
Sample 4.10:0

The most outstanding features of a low power view of a bovine hide collagen dispersion blended for 2 minutes in acetic acid, are the clumps of fibrils. Figure 6.7 shows one of these clumps, this particular clump was more than 180 μm long and 2.6 to 9.7 μm wide. It differs from the clumps seen in dispersions made at pH 7, in that many fibrils emanate from the main part of the clump. After only two minutes in acid, many of these fibrils are swollen but banded, as shown in Figure 6.8. Many narrow fibrils are also present, but even in these, the intensity of the banding has decreased. Figure 6.9 shows that the intraperiod bands have become much less distinct than those seen in the fibrils at pH 7. The histogram of Figure 6.78 shows that a large percentage of fibrils can now be found outside of the 50 to 100 nm width range. The fibrils which have widths in the 50 to 150 nm range are still intensely banded. Some strongly banded fibrils also have widths of 200 to 350 nm. However, an entirely new population of swollen fibrils, usually with weak or no banding, comprises almost half of the fibrils in this sample. The widths of these fibrils are in the 500 to 750 nm range. Although there is some variability in the actual numbers, the banding periods of these swollen fibrils are consistently 5 to 10 nm less than the banding periods of adjacent narrow fibrils. The average period for narrow fibrils is 65 nm while it is only 57 nm for swollen fibrils.

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Figure 6.7: Transmission electron micrograph (Plate #17021, Sample 4.10:0) of bovine hide collagen blended for 2 minutes in 0.05 M acetic acid, 4000 X. Stain: 0.5% PTA (pH 1.5) for 1 minute, then rinsed with distilled water.
Figure 6.8: Transmission electron micrograph (Plate #17024, Sample 4.10:0) of bovine hide collagen blended for 2 minutes in 0.05 M acetic acid, 15,000 X. Stain: 0.5% PTA (pH 1.5) for 1 minute, then rinsed with distilled water.
Figure 6.9: Transmission electron micrograph (Plate #17025, Sample 4.10:0) of bovine hide collagen blended for 2 minutes in 0.05 M acetic acid, 75,000 X. Stain: 0.5% PTA (pH 1.5) for 1 minute, then rinsed with distilled water.
Sample 4.20:0

After bovine collagen has been blended for 15 minutes in acetic acid, no large clumps of fibers are seen. A comparison of Figure 6.10 with Figure 6.7 reveals that the average fibril width has increased. Figure 6.79 shows that although there is a peak in the fibril widths at 200 nm, there is a much broader distribution of widths. At pH 7 most fibril widths were clustered around 70 nm. Now it appears that even the narrowest fibrils have swollen to three times their original widths to approximately 200 nm. Fibril widths are common up to 1100 nm, and present at up to 1600 nm. Overall, the intensity of banding has decreased (Figure 6.11), some fibrils still display the intra-period bands (Figure 6.13), but many display no banding at all. Figure 6.12 shows four types of fibrils commonly seen in the collagen samples. Narrow fibrils usually display the strongest banding. Highly swollen fibrils may or may not display banding. When the highly swollen fibrils do have a banding pattern it is usually concentrated in the central portion of the fibrils' width. Swollen fibrils without banding usually display a darker central region; and in some fibrils, the central region shows longitudinal striations. As found in the previous sample, the banding period is smaller for more swollen fibrils and is illustrated quite clearly in Figure 6.12. Narrow fibrils have an average period of 63 nm; while swollen fibrils have an average period of only 59 nm. The large swollen fibril in the center of Figure 6.12 shows striations at approximately 45° to the long
axis. These striations are due to the orientation of the microfibrils and illustrate the helical manner in which the microfibrils compose the fibrils (Petkov 1978).
Figure 6.10: Transmission electron micrograph (Plate #17226, Sample 4.20:0) of bovine hide collagen blended for 15 minutes in 0.05 M acetic acid, 4000 X. Stain: 0.5% PTA (pH 1.5) for 1 minute, then rinsed with distilled water.
Figure 6.11: Transmission electron micrograph (Plate #17227, Sample 4.20:0) of bovine hide collagen blended for 15 minutes in 0.05 M acetic acid, 15,000 X. Stain: 0.5% PTA (pH 1.5) for 1 minute, then rinsed with distilled water.
Figure 6.12: Transmission electron micrograph (Plate #17221, Sample 4.20:0) of bovine hide collagen blended for 15 minutes in 0.05 M acetic acid, 15,000 X. Stain: 0.5% PTA (pH 1.5) for 1 minute, then rinsed with distilled water.
Figure 6.13: Transmission electron micrograph (Plate #17229, Sample 4.20:0) of bovine hide collagen blended for 15 minutes in 0.05 M acetic acid, 75,000 x. Stain: 0.5% PTA (pH 1.5) for 1 minute, then rinsed with distilled water.
Sample 4.40:0

Figure 6.14 shows a transmission electron micrograph taken of bovine hide collagen which has been blended for 30 minutes in acetic acid. Figures 6.15 and 6.80 show that this sample has fibrils with somewhat wider widths than the previous sample. Figure 6.16 shows that the banding is less intense than in Sample 4.20:0. The banding period of the narrow fibrils is 62 nm and of the swollen fibrils is 53 nm.
Figure 6.14: Transmission electron micrograph (Plate #17071, Sample 4.40.0) of bovine hide collagen blended for 30 minutes in 0.05 M acetic acid, 4000 X. Stain: 0.5% PTA (pH 1.5) for 1 minute, then rinsed with distilled water.
Figure 6.15: Transmission electron micrograph (Plate # 17072, Sample 4.40:0) of bovine hide collagen blended for 15 minutes in 0.05 M acetic acid, 15,000 X. Stain: 0.5% PTA (pH 1.5) for 1 minute, then rinsed with distilled water.
Figure 6.16: Transmission electron micrograph (Plate #17073, Sample 4.40:0) of bovine hide collagen blended for 30 minutes in 0.05 M acetic acid, 75,000 X. Stain: 0.5\% PTA (pH 1.5) for 1 minute, then rinsed with distilled water.
Sample 4.50:0

Figure 6.17 presents a view of the effect of blending bovine hide collagen in acetic acid for one hour. Contrasting this micrograph with figure 6.10 reveals that fewer narrow fibers are present and that the widest fibers have swollen to an even greater extent. The wide, swollen fibers often have scalloped edges (Figure 6.18) at this stage in the swelling process. With further swelling, the scallops become less pronounced. The histogram of Figure 6.81 shows a more even distribution of fibril widths, half of the fibrils are more than 700 nm wide. A comparison of the histograms in Figures 6.79 and 6.81 show that the intensity of banding at all widths has decreased. In some of the more swollen fibrils, the banded section traverses the width of the fibril from side to side creating a dark wiggly line in the fibril (Figure 6.18). Some fibrils resist the swelling effect of the acid to such an extent, that intraperiod bands are still visible, as shown in Figure 6.19.
Figure 6.17: Transmission electron micrograph (Plate #18505, Sample 4.50:0) of bovine hide collagen blended for 1 hour in 0.05 M acetic acid, 4000 x. Stain: 0.5% PTA (pH 1.5) for 1 minute, rinsed, 1% UA (Uranyl Acetate) for 1 minute, then rinsed with distilled water.
Figure 6.18  Transmission electron micrograph (Plate #13507, Sample 4.50:0) of bovine hide collagen blended for 1 hour in 0.05 M acetic acid, 15,000 X. Stain: 0.5% PTA (pH 1.5) for 1 minute, rinsed, 1% UA for 1 minute, then rinsed with distilled water.
Figure 6.19: Transmission electron micrograph (Plate #18508, Sample 4.50:0) of bovine hide collagen blended for 1 hour in 0.05 M acetic acid, 75,000 x. Stain: 0.5% PTA (pH 1.5) for 1 minute, rinsed, 1% UA for 1 minute, then rinsed with distilled water.
Sample 4.51:0

This sample, collagen blended for 1 hour and swollen for 12 hours, shows essentially the same trend as the previous sample. The fibrils are swollen and have weak banding patterns (Figures 6.20 and 6.21). Figure 6.22 shows that the narrower fibrils maintain the most pronounced banding, while the wider fibrils have little or no banding. The narrowest banded fibrils in this sample were already 300 nm in width, so that all banded fibrils were quite swollen and showed a period of approximately 53 nm. The histogram of Figure 6.82, although based on limited data, shows that the fibril widths are essentially in the same range as those found after only one hour of blending, that is, predominantly less than 1300 nm.
Figure 6.20: Transmission electron micrograph (Plate #17075, Sample 4.51:0) of bovine hide collagen blended for 1 hour and swollen for 12 hours in 0.05 M acetic acid 4000 X. Stain: 0.5% PTA (pH 1.5) for 1 minute, then rinsed with distilled water.
Figure 6.21:  Transmission electron micrograph (Plate 17076, Sample 4.51:0) of bovine hide collagen blended for 1 hour and swollen for 12 hours in 0.05 M acetic acid, 15,000 X. Stain: 0.5% PTA (pH 1.5) for 1 minute, then rinsed with distilled water.
Figure 6.22: Transmission electron micrograph (Plate #17077, Sample 4.51:0) of bovine hide collagen blended for 1 hour and swollen for 12 hours in 0.05 M acetic acid, 75,000 X. Stain: 0.5% PTA (pH 1.5) for 1 minute, then rinsed with distilled water.
Sample 4.53:0

The transmission electron micrographic specimens of this sample and Sample 4.51:0 were made from the same dispersion. The appearance of this sample at low magnification, Figure 6.23, is quite similar to that of the previous sample. The fibers are swollen, but fewer narrow fibrils are present. The banding was, again, stronger in the narrow fibrils than in the swollen fibrils (Figure 6.25). Diligent searching of the specimens showed that low intensity banding is still present in the wide swollen fibrils (Figure 6.24). The period of the banding was found to be 62 nm for the very narrow fibrils and 54 nm for the swollen fibrils. Figure 6.83 illustrates that the additional swelling time in acid does not seem to have substantially increased the maximum fibril width.
Figure 6.23: Transmission electron micrograph (Plate #17240, Sample 4.53:0) of bovine hide collagen blended for 1 hour and swollen for 3 days in 0.05 M acetic acid, 4000 X. Stain: 0.5% PTA (pH 1.5) for 1 minute, then rinsed with distilled water.
Figure 6.24: Transmission electron micrograph (Plate #18403, Sample 4.53:0) of bovine hide collagen blended for 1 hour and swollen for 3 days, 15,000 X. Stain: 0.5% PTA (pH 1.5) for 1 minute, rinsed, 0.002 M UA for 10 minutes, then rinsed with distilled water.
Figure 6.25: Transmission electron micrograph (Plate #18404, Sample 4.53:0) of bovine hide collagen blended for 1 hour and swollen for 3 days, 75,000 X. Stain: 0.5% PTA (pH 1.5) for 1 minute, rinsed, 0.002 M UA for 10 minutes, then rinsed with distilled water.
Sample 4.52:0

Figure 6.26 shows a transmission electron micrograph of a dispersion prepared early in the study. This one-day swollen dispersion and Sample 4.54:0, a 5-day swollen sample, were prepared from the same dispersion. Figure 6.26 shows the appearance of the one-day swollen sample. The widths of swollen fibrils of this sample are grouped around 300 to 600 nm and no banding was evident in the limited number of available micrographs (see Figure 6.84).
Figure 6.26: Transmission electron micrograph (Plate 16973, Sample 4.52:0) of bovine hide collagen blended for 1 hour and swollen for 12 hours, 15,000 X. Stain: 0.5% PTA for 1 minute, then rinsed with distilled water.
Sample 4.54:0

The additional 4 days of static swelling of the dispersion of Sample 4.52:0 resulted in more swollen fibrils (see Figure 6.27). The distribution of fibril widths in Figure 6.85 shows that fibril widths of up to 1100 nm were found in the sample. As in the previous sample, the 300 to 600 nm widths are the most prevalent. Low intensity banding with a period of 56 nm was found in some of the highly swollen fibrils, while one narrow fibril with fairly distinct banding was found to have a period of 60 nm. The actual fibril widths in Samples 4.52:0 and 4.54:0 do not seem to correlate well with the previously discussed sample, in spite of the fact that the swelling trend remains similar. It is believed that this is due to the fact that no effort was made to remove bubbles from these earlier dispersions after blending, and that the presence of the numerous bubbles may have slowed the swelling process.
Figure 6.27: Transmission electron micrograph (Plate #16987, Sample 4.54:0) of bovine hide collagen blended for 1 hour and swollen for 5 days, 15,000 X. Stain: 0.5% PTA for 1 minute, then rinsed with distilled water.
Figure 6.28: Transmission electron micrograph (Plate #16988, Sample 4.54:0) of bovine hide collagen blended for 1 hour and swollen for 5 days, 75,000 X. Stain: 0.5% PTA for 1 minute, then rinsed with distilled water.
Sample 4.56:0

The microscopic appearance of a collagen dispersion which has been swollen for $2\frac{1}{2}$ months is shown in Figure 6.29. The widths of the fibrils appear essentially unchanged from those swollen for 3 days, Figure 6.23. Most fibrils are highly swollen, but a few are narrow. The fibril widths are distributed from 100 to 1300 nm, as shown in Figure 6.86. The banding intensity appears to have decreased, though; only 3% of all fibrils are banded and these display only low or very low intensity banding (See Figures 6.30 and 6.31). This compares with about 23% of all fibrils showing low intensity banding after 3 days of swelling Sample 4.53:0.

The average banding period of the weakly banded, swollen fibrils in Figure 6.31 is 48 nm. The banding period of the single narrow banded fibril found in this sample is 57 nm (Figure 6.31).
Figure 6.29: Transmission electron micrograph (Plate #17279, Sample 4.56:0) of bovine hide collagen blended for 1 hour and swollen for 2 months in 0.05 M acetic acid, 4000 X. Stain: 0.5% PTA (pH 1.5) for 1 minute, then rinsed with distilled water.
Figure 6.30: Transmission electron micrograph (Plate #17283, Sample 4.56:0) of bovine hide collagen blended for 1 hour and swollen for 2 months in 0.05 M acetic acid, 15,000 X. Stain: 0.5% PTA (pH 1.5) for 1 minute, then rinsed with distilled water.
Figure 6.31: Transmission electron micrograph (Composite of Plates #17236 and 17234, Sample 4.56:0) of bovine hide collagen blended for 1 hour and swollen for 2 months in 0.05 M acetic acid, 75,000 X. Stain: 0.5% PTA (pH 1.5) for 1 minute, then rinsed with distilled water.
Sample 4.57:0

This sample, which was swollen for 10 months in acetic acid, is shown in Figure 6.32. A distribution of fibril widths from 50 to 1350 nm was measured and is shown in Figure 6.87. Some narrow fibrils still remain even after this long time of static swelling. This sample, like Samples 4.52:0 and 4.54:0, contained many bubbles while it swelled and this may account for the fact that the fibril widths are skewed to the narrower range when compared with the two and one half month swollen sample, Sample 4.56:0. Low level banding, shown in Figure 6.33, is still present and has a period of 59 nm.
Figure 6.32: Transmission electron micrograph (Plate 16977, Sample 4.57:0) of bovine hide collagen blended for 1 hour and swollen for 10 months in 0.05 M acetic acid, 15,000 X. Stain: 0.5% PTA for 1 minute, then rinsed with distilled water.
Figure 6.33: Transmission electron micrograph (Plate #16978, Sample 4.57:0) of bovine hide collagen blended for 1 hour and swollen for 10 months in 0.05 M acetic acid, 75,000 X. Stain: 0.5% PTA for 1 minute, then rinsed with distilled water.
Sample 4.30:1

Figure 6.34 shows the appearance of a sample which has been blended, in acetic acid, for twenty minutes prior to the addition of 8% chondroitin-6-sulfate. Strongly banded fibrils with intraperiod bands, and widths of 50 to 100 nm predominate the sample, as shown in Figure 6.88. The banding period of these fibrils is 60 nm. Additionally, wispy, swollen, nonbanded fibrils are visible and have widths of 200 to 350 nm. A comparison of the histograms in Figures 6.88 and 6.79 shows that following the addition of 8% chondroitin-6-sulfate, most fibrils are de-swollen to their original widths, and returns to a strongly banded appearance.
Figure 6.34: Transmission electron micrograph (Plate #16823, Sample 4.30:1') of bovine hide collagen blended for 20 minutes in 0.05 M acetic acid, plus 8% (w/w) chondroitin-6-sulfate, 75,000 A. Stain: 0.5% PTA, no rinse.
Sample 4.20:3

This sample was blended for fifteen minutes in acetic acid before the addition of 12% chondroitin-6-sulfate. Figure 6.35 shows that this sample contains a very high percentage of strongly banded fibrils, with intraperiod bands. The banding period is 61 nm. Figure 6.89 shows that the widest fibril measured was only 150 nm. Compared to the histogram of Figure 6.79, this shows that a dramatic de-swelling and re-banding is caused by the addition of chondroitin-6-sulfate. Comparing Figures 6.89 and 6.88 shows that fewer wide, swollen fibrils are present in the sample to which more chondroitin-6-sulfate was added after spending a short time in acetic acid.
Figure 6.35: Transmission electron micrograph (Plate 16847, Sample 4.20:3') of bovine hide collagen blended for 15 minutes in 0.05 M acetic acid, plus 12% (w/w) chondroitin-6-sulfate, 75,000 x. Stain: 0.5% PTA, no rinse.
Sample 4.53:5'

Three days of swelling before the addition of 12% chondroitin-6-sulfate results in the transmission electron microscopic appearance shown in Figure 6.36. The general trend of these fibrils (Figure 6.90) is to approximately halve their widths, as compared to the widths prior to the addition of chondroitin-6-sulfate (Figure 6.83). The maximum measured fibril width for this sample is 650 nm, compared to 1350 nm for the 3-days swollen sample, Sample 4.53:0. Also, after the addition of chondroitin-6-sulfate, the 50 to 100 nm-width peak reappeared, which is the width typical of bovine hide collagen at pH 7. The banding period of the narrow sections of these fibrils is 65 nm. Figure 6.37 shows that it is common for the fibrils to undergo the transition from a strongly banded fibril, with intraperiod bands to one that is swollen and un-banded. This behaviour is seen only in samples which contain chondroitin-6-sulfate.
Figure 6.36: Transmission electron micrograph (Plate #16876, Sample 4.53.5') of bovine hide collagen blended for 1 hour and swollen for 3 days in 0.05 M acetic acid, plus 2% (w/w) chondroitin-6-sulfate, 15,000 X. Stain: 0.5% PTA, no rinse.

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Figure 6.37: Transmission electron micrograph (Plate #16878, Sample 4.53:5') of bovine hide collagen blended for 1 hour and swollen for 3 days in 0.05 M acetic acid, plus 12% (w/w) chondroitin-6-sulfate, 75,000 x. Stain: 0.5% PTA, no rinse.
Sample 4.56:1

Figure 6.38 is a low power view of a 2½ months swollen dispersion to which 12% chondroitin-6-sulfate was added. Immediately apparent is the large clump of fibrils. A co-worker, Pete Steinhagen, has shown that the clumps of fibrils in a chondroitin-6-sulfate treated dispersion average 180 \(\mu \text{m} \) in length. This clump, which is approximately 50 \(\mu \text{m} \) by 8 \(\mu \text{m} \), is quite similar in appearance to the clump seen in Figure 6.7, a sample blended only 2 minutes in acetic acid. This clearly shows that the effect of chondroitin-6-sulfate is to reverse the swelling process. Figures 6.39 and 6.40 show a return of banding to approximately 70\% of the fibrils. The banding period of these narrow fibrils is 66 nm. A comparison of Figures 6.91 and 6.86 also shows that the average fibril width has greatly decreased, following the addition of chondroitin-6-sulfate; most fibril widths are less than 500 nm after the addition of chondroitin-6-sulfate, whereas prior to the addition, more than half of the fibrils were wider than 500 nm. The banding period of the more swollen fibrils in this sample (approximately 400 nm) was 64 nm.
Figure 6.38: Transmission electron micrograph (Plate #17287, Sample 4.56:1) of bovine hide collagen blended for 1 hour and swollen for 2 months in 0.05 M acetic acid, plus 12% chondroitin-6-sulfate, 4000 X. Stain: 0.5% PTA (pH 1.5) for 1 minute, then rinsed with distilled water.
Figure 6.39: Transmission electron micrograph (Plate #17289, Sample 4.56:1) of bovine hide collagen blended for 1 hour and swollen for 2 months in 0.05 M acetic acid, plus 12% chondroitin-6-sulfate, 15,000 X. Stain: 0.5% PTA (pH 1.5) for 1 minute, then rinsed with distilled water.
Figure 6.40: Transmission electron micrograph (Plate #17292, Sample 4.56:1) of bovine hide collagen blended for 1 hour and swollen for 2 months in 0.05 M acetic acid, plus 12% chondroitin-6-sulfate, 75,000 X. Stain: 0.5% PTA (pH 1.5) for 1 minute, then rinsed with distilled water.
Sample 4.56:2

It was difficult to make transmission electron micrographs from this sample, which was formed by adding 25% chondroitin-6-sulfate to a 2½ months swollen dispersion. The reason for the difficulty is that the clumps on the grid were often too thick to be penetrated by the electron beam. Figure 6.41 shows an area near a clump (partially seen in lower right) which showed many more fibrils detached from the clump than the other specimens. The histogram of Figure 6.92 shows that the average width of these unclumped fibrils has decreased substantially from that found before the addition of the chondroitin-6-sulfate, Figure 6.86, and that half of the fibrils are banded. The banding period is 64 nm. These statistics may not truly represent the majority of the fibrils in this sample (which are in the clumps and probably narrower and more strongly banded), but are indicative of the effect of chondroitin-6-sulfate upon acetic acid swollen collagen. Figures 6.42 and 6.43 show a closer view of the banding. The banded fibrils shown in Figure 6.43 are very defined and are as close as possible to having intraperiod banding without actually having any.
Figure 6.41: Transmission electron micrograph (Plate #17861, Sample 4.56:2) of bovine hide collagen blended for 1 hour and swollen for 2 months in 0.05 M acetic acid, plus 25% chondroitin-6-sulfate, 4000 X. Stain: 0.5% PTA (pH 1.5) for 2 minutes, then rinsed with distilled water.
Figure 6.42: Transmission electron micrograph (Plate #17862, Sample 4,56:2) of bovine hide collagen blended for 1 hour and swollen for 2 months in 0.05 M acetic acid, plus 25% chondroitin-6-sulfate, 15,000 X. Stain: 0.5% PTA (pH 1.5) for 2 minutes, then rinsed with distilled water.
Figure 6.43: Transmission electron micrograph (Plate #17865, Sample 4.56:2) of bovine hide collagen blended for 1 hour and swollen for 2 months in 0.05 M acetic acid, plus 25% chondroitin-6-sulfate, 75,000 X. Stain: 0.5% PTA (pH 1.5) for 2 minutes, then rinsed with distilled water.
Sample 4.53:4

The appearance of a 3-day swollen bovine hide collagen dispersion which has been dialyzed to pH 3, Figure 6.44, is essentially the same as that before the dialysis as illustrated by Figure 6.23. This dialyzed sample has banding in approximately one-sixth of the fibrils, which is somewhat less than that found prior to dialysis (see Figure 6.45). The banding period is 53 nm for the more swollen fibrils, similar to the one in Figure 6.46, and 56 nm for the thinner fibrils. The histogram in Figure 6.93 shows that the range of fibril widths is up to 1100 nm with more than half of the fibril widths greater than 500 nm.
Figure 6.44: Transmission electron micrograph (Plate #18517, Sample 4.53:4) of bovine hide collagen blended for 1 hour and swollen for 3 days in 0.05 M acetic acid, then dialyzed to pH 3; 4000 Å. Stain: 0.5% PTA (pH 1.5) for 1 minute, rinsed, 0.002 M UA for 10 minutes, then rinsed with distilled water.
Figure 6.45: Transmission electron micrograph (Plate #18515, Sample 4.53:4) of bovine hide collagen blended for 1 hour and swollen for 3 days in 0.05 M acetic acid, then dialyzed to pH 3; 15,000 Å. Stain: 0.5% PTA (pH 1.5) for 1 minute, rinsed, 0.002 M UA for 10 minutes, then rinsed with distilled water.
Figure 6.46: Transmission electron micrograph (Plate # 18516, Sample 4.53:4) of bovine hide collagen blended for 1 hour and swollen for 3 days in 0.05 M acetic acid, then dialyzed to pH 3; 75,000 Å. Stain: 0.5% PTA (pH 1.5) for 1 minute, rinsed, 0.002M UA for 10 minutes, then rinsed with distilled water.
Sample 4.53:3

Dialysis of a 3-days swollen pH 3 dispersion to pH 7 results in much smaller fibril widths as can be seen by comparing Figures 6.47 and 6.24. The histogram in Figure 6.94 shows that the fibril widths are predominantly in the 50 to 150 nm range and that most fibrils are banded (see also Figure 6.48). It is interesting to note, that dialysis to pH 7 results in fibril widths which are quite uniform over the length of individual fibrils, whereas the addition of chondroitin-6-sulfate de-swells a fibril's width nonuniformly along its length (compare Figures 6.47 and 6.36).
Figure 6.47: Transmission electron micrograph (Plate #18414, Sample 4.53:3) of bovine hide collagen blended for 1 hour and swollen for 3 days in 0.05 M acetic acid, then dialyzed to pH 7; 15,000 Å. Stain: 0.5% PTA (pH 1.5) for 1 minute, rinsed, 0.002 M UA for 10 mintues, then rinsed with distilled water.
Figure 6.48: Transmission electron micrograph (Plate #18414, Sample 4.53:3) of bovine hide collagen blended for 1 hour and swollen for 3 days in 0.05 M acetic acid, then dialyzed to pH 7; 15,000 ×. Stain: 0.5% PTA (pH 1.5) for 1 minute, rinsed, 0.002 M UA for 10 minutes, then rinsed with distilled water.
**Sample 4.53:10**

This sample, illustrated at low power in Figure 6.49, was made by adding glutaraldehyde to a 3-days swollen dispersion. The addition of glutaraldehyde increased the fibril widths. This is evident by comparing Figure 6.49 with Figure 6.23, 3 days swelling, or Figure 6.44, 3 days swelling plus dialysis to pH 3. Also, a comparison of the histogram in Figure 6.95 with Figure 6.83 or Figure 6.93 indicates the same trend. In both Samples 4.53:4, 3 days swelling plus dialysis to pH 3, and Sample 4.53:0, 3 days of swelling, half of the fibril widths are less than 600 nm, while after the addition of glutaraldehyde all of the fibrils are wider than 600 nm and the median width is 1000 nm. The fibrils shown in Figure 6.50 show the highly swollen nature of the sample, the edges of the fibrils are quite smooth and the width is quite uniform over the length of each fibril. After an extensive search, two fibrils with very, very low intensity banding were found, one of which is shown in Figure 6.51. The banding period of this fibril is 53 nm.
Figure 6.49: Transmission electron micrograph (Plate #18410, Sample 4.53:10) of bovine hide collagen blended for 1 hour and swollen for 3 days in 0.05 M acetic acid, plus 0.5% (v/v) glutaraldehyde, 4000 X. Stain: 0.5% PTA (pH 1.5) for 10 minutes, rinsed, 0.002 M UA for 15 minutes, then rinsed with distilled water.
Figure 6.50: Transmission electron micrograph (Plate # 18406, Sample 4.53:10) of bovine hide collagen blended for 1 hour and swollen for 3 days in 0.05 M acetic acid, plus 0.5% (v/v) glutaraldehyde, 15,000 X. Stain: 0.5% PTA (pH 1.5) for 5 minutes, then rinsed with distilled water.
Figure 6.51: Transmission electron micrograph (Plate #18408, Sample 4.53:10) of bovine hide collagen blended for 1 hour and swollen for 3 days in 0.05 M acetic acid, plus 0.5% (v/v) glutaraldehyde, 75,000 X. Stain: 0.5% PTA (pH 1.5) for 5 minutes, then rinsed with distilled water.
Sample 4.53:14

Figure 6.52 is quite similar to Figure 6.49. The additional dialysis to pH 3 after the addition of glutaraldehyde does not seem to have affected the appearance of the fibrils. Figure 6.53 shows the complete absence of thin fibrils, and the presence of very wide, swollen, nonbanded fibrils. The fibril widths are mainly in the 800 to 1500 nm range, as shown in Figure 6.96, which is a little wider than those encountered in the previous sample. No banding was encountered in this specimen. Figure 6.54 is a close up of a typical fibril which shows no regular repeating pattern.
Figure 6.52: Transmission electron micrograph (Plate #18424, Sample 4.53:14) of bovine hide collagen blended for 1 hour and swollen for 3 days in 0.05 M acetic acid, plus 0.5% (v/v) glutaraldehyde, then dialyzed to pH 3; 4000 x. Stain: 0.5% PTA (pH 1.5) for 10 minutes, rinsed 0.002 M UA for 15 minutes, then rinsed with distilled water.
Figure 6.53: Transmission electron micrograph (Plate #18425, Sample 4.53:14) of bovine hide collagen blended for 1 hour and swollen for 3 days in 0.05 M acetic acid, plus 0.5% (v/v) glutaraldehyde, then dialyzed to pH 3; 15,000 x. Stain: 0.5% PTA (pH 1.5) for 10 minutes, rinsed, 0.002 M UA for 15 minutes, then rinsed with distilled water.
Figure 6.54: Transmission electron micrograph (Plate #18426, Sample 4.53:14) of bovine hide collagen blended for 1 hour and swollen for 3 days in 0.05 M acetic acid, plus 0.5% (v/v) glutaraldehyde, then dialyzed to pH 3; 75,000 x. Stain: 0.5% PTA (pH 1.5) for 10 minutes, rinsed, 0.002 M UA for 15 minutes, then rinsed with distilled water.
The addition of glutaraldehyde prior to a dialysis to pH 7 seems to exert a stabilizing effect upon the fibril widths. Figure 6.55, after dialysis to pH 7 and Figure 6.49, before dialysis, show a very similar pattern of fibril widths. This fact is also born out by a comparison of the histograms in Figures 6.97 and 6.95. Figure 6.97 should also be contrasted with Figure 6.94. These figures show that without glutaraldehyde, the dialysis to pH 7 shrinks all the fibril widths to the 100 nm range, but treatment with glutaraldehyde prior to dialysis stabilizes the fibrils, resulting in fibril widths in the 1000 nm range. Figure 6.56 shows the swollen nature of the fibrils, with no narrow fibrils present. No banding was encountered in this sample, as is shown by a high-magnification view of some fibrils in Figure 6.57.
Figure 6.55: Transmission electron micrograph (Plate #18416, Sample 4.53:13) of bovine hide collagen blended for 1 hour and swollen for 3 days in 0.05 M acetic acid, plus 0.5% (v/v) glutaraldehyde, then dialyzed to pH 7; 4000×. Stain: 0.5% PTA (pH 1.5) for 10 minutes, rinsed, 0.002 M UA for 15 minutes, then rinsed with distilled water.
Figure 6.56: Transmission electron micrograph (Plate #18417, Sample 4.53:13) of bovine hide collagen blended for 1 hour and swollen for 3 days in 0.05 M acetic acid, plus 0.5% (v/v) glutaraldehyde, then dialyzed to pH 7, 15,000 X. Stain: 0.5% PTA (pH 1.5) for 10 minutes, rinsed, 0.002 M UA for 15 minutes, then rinsed with distilled water.
Figure 6.57: Transmission electron micrograph (Plate #18418, Sample 4.53:13) of bovine hide collagen blended for 1 hour and swollen for 3 days in 0.05 M acetic acid, plus 0.5% (v/v) glutaraldehyde, then dialyzed to pH 7, 75,000 X. Stain: 0.5% PTA (pH 1.5) for 10 minutes, rinsed, 0.002 M UA for 15 minutes, then rinsed with distilled water.
Figure 6.58: Transmission electron micrograph (Plate #18510, Sample 4.53:15) of bovine hide collagen blended for 1 hour and swollen for 3 days in 0.05 M acetic acid, plus 0.5% (v/v) glutaraldehyde and 12% chondroitin-6-sulfate, 4000 X. Stain: 0.5% PTA (pH 1.5) for 10 minutes, rinsed, 0.002 M UA for 15 minutes, then rinsed with distilled water.
Sample 4.53:15

The appearance of a sample to which 12% chondroitin-6-sulfate has been added subsequent to the glutaraldehyde, as shown in Figure 6.58, is very different. Comparing this sample to Sample 4.53:10, Figure 6.49, shows that the chondroitin-6-sulfate has caused a dramatic decrease in overall fibril widths. Also, an increased tendency to clump is observed. The fibrils shown in Figure 6.59 are rather ragged looking. The edges are uneven and the widths vary along the length of the fibrils. Figure 6.60 shows that some fibrils have re-formed into very narrow, strongly banded fibrils, with intraperiod bands (compare with Figure 6.51). The banding period of the narrow fibrils is 58 nm. The histogram of Figure 6.98 when compared with Figure 6.95 illustrates the dramatic de-swelling effect of chondroitin-6-sulfate quite nicely.
Figure 6.59: Transmission electron micrograph (Plate #18512, Sample 4.53:15) of bovine hide collagen blended for 1 hour and swollen for 3 days in 0.05 M acetic acid, plus 0.5% (v/v) glutaraldehyde and 12% chondroitin-6-sulfate, 15,000 x. Stain: 0.5% PTA (pH 1.5) for 10 minutes, rinsed, 0.002 M UA for 15 minutes, then rinsed with distilled water.
Figure 6.60: Transmission electron micrograph (Plate #18513, Sample 4.53:15) of bovine hide collagen blended for 1 hour and swollen for 3 days in 0.05 M acetic acid, plus 0.5% (v/v) glutaraldehyde and 12% chondroitin-6-sulfate, 75,000 x. Stain: 0.5% PTA (pH 1.5) for 10 minutes, rinsed, 0.002 M UA for 15 minutes, then rinsed with distilled water.
Sample 4.53:18

Figure 6.61 displays a sample with a history of 3 days of swelling plus glutaraldehyde followed by chondroitin-6-sulfate and dialysis to pH 7. The appearance of these fibrils is very similar to those in the previous sample. The tendency to clump has returned. The fibrils are narrow and tagged as shown in Figure 6.62. Figures 6.63 and 6.99 show that banding has returned to a large percentage of the fibrils and that the fibril widths have returned to the 100 nm range. The banding period, 56 nm, appears to be somewhat less than that found for native collagen. This smaller banding period may be caused by the crosslinking of the highly swollen fibrils by glutaraldehyde. The swelling of fibrils results in a smaller banding period. It appears that the glutaraldehyde crosslinking has stabilized the fibril more successfully in the longitudinal direction than in the width, against the action of chondroitin-6-sulfate. The additional dialysis of this sample does not seem to have changed its appearance greatly from the appearance of Sample 4.53:15.
Figure 6.61: Transmission electron micrograph (Plate #18420, Sample 4.53:18) of bovine hide collagen blended for 1 hour and swollen for 3 days in 0.05 M acetic acid, plus 0.5% (v/v) glutaraldehyde and 12% chondroitin-6-sulfate, then dialyzed to pH 7, 4000 AL. Stain: 0.5% PTA (pH 1.5) for 10 minutes, rinsed, 0.002 M UA for 15 minutes, then rinsed with distilled water.
Figure 6.62: Transmission electron micrograph (Plate #18422, Sample 4.53:18) of bovine hide collagen blended for 1 hour and swollen for 3 days in 0.05 M acetic acid, plus 0.5% (v/v) glutaraldehyde and 12% chondroitin-6-sulfate, then dialyzed to pH 7, 15,000 ×. Stain: 0.5% PTA (pH 1.5) for 10 minutes, rinsed, 0.002 M UA for 15 minutes, then rinsed with distilled water.
Figure 6.63: Transmission electron micrograph (Plate #18423, Sample 4.53:18) of bovine hide collagen blended for 1 hour and swollen for 3 days in 0.05 M acetic acid, plus 0.5% (v/v) glutaraldehyde and 12% chondroitin-6-sulfate, then dialyzed to pH 7, 75,000 Å. Stain: 0.5% PTA (pH 1.5) for 10 minutes, rinsed, 0.002 M UA for 15 minutes, then rinsed with distilled water.
Sample 4.56:3

Figure 6.64 presents a sample which was swollen in acetic acid for 2½ months prior to the addition of glutaraldehyde. The fibrils are highly swollen and nonbanded. When compared to Figure 6.29, Sample 4.56:0, which is only acid swollen, it is evident that the fibril widths are generally larger. Figures 6.100 and 6.86 show this more graphically. The median width of the glutaraldehyde-treated dispersion is 1000 nm, whereas without the glutaraldehyde, the median is 500 nm. The distribution of widths of the 3-day swollen glutaraldehyde-treated dispersion, Figure 6.95, is very similar to that found for this sample. A few narrower fibrils were found in this sample, on specimens prepared on a holey carbon film, but no banding was found in any of the fibrils. Figure 6.65 shows the typical swollen nonbanded fibrils of this sample, which are very similar to those in Figure 6.50.
Figure 6.64: Transmission electron micrograph (Plate #17744, Sample 4.56:3) of bovine hide collagen blended for 1 hour and swollen for 2 months in 0.05 M acetic acid, plus 0.5% (v/v) glutaraldehyde, 4000 X. Stain: 0.5% PTA (pH 1.5) for 10 minutes, rinsed, 0.002 M UA for 15 minutes, then rinsed with distilled water.

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Figure 6.65: Transmission electron micrograph (Plate #17746, Sample 4.56:3) of bovine hide collagen blended for 1 hour and swollen for 2½ months in 0.05 M acetic acid plus 0.5% (v/v) glutaraldehyde, 15,000 X. Stain: 0.5% PTA (pH 1.5) for 10 minutes, rinsed, 0.002 M UA for 15 minutes, then rinsed with distilled water.
Sample 4.56:6

This sample was prepared by dialyzing the glutaraldehyde-treated dispersion of Sample 4.56:3, and is illustrated in Figure 6.66. The fibrils in this micrograph are generally similar in appearance to those in Figure 6.64, highly swollen and nonbanded. In the center right of this micrograph are a pair of narrow fibrils which are interlocked, forming an X. This pair of fibrils did exhibit banding, with a period of 58.5 nm. It is believed that the reason for the narrow-banded character of these fibrils is due to the tension created while drying on the transmission electron micrographic grid. The ends of these fibrils (see Figure 6.67) are firmly planted and nonbanded, but the central portions of the fibrils are raised and shrunk while drying, causing increased tension. This further narrowing of the fibrils, allowed the natural order to be regained and resulted in the reappearance of bands. A few fibrils like this pair were found in this sample after extensive searching, but by far the most common type of fibril is the highly swollen, nonbanded type, illustrated in Figure 6.68. A high magnification view of these fibrils, shown in Figure 6.69, shows that no banding is apparent. In spite of the presence of a few narrow fibrils, the median width is still greater than 900 nm, as evident in Figure 6.101.
Figure 6.66: Transmission electron micrograph (Plate #17857, Sample 4:56:6) of bovine hide collagen blended for 1 hour and swollen for 2½ months in 0.05 M acetic acid, plus 0.5% (v/v) glutaraldehyde, then dialyzed to pH 7, 4000 X. Stain: 0.5% PTA (pH 1.5) for 5 minutes, no rinse.
Figure 6.67: Transmission electron micrograph (Plate #17858, Sample 4.56:6) of bovine hide collagen blended for 1 hour and swollen for 2½ months in 0.05 M acetic acid, plus 0.5% (v/v) glutaraldehyde, then dialyzed to pH 7, 15,000 X. Stain: 0.5% PTA (pH 1.5) for 5 minutes, no rinse.
Figure 6.68: Transmission electron micrograph (Plate #17859, Sample 4.56:6) of bovine hide collagen blended for 1 hour and swollen for 2½ months in 0.05 M acetic acid, plus 0.5% (v/v) glutaraldehyde, then dialyzed to pH 7, 15,000 X. Stain: 0.5% PTA (pH 1.5) for 5 minutes, no rinse.
Figure 6.69: Transmission electron micrograph (Plate #17860, Sample 4.56:6) of bovine hide collagen blended for 1 hour and swollen for 2½ months in 0.05 M acetic acid, plus 0.5% (v/v) glutaraldehyde, then dialyzed to pH 7, 75,000 x. Stain: 0.5% PTA (pH 1.5) for 5 minutes, no rinse.
Sample 4.56:7

The addition of chondroitin-6-sulfate to the 2½-months swollen glutaraldehyde-treated dispersion de-swells the fibrils, as is evident by comparing Figures 6.70 and 6.64. The median width of this sample, only 400 nm as shown on Figure 6.102, is less than half of the median indicated for Sample 4.56:3 in Figure 6.100. The additional swelling time before the addition of glutaraldehyde seems to have been beneficial, though, as the fibrils resisted the de-swelling effect of chondroitin-6-sulfate much better than the 3-days swollen glutaraldehyde dispersion illustrated in Figure 6.98. Also, no banding was noticed in this dispersion. This sample and Sample 4.56:10 are the only samples with chondroitin-6-sulfate which were found to have no banded fibrils. Figure 6.71 shows that the fibrils are not very uniform in width along individual fibrils, a characteristic which is often observed in dispersions treated with chondroitin-6-sulfate. The narrow and swollen fibrils, shown at high magnification in Figure 6.72, do not exhibit any banding pattern.
Figure 6.70: Transmission electron micrograph (Plate #17735, Sample 4.56:7) of bovine hide collagen blended for 1 hour and swollen for 2½ months in 0.05 M acetic acid, plus 0.5% (v/v) glutaraldehyde and 12% chondroitin-6-sulfate, 4000 X. Stain: 0.5% PTA (pH 1.5) for 10 minutes, rinsed, 0.002 M UA for 15 minutes, then rinsed with distilled water.
Figure 6.71: Transmission electron micrograph (Plate #17737, Sample 4.56:7) of bovine hide collagen blended for 1 hour and swollen for 2½ months in 0.05 M acetic acid, plus 0.5% (v/v) glutaraldehyde and 12% chondroitin-6-sulfate, 15,000 X. Stain: 0.5% PTA (pH 1.5) for 10 minutes, rinsed, 0.002 M UA for 15 minutes, then rinsed with distilled water.
Figure 6.72: Transmission electron micrograph (Plate #17742, Sample 4.56:7) of bovine hide collagen blended for 1 hour and swollen for 2½ months in 0.05 M acetic acid, plus 0.5% (v/v) glutaraldehyde and 12% chondroitin-6-sulfate, 75,000 X. Stain: 0.5% PTA (pH 1.5) for 10 minutes, rinsed, 0.002 M UA for 15 minutes, then rinsed with distilled water.
Sample 4.56:10

After dialyzing to pH 7, the chondroitin-6-sulfate glutaraldehyde-treated dispersion showed an increased tendency to form clumps, as shown in Figure 6.73. The swollen nature of the fibrils is exhibited in Figure 6.74, but these fibrils are much thinner than those in Figure 6.65. Comparing the histograms of Figures 6.103 and 6.102 shows that the average fibril width has remained fairly constant or increased slightly during the dialysis. Figure 6.75 indicates that this sample is one of the two containing chondroitin-6-sulfate which have no detectable banding.
Figure 6.73: Transmission electron micrograph (Plate #17851, Sample 4.56:10) of bovine hide collagen blended for 1 hour and swollen for 2½ months in 0.05 M acetic acid, plus 0.5% (v/v) glutaraldehyde and 12% chondroitin-6-sulfate, then dialyzed to pH 7; 4000 A. Stain: 0.5% PTA (pH 1.5) for 5 minutes, no rinse.
Figure 6.74: Transmission electron micrograph (Plate #17854, Sample 4.56;10) of bovine hide collagen blended for 1 hour and swollen for 2½ months in 0.05 M acetic acid, plus 0.5% (v/v) glutaraldehyde and 12% chondroitin-6-sulfate, then dialyzed to pH 7; 15,000 x. Stain: 0.5% PTA (pH 1.5) for 5 minutes, no rinse.
Figure 6.75: Transmission electron micrograph (Plate #17856, Sample 4.56:10) of bovine hide collagen blended for 1 hour and swollen for 2½ months in 0.05 M acetic acid, plus 0.5% (v/v) glutaraldehyde and 12% chondroitin-6-sulfate, then dialyzed to pH 7; 75,000 x. Stain: 0.5% PTA (pH 1.5) for 5 minutes, no rinse.
Figure 6.76: Width of fibrils versus percentage of total fibril lengths for bovine hide collagen dispersed for 15 minutes and 2 hours in pH 7.0 distilled water (Samples 2.20:0 & 2.60:0).
Figure 6.77: Width of fibrils versus percentage of total fibril lengths for bovine hide collagen dispersed in physiological saline for 10 months (Sample 3.20:1').
Figure 6.78: Width of fibrils versus percentage of total fibril lengths for bovine hide collagen blended for 2 minutes in 0.05 M acetic acid (Sample 4.10:0).
Figure 6.79: Width of fibrils versus percentage of total fibril lengths for bovine hide collagen blended for 15 minutes in 0.05 M acetic acid (Sample 4.20:0).
BANDING INTENSITY

- 0 NO BANDING
- 1 WEAKLY BANDED
- 2 BANDED
- 3 STRONGLY BANDED
- 4 STRONGLY BANDED WITH INTRAPERIOD BANDING

Figure 6.80: Width of fibrils versus percentage of total fibril lengths for bovine hide collagen blended for 30 minutes in 0.05 M acetic acid (Sample 4.40:0).
Figure 6.81: Width of fibrils versus percentage of total fibril lengths for bovine hide collagen blended for 1 hour in 0.05 M acetic acid (Sample 4.50:0).
Figure 6.82: Width of fibrils versus percentage of total fibril lengths for bovine hide collagen blended for 1 hour in 0.05 M acetic acid and swollen for 12 hours (Sample 4.51:0).
Figure 6.83: Width of fibrils versus percentage of total fibril lengths for bovine hide collagen blended for 1 hour in 0.05 M acetic acid and swollen for 3 days (Sample 4.53:0).
Figure 6.84: Width of fibrils versus percentage of total fibril lengths for bovine hide collagen blended for 1 hour in 0.05 M acetic acid and swollen for 1 day (Sample 4.52:0).
Figure 6.85: Width of fibrils versus percentage of total fibril lengths for bovine hide collagen blended for 1 hour in 0.05 M acetic acid and swollen for 5 days (Sample 4.54:0).
Figure 6.86: Width of fibrils versus percentage of total fibril lengths for bovine hide collagen blended for 1 hour in 0.05 M acetic acid and swollen for 2 months (Sample 4.50:0).
Figure 6.87: Width of fibrils versus percentage of total fibril lengths for bovine hide collagen blended for 1 hour in 0.05 M acetic acid and swollen for 10 months (Sample 4.57:0).
Figure 6.88: Width of fibrils versus percentage of total fibril lengths for bovine hide collagen blended for 20 minutes in 0.05 M acetic acid, plus 8% (w/w) chondroitin-6-sulfate (Sample 4.30:1).
Figure 6.89: Width of fibrils versus percentage of total fibril lengths for bovine hide collagen blended for 15 minutes in 0.05 M acetic acid, plus 12% (w/w) chondroitin-6-sulfate (Sample 4.20:3).
Figure 6.90: Width of fibrils versus percentage of total fibril lengths for bovine hide collagen blended for 1 hour in 0.05 M acetic acid, swollen for 3 days, plus 12% (w/w) chondroitin-6-sulfate (Sample 4.53:5').
Figure 6.91: Width of fibrils versus percentage of total fibril lengths for bovine hide collagen blended for 1 hour in 0.05 M acetic acid, swollen for 2 months, plus 12% (w/w) chondroitin-6-sulfate (Sample 4.56:1).
Figure 6.92: Width of fibrils versus percentage of total fibril lengths for bovine hide collagen blended for 1 hour in 0.05 M acetic acid, swollen for 2 months, plus 25% (w/w) chondroitin-6-sulfate (Sample 4.56:2).
Figure 6.93: Width of fibrils versus percentage of total fibril lengths for bovine hide collagen blended for 1 hour in 0.05 M acetic acid, swollen for 3 days, and dialyzed to pH 3 (Sample 4.53:4).
Figure 6.94: Width of fibrils versus percentage of total fibril lengths for bovine hide collagen blended for 1 hour in 0.05 M acetic acid, swollen for 3 days, and dialyzed to pH 7 (Sample 4.53:3).
Figure 6.95: Width of fibrils versus percentage of total fibril lengths for bovine hide collagen blended for 1 hour in 0.05 M acetic acid, swollen for 3 days, plus 0.5% (v/v) glutaraldehyde (Sample 4.53:10).
Figure 6.96: Width of fibrils versus percentage of total fibril lengths for bovine hide collagen blended for 1 hour in 0.05 M acetic acid, swollen for 3 days, plus 0.5% (v/v) glutaraldehyde and dialyzed to pH 3 (Sample 4.53:14).
Figure 6.97: Width of fibrils versus percentage of total fibril lengths for bovine hide collagen blended for 1 hour in 0.05 M acetic acid, swollen for 3 days, plus 0.5% (v/v) glutaraldehyde and dialyzed to pH 7 (Sample 4.53:13).
Figure 6.98: Width of fibrils versus percentage of total fibril lengths for bovine hide collagen blended for 1 hour in 0.05 M acetic acid, swollen for 3 days, plus 0.5% (v/v) glutaraldehyde and 12% (w/w) chondroitin-6-sulfate (Sample 4.53:15).
Figure 6.99: Width of fibrils versus percentage of total fibril lengths for bovine hide collagen blended for 1 hour in 0.05 M acetic acid, swollen for 3 days, plus 0.5% (v/v) glutaraldehyde, plus 12% (w/w) chondroitin-6-sulfate and dialyzed to pH 7 (Sample 4.53:18).
Figure 6.100: Width of fibrils versus percentage of total fibril lengths for bovine hide collagen blended for 1 hour in 0.05 M acetic acid, swollen for 2½ months plus 0.5% (v/v) glutaraldehyde (Sample 4.56:3).
Figure 6.101: Width of fibrils versus percentage of total fibril lengths for bovine hide collagen blended for 1 hour in 0.05 M acetic acid, swollen for 2 months, plus 0.5% (v/v) glutaraldehyde, and dialyzed to pH 7 (Sample 4.56:6).
Figure 6.102: Width of fibrils versus percentage of total fibril lengths for bovine hide collagen blended for 1 hour in 0.05 M acetic acid, swollen for 2½ months, plus 0.5% (v/v) glutaraldehyde, and 12% chondroitin-6-sulfate (Sample 4.56:7).
Figure 6.103: Width of fibrils versus percentage of total fibril lengths for bovine hide collagen blended for 1 hour in 0.05 M acetic acid, swollen for 2½ months, plus 0.5% (v/v) glutaraldehyde, plus 12% (w/w) chondroitin-6-sulfate and dialyzed to pH 7 (Sample 4.56:10).
Discussion

The histograms and processing data for each sample are summarized in Table 6.1. The samples have been grouped so that the effect of acetic acid on collagen, the effect of chondroitin-6-sulfate on collagen and the effect of glutaraldehyde on collagen can easily be compared.

Treating bovine hide collagen with acetic acid results in four major effects. First, the fibril widths dramatically increase. Second, the dense clumps of fibrils disintegrate under the effect of the acetic acid. Third, the period of the banding decreases. And fourth, the banding intensity of the fibrils decreases.

The great increase in fibril widths caused by dispersing bovine hide collagen in acetic acid is evident after only 2 minutes (Sample 4.10:0). Formerly, at pH 7, the fibril widths were clustered about 70 nm. After two minutes in acetic acid, many fibril widths average 600 nm, almost a tenfold increase in the fibril widths. This can clearly be seen by comparing Figures 6.3 and 6.8 or Figures 6.76 and 6.78. With additional residence time in acetic acid, the fibril widths become more evenly distributed in widths of up to 1200 nm. It is interesting to note that although the bovine hide collagen fibrils had essentially the same original widths, they swelled to varying degrees under the effect of the acetic acid. This resulted in a vast distribution of
TABLE 6.1: PREDOMINANT FIBRIL WIDTHS OF COLLAGEN SAMPLES. 50 NM. BLOCKS OF FIBRIL WIDTHS POPULATED BY MORE THAN 4% OF ALL SAMPLE FIBRILS ARE INDICATED BY HEAVY LINES.

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1. CHONDROITIN-4-SULFATE
2. AIR DRIED COLLAGEN FROM A PH5B MEMBRANE DISPERSED IN PHYSIOLOGICAL SALINE.
fibril widths. The underlying cause of this may be the variations in density of the physiological crosslinks.

Collagen at pH 7 is very tightly compacted into clumps, as shown in Figure 6.2. Figure 6.7 shows the immediate break up of such a clump under the action of the acetic acid. After 15 minutes in acetic acid, no clumps are visible (Figure 6.10). The samples are made up of individual fibrils which are loosely intertwined on the grid. The dissociation of the clumps into individual swollen fibrils accounts for the viscous, homogenous macroscopic appearance of the collagen acetic acid dispersions.

The banding period decreases in the presence of acetic acid. Sample 4.10:0, which was exposed to acid for only two minutes, had many swollen fibrils with a banding period averaging 57 nm, compared with a period of 65 nm for the non-swollen fibrils in this sample. Similar values were observed for the other swollen samples. The reduction in banding period means that there is some longitudinal contraction in conjunction with the swelling of the widths. A measurement of the adjacent left most fibrils in Figure 6.12 also illustrates the decreased banding period caused by swelling in acetic acid.

The intensity of the banding, in the samples represented by Figures 6.78 to 6.87, is obviously much less than the natively banded specimens prepared at pH7. The intraperiod bands are the first to disappear, reducing the banding
pattern to a simple light-dark pattern. The intraperiod bands are still visible in a few fibrils after 15 minutes in acid (Figure 6.13), and become very occasionally present and indistinct with acid residence times of more than 1 hour (Figures 6.17 to 6/19). After this length of time, only the major periodicity is visible, as shown in the fibril in Figure 6.22. Even this low level banding disappeared in most fibrils, as the residence time in acetic acid was extended past 12 hours to 3 days or longer. In bovine hide collagen samples swollen in acetic acid as long as 10 months, banding was still present in a small percentage of the fibrils. It appears that the pH 3 acetic acid treatment of such a physiologically crosslinked specimen is insufficient to completely destroy all banding.

The dialysis of an acid swollen collagen dispersion caused a decrease of the fibril widths to essentially their original widths (compare Figures 6.94 and 6.83). The dialysis also intensified the banding pattern. The width of each fibril, following dialysis, was essentially uniform along its length.

The samples represented by Figures 6.88 to 6.92 reveal the effect which chondroitin-6-sulfate has on bovine hide collagen. The general effect of the chondroitin-6-sulfate on an acetic acid swollen collagen sample appears to be to reverse the swelling process. The fibril widths are decreased, the clumps of fibrils may appear, the banding
intensity is increased, and the banding period is increased to the normal range (if the sample has not been stabilized by glutaraldehyde).

Samples 4.30:1' and 4.20:3' show the effect of the addition of chondroitin-6-sulfate to collagen which has been blended in acetic acid for 15 minutes. The appearance of a 15 minute blended dispersion is shown in Figures 6.10 to 6.12. The swollen character of virtually all fibrils is reversed when 12% chondroitin-6-sulfate is added (Sample 4.20:3', Figures 6.35 & 6.89) and reversed for most fibrils when 8% chondroitin-6-sulfate is added (Sample 4.30:1', Figures 6.34 & 6.88). Clumping of fibrils (not shown) was also present in Samples 4.20:3' and 4.30:1'. These findings correlate well with macroscope observations. Collagen-acetic acid dispersions with less then 10% chondroitin-6-sulfate are fairly homogenous and viscous (due to the presence of the swollen fibrils), while dispersions with more than 10% chondroitin-6-sulfate are fibrous and have larger particles (clumps of fibrils) which can settle out.

Comparing Figures 6.37 & 6.90 (Sample 4.53:5) to Figures 6.25 & 6.83 (Sample 4.53:0) shows that the addition of the chondroitin-6-sulfate to a 3 day swollen dispersion greatly reduces the width of all fibrils and intensifies the banding to reveal the intraperiod bands. The banding returns to isolated segments of each fibril (Figure 6.36) resulting
in large fibril width variations along individual fibrils. This is in contrast to samples which were de-swollen by dialysis from acid to neutral pH (Sample 4.53:3). That sample showed a general decrease in fibril widths as well, but with relatively uniform widths along each fibril's length.

Comparing Figures 6.38 & 6.91 (Sample 4.56:1) and 6.41 & 6.92 (Sample 4.56:2) with Figures 6.29 & 6.86 (Sample 4.56:0) shows that even after 2 months of prior swelling in acetic acid, the collagen fibrils are de-swollen by the addition of chondroitin-6-sulfate. Both Samples 4.56:1 and 4.56:2 had many regions of clumped fibrils, one of which is illustrated in Figure 6.38. The clump in this micrograph is very similar in appearance to the clumps observed in 2 minute acid swollen dispersion (Figure 6.7). The clump has dense areas with many fibrils emanating from the main body of the clump. The banding was also much more obvious in the samples to which chondroitin-6-sulfate had been added (Figures 6.40 & 6.43), but not as pronounced as when added after shorter acid swelling times (Figure 6.38).

The treatment of acetic acid swollen bovine hide collagen dispersions with glutaraldehyde stabilizes the fibrils against the effects of air-drying, changes of pH and, to a lesser extent, chondroitin-6-sulfate. Figures 6.50
& 6.95 (Sample 4.53:10) describe a 3 day swollen dispersion which was treated with glutaraldehyde. The fibril widths are distributed at higher values than the comparable sample which was not treated with glutaraldehyde (Sample 4.53:0, Figures 6.24 & 6.83). This difference is attributed to the de-swelling effect of the air-drying of specimens on the transmission electron micrographic grid. It is felt that the fibril widths and appearances obtained by viewing the gluteraldehyde treated dispersions are a closer approximation to what is actually present in the dispersion. Sample 4.56:3 (Figures 6.65 & 6.100) and 4.56:0 (Figures 6.30 & 6.86) also show the same trend for samples with much longer swelling times. Further exposure to acetic acid after glutaraldehyde treatment, such as by dialysis to pH 3, does not change the appearance of the fibrils (compare Figures 6.53 & 6.96 with 6.51 & 6.95).

All of the above glutaraldehyde treated samples above had either no banding (Figure 6.54) or very, very low level banding in rare fibrils (Figure 6.51). Dialysis of the glutaraldehyde treated samples to pH 7 did not substantially change the appearance of the fibrils or the distribution of their widths. This is evidenced by contrasting Figures 6.56 & 6.97 (Sample 4.53:13) with Figures 6.50 & 6.95 (Sample 4.53:10) and Figures 6.68 & 6.101 (Sample 4.56:6) with Figures 6.65 & 6.100 (Sample 4.56:3). It should be recalled that dialysis of dispersions untreated
with glutaraldehyde causes the fibrils to de-swell to their original widths.

The addition of chondroitin-6-sulfate to the glutaraldehyde treated dispersion did cause some de-swelling of the fibrils. The effect was fairly pronounced when the time of swelling before the addition of the glutaraldehyde was short, as is shown by a comparison of Figures 6.6 & 6.98 (Sample 4.53:15) with Figures 6.51 & 6.95 (Sample 4.53:10). Strong banding reappeared in Sample 4.53:15 but the period seemed to less that the value found for natively banded collagen even after dialysis to pH 7 (Figure 6.63, Sample 4.53:18).

The fibril widths also decreased, following the addition of chondroitin-6-sulfate to dispersions which had been swollen for long periods of time, as shown by comparing Figures 6.71 & 6.102 (Sample 4.56:7) with Figures 6.65 & 6.100 (Sample 4.56:3). The distribution of fibril widths in Sample 4.56:7 were not substantially changed by dialysis to pH 7 (Sample 4.56:10, Figure 6.103). However, the dialysis did seem to cause increased clumping of the fibrils. The widths of the fibrils were quite nonuniform along the length of each fibril, as was found with other chondroitin-6-sulfate treated samples (Sample 4.53:5, Figure 6.36). Samples 4.56:7 and 4.56:10 are the only two samples containing chondroitin-6-sulfate which also exhibited no banding (see Figures 6.72 and 6.75).
In summary, the major findings derived from the transmission electron micrographs involve the effects of acetic acid, chondroitin-6-sulfate and glutaraldehyde on bovine hide collagen. Acetic acid causes rapid swelling of the fibrils to widths an order of magnitude greater than the original widths. All clumps of fibrils are disintegrated, the banding intensity is greatly reduced and the major period of the bands is reduced. The addition of chondroitin-6-sulfate reverses the swelling trends. The fibrils are de-swollen to their original widths, clumps of fibrils tend to form, the banding intensity is increased and the period is returned to its original value. Treating a swollen collagen dispersion with glutaraldehyde stabilizes the fibrils against the effects of air-drying, changes of pH and, to a limited extent, chondroitin-6-sulfate. Several samples which were treated with glutaraldehyde exhibited no banding, even if dialyzed to pH 7.
CHAPTER VII

Blood Compatibility Testing of the Collagen Composite Materials

Introduction

Platelets play an integral part in the thrombosis mechanism, which is the most serious initial complication of an implant which contacts blood. Therefore, platelet aggregation tests were performed to test the thrombogenic activity of the materials. The measurement of the degree of platelet aggregation works on the principle that platelet rich plasma (PRP, whole blood minus the red and white blood cells) transmits less red light than platelet poor plasma (PPP, whole blood minus most platelets, red and white blood cells). If an agent is added to platelet rich plasma, causing the platelets to aggregate, the platelet rich plasma is rapidly reduced to platelet poor plasma. The resulting change in optical density is measured as an indication of the degree of thrombogenic activity of the tested material.

One complication of performing these experiments is that untreated whole blood will coagulate rapidly when removed from the donor. In glass containers the coagulation time is 9 to 15 minutes, and in siliconized containers, this time is extended to 20 to 60 minutes, still too short a time to perform the desired experiments. An anticoagulant is added to the blood during collection, to increase the time over which experiments can be performed. The anticoagulant used, in this case, is sodium citrate \( \text{C}_6\text{H}_5\text{O}_7\text{Na}_3 \) which acts on the blood by removing...
the calcium ions (Ca\(^{++}\)), a necessary component in the coagulation cascade which does not interfere with platelet aggregation. The anticoagulated blood is centrifuged to separate the platelet rich plasma from the red and white blood cells. Then the platelet rich plasma is used in an aggregometer, a device which measures the change in optical density when a sample is added to the platelet rich plasma. A more detailed account of the experimental procedure follows.

The Platelet Aggregation Experimental Procedure

The first step of performing platelet aggregation experiments was to obtain suitable anticoagulated blood. The phlebotomies were performed with a 19-guage scalp needle attached to a 12-inch piece of polypropylene tubing. The venous blood was drained by gravity, discarding the first 10 ml of blood. The next 36 ml of blood were drained into a 50 ml polypropylene centrifuge tube containing 4 ml of 0.13 M (3.8% w/v) sodium citrate. This resulted in 40 ml of anticoagulated blood with a ratio of anticoagulant to blood of 1:9.

The platelet rich plasma was obtained by centrifuging the anticoagulated blood for 20 minutes in a Model HN International Equipment Corporation centrifuge at 250 g (800 RPM on the dial) at room temperature. The straw-coloured liquid in the upper half of the centrifuge tube, the platelet rich plasma, typically contained 450,000 platelets/mm\(^3\). The lower half of the centrifuge tube, composed mainly of packed red blood cells, was not required for platelet aggregation testing.
The platelet poor plasma was obtained by centrifuging 1 ml of the platelet rich plasma for two minutes, at room temperature, in a Model #3200 Eppendorf Centrifuge, at approximately 11,000 g. The platelets formed a compact ball at the bottom of the centrifuge tube, typically leaving less than 15,000 platelets/mm$^3$ in the supernatant.

The platelet rich plasma, platelet poor plasma and sample were all measured with Pipettenman automatic pipettes with disposable tips. The platelet rich and platelet poor plasma were measured with a 1000 $\lambda$ pipette set to 500 $\lambda$ ($1000 = 1$ ml). The samples of material and epinephrine were measured with a 20 $\lambda$ pipette set from 5 to 20 $\lambda$.

After obtaining specimens of platelet rich and platelet poor plasma the calibration of the Chrono-Log Corporation Model #300 Aggregometer was performed. Prior to the calibration, the aggregometer and 37°C water bath were allowed to warm up for half an hour. The 500 $\lambda$ each of platelet rich plasma and platelet poor plasma were pipetted into siliconized Chrono-Log tubes. A teflon coated stirring bar (flea) was placed in each tube before preheating the tubes in the 37°C water bath for at least 2 minutes. The platelet rich plasma was covered tightly to exclude air, when not in use. The preheated Chrono-Log tube of platelet rich plasma was dried and placed in the sample holder of the aggregometer, with the coarse bias adjusted to keep the pen of the chart recorder on scale (approximately 6). The fine bias was adjusted until the pen read 20% of full
scale. The preheated platelet poor plasma replaced the platelet rich plasma in the sample holder, while the gain was adjusted until the pen of the chart recorder read 100% of full scale. The adjustment of the bias and gain was iterated as described above, until the platelet rich plasma showed a reading of 20% of full scale and the platelet poor plasma showed a reading of 100% of full scale. This calibration procedure was repeated for each new sample of platelet rich plasma prior to each experimental run.

It was important to test each donor's platelets for normal platelet activity. The blood was tested by placing a 500 \( \lambda \) sample of platelet rich plasma in the aggregometer and subsequently adding 5 \( \lambda \) of 1 mg/ml epinephrine solution. If the resulting aggregation curve was normal, its appearance was similar to that shown in Figure 7.1. If a normal aggregation curve did not result, then the donor's blood could not be used for the aggregation tests, and a new donor was found. A common reason for the decreased platelet activity of the donor was due to ingestion of aspirin within 7 days prior to the extraction of the blood. This usually resulted in an aggregation curve with a single low hump which gradually decayed. Adenosine diphosphate was also occasionally used to test the platelets instead of epinephrine. Both the adenosine diphosphate and epinephrine solutions were placed in ice to help retain their potency between tests.

As another check on the platelet aggregating activity,
Figure 7.1  Typical Epinephrine Aggregation Curve, Test 1, Series B.

Figure 7.2  Typical Horm Collagen Aggregation Curve, Test 2, Series B.
an independant source of collagen was used to test the platelets. One part of the 1 mg/ml stock solution of Horm collagen (Hormon Chemie, Germany) was diluted with 9 parts of the pH 2.8 Horm Buffer. Usually, 5 λ of this solution was added to 500 λ of preheated platelet rich plasma. Normal platelets aggregated after the addition of the collagen, yielding a single humped curve after about 5/8 minutes delay, as shown in Figure 7.2.

The experimental runs of the collagen composite materials were performed in a manner similar to the blood testing procedures. 500 λ of platelet rich plasma and a flea were placed in a siliconized Chrono-Log tube and preheated for two minutes in the water bath (usually during the last part of the previous experimental run). Usually, 20 λ of the experimental material was pipetted into the Chrono-Log tube, and the response of the platelets was monitored by the aggregometer. Often large particles were present in the experimental samples. For such samples, the end of the disposable tip of the pipette was cut off to allow the larger particles to be pipetted. In cases where a large Decrease (in light transmission) due to the Insertion of the Sample (DIS) was anticipated, the baseline of the platelet rich plasma was shifted upwards, approximately 50% of full scale, by shifting the coarse bias one division. At the end of each Run where no platelet reaction appeared, epinephrine was added to check the viability of the platelets.
Periodically, and at the end of each day's experiments, epinephrine and Horm collagen tests were performed. The resulting curves were compared with those generated at the beginning of the experiments, to ascertain whether or not the platelet activity was dwindling. Experiments were generally carried out with the most unreactive samples performed first and the more reactive ones later to lessen any effect of dwindling platelet activity.

Analysis of the Platelet Aggregation Experiments

The experiments were carried out according to the procedure described in the previous section. Table 7.1, in the next section, summarizes the data extracted from the experimental curves. In this table, the word "Test" is reserved for experiments performed with control materials, such as ADP, epinephrine or Horm collagen, while the word "Run" is reserved for experiments performed on materials with unknown platelet aggregation properties, such as the samples described in Chapter V. The word "Check" refers to a test made on platelet rich plasma during a Run; that is, epinephrine is added to platelet rich plasma to which a Sample has already been added. A Check is performed to verify that the platelets are still capable of aggregating in experiments where the presence of the Sample did not initiate this reaction.

The easiest way to illustrate the meaning of the other terms used in the table is pictorially. Figure 7.3 shows a typical platelet aggregation curve. Prior to performing each
Figure 7.3 A typical aggregation curve, showing the definition of terms used in the tables.

Figure 7.4 An aggregation curve with a double hump, typical of epinephrine and ADP test. Both Slopes 1 and 2 are recorded under the Maximum Slope column in the tables.
experiment, the calibration was checked with samples of platelet rich plasma and platelet poor plasma. The range between these samples represents 100% aggregation and is set to approximately 80 divisions on the chart paper, from 20 to 100 divisions. The number of divisions which actually equaled 100% aggregation varied slightly with the individual experiment.

The time lag is defined as the time between the insertion of the sample and the beginning of aggregation. A mark was made on the chart paper (moving at 1 inch/minute) to indicate the time that the sample was pipetted into the platelet rich plasma. Where the beginning of aggregation was difficult to determine specifically, the baseline and slope-lines were extrapolated to define a point.

The asymptote is defined as the maximum value of the curve, with a slope of zero. In cases where there was a slight slope at the time the experiment was terminated, a plus (+) sign was noted after the value of the asymptote in the table to indicate that the curve had not completely leveled off.

The maximum slope is recorded to give an indication of the rapidity of the aggregation. If there were two humps in the curve (Figure 7.4), as often happened with epinephrine curves, the maximum slope for each section was listed.

In some samples, due to their particulate nature, the light transmission decreased upon insertion of the sample. In
these cases, 100% aggregation was said to be equal to the calibrated value, but shifted down by the value of the Decrease due to Insertion of the Sample (DIS). (See Figure 7.5). This simplistic method of correction seemed to work well for small DIS values, but seemed to be misleading for very large values (i.e. 50%), this will be considered more fully in the Discussion. Also, the presence of the particles caused the curve to exhibit a high frequency noise component. This was integrated by eye, where necessary, to yield values for the asymptote and maximum slope. The decrease in light transmission caused by the sample connotes the number of particles inserted. Therefore, the DIS may be considered as an indication of the relative dose, and is used extensively in the analysis of the data.

The duration of the experiment varied and was determined by the operator. When it was felt that no further significant reaction would take place, the experiment was terminated, usually after about 8 minutes for marginally reactive or unreactive samples.

In runs where little or no reaction was observed, epinephrine was added to the PRP to determine whether or not the platelets were still active (this is referred to as a "Check"). The normal response of the platelets to the epinephrine, in spite of the fact that they are contacting the foreign material of the sample, gives the assurance that the platelets are still active. The significance of the normal response is that the material is then said to be bland, a material which
Figure 7.5 An aggregation curve with a Decrease in light transmission due to the Insertion of the Sample (DIS). The baseline shifts down by the value of the DIS, but the magnitude of 100% aggregation is assumed to be unchanged.
neither activates nor inactivates the thrombogenic nature of the platelets. The magnitude of the asymptote of the epinephrine check is recorded as a previous rise due to the sample plus the rise caused by the epinephrine.

Results of the Platelet Aggregation Experiments

Ideally, the results from the platelet aggregation experiments would either be unequivocally positive or negative. However, although many experiments did clearly show platelet aggregation or the lack of aggregation, there were a few samples which did exhibit a weak reaction. The results were, therefore, classified into three categories: reactive, unreactive and marginally reactive, denoted by +, - and ~+.

The reactive samples were relatively easy to determine. The aggregation started almost immediately after the insertion of the sample, proceeded rapidly with a steep slope to a high asymptote and was completed after only a few minutes. Typical values for the time lag, maximum slope, asymptote and duration were 0.5 minutes, 100% / minute, 80% and 6 minutes.

The unreactive samples were also usually straightforward to identify. After the insertion of the sample, a straight line was drawn by the chart recorder indicating no reaction to the material by the platelets (see Figure 7.6). The epinephrine check performed on these materials usually rose to about 70% above the new baseline caused by the insertion of the sample.
Figure 7.6 A typical aggregation curve with an epinephrine check for an unreactive sample. Note that the asymptote of the sample equals zero.

Figure 7.7 A typical aggregation curve with an epinephrine check for a marginally reactive sample. Note that the asymptote of the sample rises above the old baseline.
The marginally reactive samples showed a little more variation in their characteristics. The time lag was usually about 1 minute but could be from 0 to 5 minutes. The maximum slope was often less than 10%/minute but could be as high as 40%/minute. As shown in Figure 7.7, the asymptote was variable but seemed to be 5 to 10% higher than the DIS. Another interesting observation is that the asymptote of the epinephrine check was often about 70% greater than the magnitude of the DIS.

An overview of the reaction of the platelets to the different types of materials, in view of their preparation procedures, yielded a remarkably coherent picture. Tables 7.1 and 7.2 summarize the results from the platelet aggregation experiments and the checks and tests which were performed on the samples. They are ordered approximately in the numerical order of their sample numbers; that is, travelling left to right and top to bottom on Figure 5.1, the Sample Preparation Flow Chart.

Bovine hide collagen in physiological saline (Samples 1.10:0 and 1.10:0'), not surprisingly, aggregated platelets quite strongly. When the bovine hide collagen was first dispersed in water at pH 5.8, air-dried, Wiley-milled and placed in physiological saline (Samples 3.20:1 and 3.20:1'), a strong platelet aggregation resulted. The maximum slope of this sample, when freshly prepared, was much less than that found for the previous sample. Sample 3.20:1a was simply the air-dried Wiley-milled particles used to make sample 3.20:1
which were added, dry, to the platelet rich plasma. Because the sample was not previously wetted with the physiological saline, the platelets could not react well with the material. One other sample was prepared at pH 7. Sample 2.20:1 was prepared by blending bovine hide collagen for 15 minutes at pH 7, forming a membrane, drying, crosslinking in a glutaraldehyde bath, Wiley-milling and dispersing in physiological saline. In spite of this treatment, this sample showed a clearly positive platelet reaction, but with a slightly longer time lag (1.2 minutes) than most reactive samples.

The remainder of the samples used for the platelet aggregation experiments were all initially treated with 0.05M acetic acid at pH 3. A positive platelet aggregation result was elicited by Sample 4.20:1. This sample was prepared by blending bovine hide collagen in 0.05 M acetic acid for 15 minutes, forming a membrane, air-drying, Wiley-milling to a 60 mesh particle size and dispersing in physiological saline. Sample 4.20:2, however, which was prepared by the same procedure except for a glutaraldehyde crosslinking step after forming the membrane, did not cause any platelet aggregation.

More extensive treatment with acetic acid resulted in a decreased tendency for the platelets to react with the material. Sample 4.53:1, which was prepared by blending bovine hide collagen for 1 hour in 0.05 M acetic acid, swelling for 3 days, forming a membrane, air-drying, Wiley-milling and
dispersing in physiological saline, resulted in a classic marginal platelet reaction. The reaction of the platelets to this sample after 9\frac{1}{2} months in physiological saline (Sample 4.53:1') was variable. At lower doses no reaction occurred; at medium doses, a marginal reaction occurred; and at high doses a positive reaction resulted. Sample 4.53:2 was prepared in a similar manner to Sample 4.53:1, except that a crosslinking step was inserted after forming the membrane. Sample 4.53:2 did not cause any platelet reaction even after it had been in contact with physiological saline for 9\frac{1}{2} months (Sample 4.53:2').

One of the dispersions used to make the above samples was also used to make Samples 4.54:0 and 4.57:0. Sample 4.54:0 was made by blending bovine hide collagen for 1 hour and swelling for 5 days in acetic acid. One dispersion prepared in the same manner gave a marginal platelet reaction, but a second dispersion prepared in the same manner gave a strongly positive reaction. Retesting of the marginally reactive dispersion 9\frac{1}{2} months later (Sample 4.57:0), however, gave a strong positive reaction. Dialysis of the 1 hour blended, 3 day swollen dispersion to pH 3 (Sample 4.53:4) and to pH 7 (Sample 4.54:3) also resulted in samples which strongly aggregated platelets.

Chondroitin-6-sulfate was also a component in the preparation procedure of many samples. When dispersions with a ratio of 8 or 12% (w/w) chondroitin-6-sulfate to bovine hide

-253-
collagen (Samples 4.30:1 and 4.20:3) were added to platelet rich plasma, the result was a strong platelet reaction. This reaction was still present when these dispersions were tested 10 months later (Samples 4.30:1' and 4.20:3'). Sample 4.53:5 was prepared by blending bovine hide collagen for 1 hour in 0.05 M acetic acid, swelling for 3 days and adding 12% chondroitin-6-sulfate. One dispersion prepared by this method elicited no platelet response. (This was made from the same dispersion as in Sample 4.54:0, which gave only a marginal reaction). Another dispersion prepared by the identical method, yielded a strongly positive response. However, retesting of the initially unreactive dispersion 9 1/2 months later (Sample 4.53:5'), resulted in a strong platelet aggregation response. Dialysis of Sample 4.53:5 to pH 7.0, forming Sample 4.53:8, also resulted in a platelet aggregating material.

Membranes which were formed from chondroitin-6-sulfate dispersions were less reactive than the dispersions from which they were made. Sample 4.53:6 was made from a 1 hour blended, 3 day swollen, 12% chondroitin-6-sulfate treated bovine hide collagen dispersion which was formed into a membrane, dried, Wiley-milled and dispersed in physiological saline. This sample was initially marginally reactive with platelets, and exhibited this same level of reactivity after 9 1/2 months of contact with physiological saline (Sample 4.53:6'). A membrane prepared in the same manner as 4.53:6 but crosslinked
after the membrane was formed (Sample 4.53:7), was initially unreactive, but became marginally reactive after 9.5 months of contact with physiological saline (Sample 4.53:7'). Sample 4.55:1 was prepared from a 1 hour blended, 2 week swollen, 12% chondroitin-6-sulfate bovine hide collagen pH 3 dispersion, which was formed into a tube, crosslinked, stored in 70% ethanol, dried, Wiley-milled and dispersed in physiological saline. This sample elicited a marginal reaction from most platelet aggregation runs but one experiment caused a positive platelet platelet reaction.

The remaining samples were prepared by adding glutaraldehyde to the collagen while it was still in the dispersed state. The final concentration of glutaraldehyde to the dispersion was set to 0.5% (v/v). The free glutaraldehyde was dialyzed or washed from each of the samples to prevent fixing of the platelets and subsequent erroneous platelet aggregation responses. The epinephrine checks (which were always positive for all previously discussed samples) were used to verify the activity of the platelets. Two checks made on Sample 4.56:9 during Runs 3B/H and 3R/H were the only negative checks encountered. Following dialysis against physiological saline to remove the free glutaraldehyde, this sample was reused in Runs 4B/J and 4R/J, where a negative platelet reaction was recorded. The positive checks on these runs indicated that the platelets were unharmed by the presence of the sample.

Dispersions which were made by blending bovine hide collagen for 1 hour in 0.05 M acetic acid, swelling for 3 days,
adding glutaraldehyde and then either dialyzing to pH 3 (Sample 4.53:14) or to pH 7 (Sample 4.53:13) showed platelet aggregation ability. If chondroitin-6-sulfate was added before dialyzing to pH 3 (Sample 4.53:19) or to pH 7 (Sample 4.53:18) a positive platelet reaction was also exhibited.

The membranes made from glutaraldehyde treated dispersions, however, were quite unreactive. Sample 4.53:11 was made from a 1 hour blended, 3 day swollen glutaraldehyde treated dispersion from which a membrane was formed, air-dried, Wiley-milled and dispersed in physiological saline. Sample 4.53:12 was similar to Sample 4.53:11, except that the membrane was crosslinked in a 5% glutaraldehyde bath. Both Samples 4.53:11 and Sample 4.53:12 were unreactive samples. Samples 4.53:16 and 4.53:17 were made by procedures similar to those for 4.53:11 and 4.53:12 but with the addition of 12% chondroitin-6-sulfate to the dispersion after the glutaraldehyde treatment. Neither Sample 4.53:16 nor Sample 4.53:17 elicited any platelet reaction when added to platelet rich plasma.

The glutaraldehyde treated samples made from 2½ month swollen dispersions showed a similar trend. Sample 4.56:6, a pH 3 2½ month swollen glutaraldehyde treated dispersion, dialyzed to pH 7, and Sample 4.56:10 which was identically prepared except for the addition of 12% chondroitin-6-sulfate after the glutaraldehyde; both caused platelets to aggregate. However, no platelet reaction was caused by a membrane formed
from a glutaraldehyde treated dispersion which was either air-dried (Sample 4.56:4) or crosslinked and air-dried (Sample 4.56:5), before Wiley-milling and dispersing in physiological saline. Similarly, no platelet reaction was caused by Samples 4.56:8 and 4.56:9 which were prepared like Samples 4.56:4 and 4.56:5 but with chondroitin-6-sulfate added to the glutaraldehyde dispersions.

Foam 79013 was prepared by freezedrying a 1 hour blended, 8% chondroitin-6-sulfate bovine hide collagen dispersion. The foam was then dehydrothermally treated, crosslinked with glutaraldehyde, freezedried, Wiley-milled and dispersed in physiological saline. This Foam did not cause any platelet reaction. Foams 79015 and 79016, which were left in the acetic acid for 4 days prior to the addition of chondroitin-6-sulfate, also caused no platelet reaction.

In summary, only bovine hide collagen samples which were prepared from acetic acid swollen dispersions and either air-dried and crosslinked, or glutaraldehyde treated and air-dried, caused no platelet aggregation. All dispersions made at any pH and all membranes made at pH 5.8 or 7 aggregated platelets. Membranes made from acetic acid swollen dispersions which were only air-dried, especially those treated with chondroitin-6-sulfate, exhibited marginal platelet reactivity.
Table 7.1  Platelet Aggregation Experiments

<table>
<thead>
<tr>
<th>Sample Number and Description</th>
<th>Run (#/series)</th>
<th>Performance Short-hand$^1$</th>
<th>Dose DIS$^2$ (%)</th>
<th>Time Lag (min)</th>
<th>Maximum Slope (%/min)</th>
<th>Asymptote (%)</th>
</tr>
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<tbody>
<tr>
<td>1.10:0, BHC$^3$ dispersed in PS$^4$</td>
<td>8/A</td>
<td>+</td>
<td>20</td>
<td>45</td>
<td>0.1</td>
<td>≈250</td>
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<td>+</td>
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<td>0.4</td>
<td>250</td>
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<td>+</td>
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<td>0</td>
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<tr>
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<td>3.20:1a, Sample 3.20:1 added dry</td>
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<td>+</td>
<td>20</td>
<td>16</td>
<td>0.5</td>
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<tr>
<td></td>
<td>1/B</td>
<td>+</td>
<td>20</td>
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<td>4.20:3', Retested 4.20:3 10 months later</td>
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<td>-</td>
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<td>47</td>
<td>2.4</td>
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<td>Sample Number and Description</td>
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<td>Performance Short-Hand</td>
<td>Dose (λ) (%)</td>
<td>DIS (min)</td>
<td>Time Lag (min)</td>
<td>Maximum Slope (%/min)</td>
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<td>4.54:0, pH 3 BHC disp., swollen 5 days</td>
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<tr>
<td>4.57:0, pH 3 BHC disp., swollen 10 months</td>
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<tr>
<td>4.53:2', Retested 9½ months later</td>
<td>5B/C -</td>
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<td>4.53:2', Retested 9½ months later</td>
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<td>--</td>
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<td>4.53:2, pH 3 BHC disp., swollen 3 days, dried, X-linked, WM, in PS</td>
<td>4/B -</td>
<td>20 23</td>
<td>--</td>
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## Platelet Aggregation Experiments

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<th>Sample Number and Description</th>
<th>Performance</th>
<th>Maximum</th>
<th>Asymptote</th>
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<tbody>
<tr>
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<td>Dose ((\lambda))</td>
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<td>4.53:2 9½ months later</td>
<td>7R/C</td>
<td>-</td>
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<tr>
<td>4.53:5', Retested 4.53:5 9½ months later</td>
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<td>-</td>
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<td>4.53:5 9½ months later</td>
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<td>-</td>
<td>40</td>
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<tr>
<td>4.53:8, pH 3</td>
<td>7B/D</td>
<td>-</td>
<td>40</td>
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<tr>
<td>4.53:6, pH 3</td>
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<td>-</td>
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<tr>
<td>4.53:6'</td>
<td>1B/E</td>
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</tr>
<tr>
<td>4.53:6</td>
<td>1R/E</td>
<td>-</td>
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<td>4.56:6', Retested 4.56:6 9½ months later</td>
<td>2B/E</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td>4.56:6 9½ months later</td>
<td>2R/E</td>
<td>~+</td>
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<td>4.53:5, pH 3</td>
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<td>-</td>
<td>40</td>
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<tr>
<td>4.53:5'</td>
<td>2B/J</td>
<td>+</td>
<td>20</td>
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<tr>
<td>4.53:6', Retested</td>
<td>2R/J</td>
<td>+</td>
<td>20</td>
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<tr>
<td>4.53:8, pH 3</td>
<td>6B/E</td>
<td>+</td>
<td>20</td>
</tr>
<tr>
<td>4.53:7'</td>
<td>6R/E</td>
<td>+</td>
<td>20</td>
</tr>
<tr>
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<td>11/A</td>
<td>+</td>
<td>1 piece, 0</td>
</tr>
<tr>
<td>disp., swollen 3</td>
<td>fragmented instantly</td>
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<td></td>
</tr>
<tr>
<td>days, plus 12% C6S, dialyzed to pH 7</td>
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<tr>
<td>4.53:7</td>
<td>3/A</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td>disp., swollen 3</td>
<td>5/A</td>
<td>~+</td>
<td>20</td>
</tr>
<tr>
<td>days, 12% C6S, dried, WM, in PS</td>
<td>6/A</td>
<td>~+</td>
<td>20</td>
</tr>
<tr>
<td>4.56:6', Retested</td>
<td>16/A</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td>4.56:6 9½ months later</td>
<td>5/B</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td>4.53:7</td>
<td>6/B</td>
<td>~+</td>
<td>20</td>
</tr>
<tr>
<td>disp., swollen 3</td>
<td>7/B</td>
<td>~+</td>
<td>20</td>
</tr>
<tr>
<td>days, 12% C6S, dried, X-linked, WM, in PS</td>
<td>8/B</td>
<td>~+</td>
<td>20</td>
</tr>
<tr>
<td>4.53:7</td>
<td>9/B</td>
<td>~+</td>
<td>40</td>
</tr>
<tr>
<td>4.53:7', Retested 4.53:7 9½ months later</td>
<td>6B/D</td>
<td>~+</td>
<td>20</td>
</tr>
<tr>
<td>4.53:7 9½ months later</td>
<td>6R/D</td>
<td>~+</td>
<td>20</td>
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<tr>
<td>4.53:7, pH 3</td>
<td>7/A</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td>disp., swollen 3</td>
<td>12/B</td>
<td>-</td>
<td>20</td>
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(continued)
Table 7.1 Platelet Aggregation Experiments

<table>
<thead>
<tr>
<th>Sample Number and Description</th>
<th>Run (/#/series)</th>
<th>Performance Short-hand</th>
<th>Dose ((\mu))</th>
<th>DIS (%)</th>
<th>Time Lag (min)</th>
<th>Maximum Slope (%/min)</th>
<th>Asymptote (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.53:14, pH 3 BHC disp., swollen 3 days, Glut, dialyzed to pH 3</td>
<td>6/I +</td>
<td>20</td>
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<tr>
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<td>9/I +</td>
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<td>0</td>
<td>96</td>
<td>99</td>
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</tr>
<tr>
<td>4.53:11, pH 3 BHC disp., swollen 3 days, Glut, dried, WM, in PS</td>
<td>7B/J -</td>
<td>40</td>
<td>38</td>
<td>(\infty)</td>
<td>0</td>
<td>0</td>
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</tr>
<tr>
<td>4.53:12, pH 3 BHC disp., swollen 3 days, Glut, dried, X-linked, WM, in PS</td>
<td>5B/J -</td>
<td>40</td>
<td>38</td>
<td>(\infty)</td>
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<td>85</td>
<td>88</td>
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<td>4.55:1, pH 3 BHC disp., swollen 2 weeks, formed tube, 2B/F X-linked, stored in 2R/F ethanol, dried, WM, in PS</td>
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<td>4.8</td>
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<td>Dose</td>
<td>DIS</td>
<td>Time Lag</td>
<td>Maximum</td>
<td>Asymptote</td>
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<td>(x)</td>
<td>(%</td>
<td>(min)</td>
<td>Slope (%/min)</td>
<td>(%)</td>
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<td>4B/J</td>
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<tr>
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<td>3R/E</td>
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<td>20</td>
<td>5</td>
<td>∞</td>
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<td>Foams 79015 &amp; 79016, pH 3 BHC disp., swollen 4 days, 8% C6S, centrifuged</td>
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<td>6</td>
<td>∞</td>
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Footnotes

1 Performance Shorthand: + for a strong platelet reaction, ~+ for a weak (or marginal) platelet reaction, and - for no platelet reaction.

2 DIS: Decrease of light transmission due to Insertion of Sample.

3 BHC: Bovine Hide Collagen.

4 PS: Physiological Saline.

5 WM: Wiley Milled.

6 X-linked: Crosslinked in a glutaraldehyde bath.

7 disp.: Dispersion. Unless otherwise noted, pH 3 collagen dispersions are made at 1% (w/v) ratios; that is, 0.25 gm (dry weight) of collagen per 100 ml of 0.05 M acetic acid.

8 C6S: Chondroitin-6-Sulfate. The percentage refers to the ratio of the weight of chondroitin-6-sulfate to the weight of the collagen.

9 Glut: Glutaraldehyde added to the dispersion to make the final concentration 0.5% (v/v).

10 FD: Freeze-dried.

11 DHT: Dehydrothermal Treatment. This treatment consists of placing the material in a vacuum oven at 105°C for 24 hours.
## Table 7.2 Platelet Aggregation Tests and Checks

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Platelet Aggregation Tests and Checks

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<td>1st 25</td>
<td>0+64</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C8B/J</td>
<td>+</td>
<td>20 R 35</td>
<td>0</td>
<td>1st 40</td>
<td>0+69</td>
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<tr>
<td></td>
<td>C8R/J</td>
<td>+</td>
<td>20 R 40</td>
<td>0</td>
<td>1st 29</td>
<td>0+61</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T3/J</td>
<td>+</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>1st 29</td>
<td>62</td>
</tr>
</tbody>
</table>

Horm Collagen (1 mg/ml)

<table>
<thead>
<tr>
<th>Performance Shorthand</th>
<th>Dose (µm)</th>
<th>DIS (%)</th>
<th>Time Lag (min)</th>
<th>Maximum Slope (%/min)</th>
<th>Asymptote (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2/B</td>
<td>+</td>
<td>5</td>
<td>0</td>
<td>0.6</td>
<td>110</td>
</tr>
<tr>
<td>T5/B</td>
<td>+</td>
<td>5</td>
<td>0</td>
<td>0.9</td>
<td>101</td>
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<tr>
<td>T2/C</td>
<td>+</td>
<td>5</td>
<td>0</td>
<td>0.6</td>
<td>85</td>
</tr>
<tr>
<td>T2/D</td>
<td>+</td>
<td>5</td>
<td>0</td>
<td>0.4</td>
<td>94</td>
</tr>
<tr>
<td>T4/D</td>
<td>+</td>
<td>20</td>
<td>0</td>
<td>0.7</td>
<td>59</td>
</tr>
<tr>
<td>T5/D</td>
<td>+</td>
<td>20</td>
<td>0</td>
<td>0.5</td>
<td>124</td>
</tr>
<tr>
<td>T2/E</td>
<td>+</td>
<td>20</td>
<td>0</td>
<td>0.6</td>
<td>98</td>
</tr>
<tr>
<td>T4/E</td>
<td>+</td>
<td>20</td>
<td>0</td>
<td>0.6</td>
<td>65</td>
</tr>
<tr>
<td>T4/F</td>
<td>+</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>144</td>
</tr>
<tr>
<td>C2B/F</td>
<td>+</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>C2R/F</td>
<td>+</td>
<td>20</td>
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Table 7.2  Platelet Aggregation Tests and Checks

<table>
<thead>
<tr>
<th>Testing Material</th>
<th>Test or Check (#/series)</th>
<th>Performance Shorthand</th>
<th>Dose (x)</th>
<th>DIS (%)</th>
<th>Time Lag (min)</th>
<th>Maximum Slope (%/min)</th>
<th>Asymptote (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horm Collagen</td>
<td>T6/F</td>
<td>+</td>
<td>20</td>
<td>0</td>
<td>0.5</td>
<td>138</td>
<td>90</td>
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<tr>
<td>0.1 mg/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>T2/G</td>
<td>+</td>
<td>10</td>
<td>0</td>
<td>0.6</td>
<td>158</td>
<td>75</td>
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<td></td>
<td>T3/G</td>
<td>+</td>
<td>5</td>
<td>0</td>
<td>0.7</td>
<td>185</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>T4/G</td>
<td>+</td>
<td>2½</td>
<td>0</td>
<td>0.7</td>
<td>169</td>
<td>83</td>
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<tr>
<td></td>
<td>T6/G</td>
<td>+</td>
<td>5</td>
<td>0</td>
<td>1.0</td>
<td>71</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>T2/H</td>
<td>+</td>
<td>5</td>
<td>0</td>
<td>0.7</td>
<td>46</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>T3/H</td>
<td>+</td>
<td>20</td>
<td>0</td>
<td>0.3</td>
<td>112</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>T5/H</td>
<td>+</td>
<td>20</td>
<td>0</td>
<td>0.4</td>
<td>123</td>
<td>75</td>
</tr>
<tr>
<td>BHC in PS</td>
<td>T3/I</td>
<td>+</td>
<td>20</td>
<td>5</td>
<td>0.1</td>
<td>55</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>T4/I</td>
<td>+</td>
<td>20</td>
<td>20</td>
<td>0</td>
<td>70</td>
<td>86</td>
</tr>
<tr>
<td>ADP 5 x 10⁻⁴ molar in PS</td>
<td>T3/B</td>
<td>+</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>1st 108</td>
<td>86</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>2nd 18</td>
<td></td>
</tr>
</tbody>
</table>

1. Performance Shorthand: + for a strong platelet reaction, ~+ for a weak (or marginal) platelet reaction, and - for no platelet reaction.

2. DIS: Decrease of light transmission due to Insertion of Sample.

3. The asymptotes of the epinephrine checks are given as the asymptote of the run just prior to the addition of the epinephrine plus the increase caused by the epinephrine check.

4. T: stands for "Test". "Test" denotes experiments which were carried out using control materials such as epinephrine, Horm collagen and ADP.

5. C: stands for "Check". "Check" denotes a Test made on platelet rich plasma to which a sample had already been added.

6. R: Indicates that the DIS values given for epinephrine checks are the DIS values for the insertion of the sample at the beginning of the Run. The DIS for all epinephrine samples is zero.

7. BHC: Bovine Hide Collagen.

8. PS: Physiological Saline.

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Discussion of the Platelet Aggregation Experimental Results

The purpose of the platelet aggregation experiments is not only to determine which materials are non-thrombogenic, but also which procedures in the production of these materials are responsible for creating a non-thrombogenic material. Since the raw material of the samples is clearly thrombogenic and the finished product is non-thrombogenic, a logical comparison of the reactivities of the intermediate materials should yield valuable information.

Before entering into a discussion of the critical steps in the material preparation, an examination of the meaning of each type of test result is in order. As mentioned previously, the platelet aggregation results are classified as reactive (+), unreactive (−) or marginally reactive (∼+). A reactive material is one which clearly and immediately causes platelet aggregation and is therefore unsuitable for use in vascular prostheses. An unreactive material is one which does not interact with platelets in anyway; it neither hinders nor enhances the platelet aggregation reaction. This is a desirable material for vascular prostheses, since it will not initiate the coagulation cascade and will not impair the response of the platelets to a trauma.

The response which marginally reactive samples elicit from platelets is a little more difficult to decipher. There are several relevant experimental observations. First, epinephrine tests result in asymptotes averaging 78%. Second,
unreactive samples cause asymptotes of less than a few percent. Third, the epinephrine checks made on unreactive samples rise to a level averaging 70% above the new baseline caused by the insertion of the sample. This asymptote is independant of the DIS value. Fourth, the marginally reactive samples cause a rise to an intermediate asymptote which is about 5% more than the value of the DIS. And finally, the checks performed on the marginally reactive samples rise to asymptotes of approximately DIS = 70%. Figure 7.6 should also be recalled, which shows that the old baseline, established before the addition of the sample, is at a level equal to the DIS value above the new baseline, established after the insertion of the sample. It is believed that these facts show that the platelets respond to the marginally reactive samples by adhering to the sample particles without releasing the contents of their granules.

Figure 7.8 shows the relationship of the asymptote of the light transmittance response of the platelet rich plasma to the presence of the sample versus the decrease in light transmission due to the insertion of the sample (DIS). This plot uses the data from all 18 different types of particulate samples which exhibited marginally reactive or unreactive experimental runs. This graph shows that virtually all of the points either fall above the asymptote=DIS line (the marginally reactive runs), or below the asymptote=11% line (the unreactive runs). Initially, experiments were performed
Figure 7.3  Asymptote versus the Decrease of light transmission due to the Insertion of the Sample (DIS) for marginally reactive and unreactive runs of particulate samples.
at low values of DIS, where a distinction between the marginally reactive and unreactive samples cannot be made. Only by giving massive doses of the samples to the platelet rich plasma can a clear distinction between the two types of samples be made. It was found that the range of DIS values between 20% and 45% are the best to use for differentiating between marginally reactive and unreactive samples.

The intermediate response of the platelets to a marginally reactive material is not a typical response. Normally, platelets either ignore their surroundings completely, or respond to a stimulus by aggregating immediately and completely. For example, the response of platelets to reactive samples averages over 80% regardless of the DIS, and the response of platelets to epinephrine averages 78%.

The response of the platelets to the marginally reactive samples is also quite slow. Many minutes are required before the final asymptote is reached. The response of the platelets to the marginally reactive samples is, therefore, quite different than the response elicited by low doses of ADP. A low dose of ADP can cause a rapid rise to an intermediate peak, but this is followed by a gradual return to the baseline. The response of the platelets in this case is actually aggregation caused by the extracellular ADP, followed by disaggregation since the granules did not release any more ADP. This is clearly a different response than the slow steady rise to an asymptote which is produced by marginally
reactive samples.

Figure 7.9 shows the asymptotes of the epinephrine checks of the marginally reactive and unreactive samples. The trend of the checks of the unreactive samples is to remain roughly constant, with respect to the DIS values, at about 70%. On the other hand, the trend for the asymptotes of the checks on marginally reactive samples is upward, roughly centered on the asymptote = (DIS+70%) line.

The fact that the unreactive samples do not create any significant asymptote is the first sign of their bland nature toward platelets. The second indication is that the asymptotes of the epinephrine checks are very nearly the same as for the epinephrine tests. This means that, in spite of the presence of the sample particles, the platelets aggregate independently, without significant interaction with the sample particles. The only effect which the unreactive samples have is to increase the background optical density, resulting in a shift of the baseline downward.

The experimental observations of marginally reactive samples indicate that there must be some interaction between the platelets and the sample particles. The first indication that there is some interaction is the fact that the asymptote rises to about 12% higher than the old baseline. This could only occur if some platelets either aggregated or were removed from the platelet rich plasma. A separate test of a marginally reactive sample in platelet poor plasma showed that
Figure 7.9 Asymptote of the Epinephrine Checks versus the Decrease of light transmission due to the Insertion of the Sample (DIS) for marginally reactive and unreactive runs of particulate samples.
the sample particles themselves showed no propensity to aggregate or otherwise cause an increase in light transmission from the new baseline. The second indication that the platelets and particles must be interacting is the fact that the asymptotes of the epinephrine checks are at about 70% above the old baseline. In other words, the asymptotes of the epinephrine checks are at values which would be expected if the sample particles were not present or had in some way aggregated. For example, Sample 4.53:1 during Run 10/B, exhibited a DIS of 27%, an asymptote of 37% and an asymptote for the epinephrine check of 106%. Since the asymptote of the check exceeds 100%, the involvement of the sample particles in the aggregation is certain. The asymptote of this check is 79% above the old baseline, which is typical of an epinephrine test (that is epinephrine in platelet rich plasma with no sample added prior to the epinephrine).

An explanation for these facts is provided if one assumes that the platelets adhere to the marginally reactive sample particles but do not release the contents of their granules. The adhesion of the platelets to the particles would explain the slower rise to an intermediate asymptote. The adhesion process would not be autocatalytic, unlike an aggregation reaction, and would therefore proceed at a slower rate. The dependence of the magnitude of the asymptote upon the DIS could also be explained. The platelets would adhere to the surface of the particles at an approximately constant density
per unit area. The area of the surface of the sample particles is dependant upon the number of sample particles, which, in turn, is indicated by the DIS value. The number of platelets which adhere to the sample particles and disappear from the path of the light, would, therefore, be dependent upon the DIS value.

A large asymptote for the epinephrine check would also be expected if the platelets first adhered to the sample particles. The addition of epinephrine would then precipitate the aggregation reaction, causing the platelets on the sample particles to adhere to platelets on other sample particles, in effect creating aggregates of sample particles. The free platelets would aggregate amongst themselves as well as with platelets already adhering to the sample particles. The net effect of the aggregation of the sample particles would be to cause a much smaller optical density due to the sample particles than was originally presented by the DIS. This would effectively raise the baseline almost to the level of the old baseline and result in a much higher asymptote for the epinephrine check than would be anticipated without platelet-particle interactions. Silver (1977) presents some electron micrographic data which suggests that the adhesion of platelets without aggregation has been observed on materials similar to the marginally reactive materials in this thesis.

The marginally reactive materials are probably not desirable for use as vascular prostheses, but understanding the
response of the platelets to these materials aids in the determination of truly unreactive samples. The zero or very low asymptotes of the unreactive samples indicate that the platelets are not adhering to the sample particles or releasing the contents of their granules to initiate aggregation. The asymptotes of the epinephrine checks on truly unreactive samples are independent of the DIS values, further strengthening the claim of unreactivity. The asymptotes of the checks average about 70% above the new baseline, indicating that the platelets aggregate independently, without interacting with the sample particles. The differentiation between the marginally reactive and unreactive samples by the use of an epinephrine check, helps to state with certainty that the unreactive samples neither cause adhesion nor aggregation of platelets.

The understanding of the response of the platelets to the various types of materials clears the way for a discussion of the critical steps in the material preparation procedures. The steps used for sample preparation are listed below in the order which they would be carried out. Of course, not all of these steps would be used on any one sample. The sample preparation begins by Wiley-milling the raw bovine hide collagen to 60 mesh sized particles, followed by blending in water (pH 7.0 or pH 5.8), physiological saline (pH 7.0) or 0.05 M acetic acid (pH 3.0). The blending is performed for up to 1 hour followed by static swelling at 4°C for up to 10 months.
Glutaraldehyde is added to some dispersions to make the concentration of glutaraldehyde 0.5% (v/v). Chondroitin-6-sulfate can also be added at this point in ratios of 8% or 12% (w/w) with respect to the dry weight of collagen in the dispersion. At this point some dispersions were dialyzed to pH 3 or pH 7 to remove free glutaraldehyde before immediate use in the platelet aggregation tests. Many dispersions were filtered on a Buechner funnel to create thin membranes. These membranes were air-dried, or crosslinked in 0.5% or 5% glutaraldehyde baths before air-drying. These dried membranes were Wiley-milled and dispersed in physiological saline to facilitate platelet aggregation testing. Some samples were exposed to physiological saline for long periods of time to simulate a long term residence of the material in the body.

The results of the platelet aggregation experiments show that three steps in the fabrication procedure are necessary to eliminate the thrombogenic nature of the collagen. The required steps are swelling in acetic acid, air-drying and glutaraldehyde crosslinking. It appears that the order of the air-drying and glutaraldehyde crosslinking steps can be interchanged, but the acid swelling must always precede both of these steps.

The importance of the fabrication steps is realized by first comparing Samples 4:20:2, 4:20:1, and 2:20:1. Sample 4:20:2 was prepared by blending bovine hide collagen for 15 minutes in 0.05 M acetic acid, forming a membrane,
crosslinking in glutaraldehyde, air-drying, Wiley-milling and dispersing in physiological saline. Sample 4.20:2 was unreactive. Sample 2.20:0 was prepared by the same method, but used water as the dispersing medium. Since this sample was reactive, a comparison between the two shows the importance of dispersing the bovine hide collagen in an acid medium. Sample 4.20:1 was prepared in a manner similar to Sample 4.20:2 which only omitted the glutaraldehyde crosslinking step. Since Sample 4.20:1 also caused platelet aggregation, the importance of the glutaraldehyde treatment is also evident.

The air-drying process is also recognized as an important step after examining Samples 4.53:14 and 4.53:11. Sample 4.53:14 was made from a 1 hour blended, 3 day swollen pH 3 bovine hide collagen dispersion to which glutaraldehyde was added, followed by a dialysis at pH 3 to remove the free glutaraldehyde. Although this sample contained the important acid swelling and glutaraldehyde treatment steps, it still caused platelets to aggregate. Sample 4.53:11, was made by forming and air-drying a membrane made from the same dispersion, prior to its dialysis, followed by Wiley-milling and dispersing in saline. This sample elicited no detectable reaction from viable platelets, confirming the importance of the air-drying step.

It is interesting to note that air-drying, alone, can retard the platelet aggregation reaction. Sample 1.10:0,
which is simply Wiley-milled bovine hide collagen dispersed in physiological saline, educed a much more rapid platelet aggregation response than Sample 3.20:1. Sample 3.20:1 was made by blending bovine hide particles in water for 15 minutes, forming a membrane, air-drying, Wiley-milling and dispersing in physiological saline. Both of these samples caused platelet aggregation, but the maximum slope of the aggregation curve of Sample 1.10:0 was five times the maximum slope of Sample 3.20:1. The air-drying appears to be the step responsible for the decreased reaction rate of the platelet aggregation process.

Increasing the residence time of the collagen in the acetic acid also seems beneficial. Samples 4.20:1 and 4.20:2, described earlier, were blended for only 15 minutes in acetic acid, and the filtering of the membrane was completed in less than 1½ hours. Even with this short residence time in acetic acid, the crosslinked sample, Sample 4.20:2, showed a negligible platelet reaction. However, the non-crosslinked sample, Sample 4.20:1, evoked an emphatic aggregation response. A similar pair of samples, Samples 4.53:2 and 4.53:1, were prepared by blending bovine hide collagen in acetic acid for 1 hour and swelling for 3 days before forming the membrane and either crosslinking and air-drying or simply air-drying. The finished membranes were Wiley-milled and dispersed in physiological saline, as usual, for use in the platelet aggregation experiments. Sample 4.53:2, the crosslinked
sample, gave no indication of causing platelet aggregation, even after 9½ months of contact with physiological saline. Sample 4.53:1, the air-dried sample, elicited only a marginal platelet reaction, initially and after 9½ months of contact with physiological saline. Comparing the positive reaction of Sample 4.20:1 with the marginal reaction of Sample 4.53:1, yields the conclusion that the additional 3 days of swelling in acetic acid were beneficial in reducing the thrombogenic nature of the bovine hide collagen.

The comparison of Samples 4.53:2 and 4.53:11 shows the order of glutaraldehyde treatment and air-drying steps can be interchanged. Sample 4.53:2 was first formed into a membrane before crosslinking with glutaraldehyde. Sample 4.53:11 was made by adding glutaraldehyde to the acid swollen dispersion and then air-drying. Both samples showed no tendency to aggregate platelets, indicating that the order of the air-drying and crosslinking are unimportant, but that both must follow the acid swelling step.

The presence of chondroitin-6-sulfate does not seem to be a positive factor in creating a non-thrombogenic material from bovine hide collagen. There is some conflicting evidence, however. The first platelet aggregation tests of a 1 hour blended, 5 day swollen bovine hide collagen dispersion, Sample 4.54:0; and a 1 hour blended, 3 day swollen dispersion with 12% chondroitin-6-sulfate added, Sample 4.53:5, were marginally reactive and unreactive, respectively. Retesting
the samples 9.5 months later as Samples 4.57:0 and 4.53:5' resulted in positive tests. New dispersions, which were made using the identical procedures for Samples 4.54:0 and 4.53:5, caused aggregation of the platelets. The reason for this discrepancy has not been determined, and it has not been possible to repeat the negative platelet response to a dispersion containing chondroitin-6-sulfate. Samples 4.20:3 and 4.30:1 are two more dispersions containing chondroitin-6-sulfate, added after a short period of swelling in acid, which also caused platelet aggregation.

The effect of chondroitin-6-sulfate in a membrane can be determined by comparing Samples 4.53:1, 4.53:2, 4.53:6, 4.53:7 and their primed counterparts, representing retesting of the same samples after 9.5 months in physiological saline. Sample 4.53:1 was made by blending bovine hide collagen for 1 hour, swelling for 3 days, forming a membrane, air-drying and dispersing in physiological saline. Sample 4.53:2 was made by the same procedure with the addition of glutaraldehyde crosslinking after forming the membrane. Sample 4.53:6 and 4.53:7 were made following procedures parallel to Samples 4.53:1 and 4.53:2, but with the addition of 12% (w/w) chondroitin-6-sulfate to the 3 day swollen dispersion. Sample 4.53:1 elicited a marginally reactive response from the platelets, as did Sample 4.53:1'. Samples 4.53:2 and 4.53:2' both caused no platelet aggregation. The platelet aggregation responses to Samples 4.53:6, and 4.53:7 were also
marginally reactive and unreactive, respectively. The comparison between the samples treated with chondroitin-6-sulfate and those which were not, seems to indicate that the presence or absence of the chondroitin-6-sulfate is unimportant. However, although Sample 4.53:6', an air-dried membrane containing chondroitin-6-sulfate which was in contact with physiological saline for 9½ months, remained marginally reactive, Sample 4.53:7' was more reactive than 4.53:7. Sample 4.53:7', a crosslinked membrane containing chondroitin-6-sulfate which was in contact with physiological saline for 9½ months, was marginally reactive, whereas Sample 4.53:7 did not evoke a platelet reaction. It appears that the 9½ months of contact with physiological saline, simulating long term residence in the body, caused a return of the thrombogenic nature of the collagen. Since this did not happen in Samples 4.53:2 and 4.53:2' which were free of chondroitin-6-sulfate, this indicates that the presence of chondroitin-6-sulfate may actually be detrimental to maintaining a non-thrombogenic bovine hide collagen material.

The chondroitin-6-sulfate may be desirable if it is used to de-swell the collagen to a limited extent, allowing a greater density of crosslinks when exposed to glutaraldehyde. All membranes derived from dispersions 4.53:10 and 4.56:3 caused no platelet aggregation, regardless of whether or not chondroitin-6-sulfate was present. The success of these membranes is due to the addition of glutaraldehyde to the
dispersion prior to the addition of chondroitin-6-sulfate or forming a membrane. Sample 4.53:11 was made by blending bovine hide collagen for 1 hour, swelling for 3 days, adding glutaraldehyde to the dispersion, making a membrane, air-drying, Wiley-milling and dispersing in physiological saline. Sample 4.53:12 contained a second treatment with 5% glutaraldehyde after forming the membrane. Samples 4.53:16 and 4.53:17 were prepared in a similar manner to Samples 4.53:11 and 4.53:12, but with the addition of chondroitin-6-sulfate to the dispersion after the glutaraldehyde. All of these samples cause no platelet reaction and strictly from a thrombogenic point of view, all would be acceptable for use as vascular prostheses.

The preparation of Samples 4.56:4, 4.56:5, 4.56:8 and 4.56:9 corresponds to the procedure used for Samples 4.53:11, 4.53:12, 4.53:16 and 4.53:17 except that a 9 to 12 week swelling time was used prior to the addition of glutaraldehyde. All of these samples, again, caused no platelet aggregation. The use of chondroitin-6-sulfate in the above samples neither enhanced nor hindered the non-thrombogenic nature, because of the prior addition of glutaraldehyde. Also, since the presence of free chondroitin-6-sulfate, alone, is unimportant to platelets, it appears that the use of chondroitin-6-sulfate could be justified for reasons of strength without jeopardizing non-thrombogenicity.

Sample 4.55:1 was made by blending bovine hide collagen
for 1 hour, swelling for 2 weeks, adding 12% chondroitin-6-
sulfate, forming a tube and crosslinking in glutaraldehyde.  
At this point the tube was stored in a 70% ethanol and 30%  
water solution to prevent bacterial growth. Subsequently  
the tube was air-dried, Wiley-milled and dispersed in physio-
logical saline. Although this preparation was very similar  
to Sample 4.53:7, which elicited no platelet reaction, Sample  
4.53:1 caused a marginal platelet reaction in most experiments  
and a platelet aggregation in one experiment. Another sample  
which was stored in ethanol after preparation by a coworker,  
was tested by the author (this sample is not listed in Table  
7.2) and unexpectedly gave a marginal platelet reaction.  
Although the evidence is not conclusive, the storage of these  
samples in ethanol may have been detrimental to their non-
thrombogenic character.

The foams, which were tested, are interesting materials  
because of their freeze-drying and dehydrothermal treatment 
steps. Foam 79013 was made from a 1 hour blended bovine hide 
collagen dispersion to which 8% chondroitin-6-sulfate was  
added, followed by centrifugation and freeze-drying. The de-
hydrothermal treatment was performed next. It consists of  
heating the dried material to 105°C in a vacuum to further  
remove the residual water. This process is, in effect, an  
ultimate form of the air-drying step used for most samples.  
The foam was then crosslinked in a glutaraldehyde bath,  
freezedried, Wiley-milled and dispersed in physiological
saline. Foams 79015 and 79016 were prepared in the same fashion, but with the insertion of a 4 day swelling period before the addition of the chondroitin-6-sulfate. All three of these samples contained the steps believed to be essential for a non-thrombogenic material: acid swelling, air-drying or dehydrothermal treatment, and glutaraldehyde crosslinking. Not surprisingly, these foams caused no platelet reaction, and consequently, conformed with and supported the experimental results of the other samples.

When viewed as a whole, the platelet aggregation experiments present a thoroughly coherent picture. The differentiation between marginally reactive samples and unreactive samples allows the assurance that platelets neither adhere to the unreactive samples nor aggregate in their presence. The experiments show that three steps: acid swelling, air-drying and glutaraldehyde treatment, are necessary to convert bovine hide collagen to a non-thrombogenic material. The glutaraldehyde treatment may precede the air-drying, but both must follow the acid swelling step. A longer swelling time in acid seems beneficial. The air drying step can probably be replaced by the more severe dehydrothermal treatment. Chondroitin-6-sulfate is probably slightly detrimental to the non-thrombogenic nature of the bovine hide collagen due to its de-swelling effect. The adverse effects of chondroitin-6-sulfate can be eliminated if the dispersion is first treated with glutaraldehyde before the addition of chondroitin-
6-sulfate. And finally, there is a possibility that storing the collagen materials in a 70% ethanol solution is detrimental to their non-thrombogenic character. The relationship of these conclusions to other findings such as the transmission electron micrographic appearance of the samples, will be discussed in the final chapter.

Comparison of the Platelet Aggregation Experimental Results

The discussion of the platelet aggregation results would not be complete without a comparison to earlier results. Silver (1977) performed most of the previous testing of possible non-thrombogenic materials for this laboratory. Table 7.3 summarizes the experiments that Silver performed on bovine hide collagen materials. A few conversions were necessary to compare the data presented by Silver with the data in this report. The platelet aggregation curves of Silver are shown in light transmission units of 1 to 9, corresponding to 0% and 100% aggregation. Although not specifically stated by Silver, $M_c$ (Molecular weight between crosslinks; a low number means a high crosslink density) values of approximately 20,000 were made by dehydrothermal treatment, and $M_c$ values of approximately 2,000 were made by gluteraldehyde crosslinking. A further complication is that no mention has been made of the relative dosage used for the particulate samples. Also, a 20-mesh particle size was used, which is too large to easily enter the pipette tip, thereby ensuring relatively low doses. In spite of the different presentation
### Table 7.3 Summary of Platelet Aggregation Results of Silver

<table>
<thead>
<tr>
<th>Description of Sample</th>
<th>Figure Number and symbol of Samples (Silver)</th>
<th>Approximate Corresponding Sample Number (Forbes)</th>
<th>Dose (x)</th>
<th>Time Lag (min)</th>
<th>Maximum Slope (%/min)</th>
<th>Asymptote (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHC&lt;sup&gt;4&lt;/sup&gt;, WM&lt;sup&gt;5&lt;/sup&gt;, in S&lt;sup&gt;6&lt;/sup&gt;</td>
<td>3.34 ○</td>
<td>1.10:0</td>
<td>UN&lt;sup&gt;7&lt;/sup&gt;</td>
<td>0.5</td>
<td>40</td>
<td>50</td>
</tr>
<tr>
<td>BHC blended 2 min at pH 3, cast&lt;sup&gt;8&lt;/sup&gt;, WM, in S</td>
<td>3.34 ○</td>
<td>4.20:1</td>
<td>UN</td>
<td>UN</td>
<td>10</td>
<td>18</td>
</tr>
<tr>
<td>BHC blended 2 min at pH 3, cast, DHT&lt;sup&gt;9&lt;/sup&gt;, WM, in S</td>
<td>3.39 △</td>
<td>4.20:1</td>
<td>UN</td>
<td>UN</td>
<td>14</td>
<td>19</td>
</tr>
<tr>
<td>BHC blended 2 min at pH 3, cast, X-linked&lt;sup&gt;10&lt;/sup&gt;, WM, in S</td>
<td>3.39 △</td>
<td>4.20:2</td>
<td>UN</td>
<td>UN</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BHC blended 2 min at pH 3, 1.8% C6S&lt;sup&gt;11&lt;/sup&gt;, dried&lt;sup&gt;12&lt;/sup&gt;, WM, in S</td>
<td>3.36 ○</td>
<td>4.20:1</td>
<td>UN</td>
<td>UN</td>
<td>12</td>
<td>22</td>
</tr>
<tr>
<td>BHC blended 2 min 3.36 x at pH 3, 6% C6S, dried, WM, in S</td>
<td>--</td>
<td>--</td>
<td>UN</td>
<td>UN</td>
<td>13</td>
<td>9</td>
</tr>
<tr>
<td>BHC blended 2 min at pH 3, 10% C6S, dried, DHT, WM, in S</td>
<td>3.41 x</td>
<td>[4.53:6]</td>
<td>UN</td>
<td>UN</td>
<td>10</td>
<td>18</td>
</tr>
<tr>
<td>BHC blended 2 min at pH 3, 10% C6S, dried, X-linked, WM, in S</td>
<td>3.41 ○</td>
<td>[4.53:7]</td>
<td>UN</td>
<td>UN</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BHC blended 2 min at pH 3, 15% C6S, dried, DHT, WM, in S</td>
<td>3.41 △</td>
<td>[4.53:6]</td>
<td>UN</td>
<td>UN</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>BHC blended 2 min at pH 3, 15% C6S, dried, X-linked, WM, in S</td>
<td>3.41 □</td>
<td>[4.53:1]</td>
<td>UN</td>
<td>UN</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
(continued) Table 7.3 Summary of Platelet Aggregation Results of Silver

Footnotes


2 The figure numbers and symbols correspond to those used by Silver in the above reference.

3 The samples in this report which relate closely to those made by Silver are listed in this column. The numbers enclosed by parentheses are only distantly related.

4 BHC: Bovine Hide Collagen

5 WM: Wiley milled to a 20 mesh particle size.

6 S: Saline, 0.9% w/v NaCl in water, pH not specified.

7 UN: Unknown

8 cast: Formation of a membrane by allowing the solvent from a dispersion to evaporate completely.

9 DHT: Dehydrothermal treatment. Consists of placing a membrane in a vacuum oven at 90°C to 120°C for 1 to 2 days.

10 X-link: Consists of immersion of membrane in a 0.025% glutaraldehyde bath at pH 7.4.

11 C6S: Chondroitin-6-sulfate. Dissolved in 0.05 M acetic acid and added to the dispersion over 2 minutes of blending.

12 dried: Formation of a membrane by filtering on a Buechner funnel and air-drying.
of the data, the platelet aggregation experiments of Silver support the conclusions of the previous section.

The control sample of bovine hide collagen in saline caused a fairly sluggish platelet reaction, as the asymptote was only 50% and the maximum slope was only 40%/minute. This may have been due to a low dose, since the same preparation from the same raw material gave a strong platelet aggregation response.

Silver's samples 3.34x, 3.390, 3.39A, 3.360, 3.36x, 3.41x and 3.41A were all prepared from a 0.05M acetic acid dispersion that was blended only 2 minutes. Chondroitin-6-sulfate was added to the last 5 samples in ratios ranging from 1.8%(w/w) to 15%(w/w) with respect to collagen. All of these samples were then cast or formed into membranes, air-dried or dehydrothermally treated, Wiley milled, and dispersed in saline. Casting a membrane means simply allowing the solvent to completely evaporate from a dispersion, forming a film. Forming a membrane means using a Buechner funnel to de-water the dispersion followed by air-drying to form a membrane.

The asymptotes of these samples average 19%. The low asymptotes of these samples, coupled with their typical maximum slope of 12%/minute, are highly suggestive of the platelet adhesion reaction believed to occur in marginally reactive samples. The marginal platelet reaction is a typical response to bovine hide collagen samples that have been exposed to acetic acid and air-dried, but have not been crosslinked with
glutaraldehyde, such as Samples 4.53.1 and 4.53.2. It is remarkable that these samples elicited only a marginal reaction, as the exposure of the bovine hide collagen to acetic acid was very brief. The asymptote of Silver's samples do not seem to suggest any dependence upon the chondroitin-6-sulfate content. For example, 3.36x, which contains only 6% chondroitin-6-sulfate and is only air-dried, has an asymptote of 9%, which is only half of that for 3.41x, which contains 10% chondroitin-6-sulfate and was dehydrothermally treated. The variation between these two experiments cannot be considered significant since the relative dose is not stated and could be within experimental variation. It is interesting to note that Silver shows platelet adhesion to a rat tail tendon collagen sample treated with chondroitin-6-sulfate. Although the sample is not the same as Silver's samples above or any samples in this report, it is related, and shows that a sample can cause platelet adhesion without causing aggregation.

None of the highly crosslinked samples that Silver prepared showed any platelet aggregation. Silver's sample 3.39x, which is similar to samples 4.20.2 or 4.53.2 was prepared by blending bovine hide collagen for 2 minutes in acetic acid, casting into a film, crosslinking in gluteraldehyde, air-drying, Wiley milling, and dispersing in saline. The casting process allowed several days of swelling in acetic acid before completely air-drying. Silver's samples
3.410 and 3.410 were made by adding 10% and 15% chondroitin-6-sulfate to a 2 minute blended dispersion, forming a membrane, crosslinking in gluteraldehyde, air-drying, Wiley milling, and dispersing in saline. These experiments show that a bovine hide collagen sample that has been exposed to acetic acid, air-dried and gluteraldehyde crosslinked does not cause a platelet reaction, regardless of whether or not any chondroitin-6-sulfate is present. In conclusion, the experiments performed by Silver support the findings and conclusions presented in the previous section.
CHAPTER VIII

The Crossflow Filtration Apparatus

Introduction

The promise of a material which is non-thrombogenic yields a desire to form seamless tubing for use as vascular prosthesis. Tubes can be manufactured by a variety of methods. The methods commonly used for plastics, extrusion and casting, usually require heating of a polymer, followed by cooling to form the desired shape. Heating of collagen is not possible, as it quickly denatures to gelatin. The extrusion process, however, can be modified to make such articles as surgical sutures or edible sausage casings from collagen (Lieberman 1964). These are fabricated by extruding a dispersion of collagen into a collagen non-solvent, causing the solvent of dispersion to leave the collagen, and resulting in a de-swollen solid collagen article. The disadvantage of this process is that the deswelling of the collagen is detrimental to the formation of a non-thrombogenic collagen material. It is possible to form tubes from a collagen dispersion by centrifugal force, but the required angular velocities become prohibitively high for the small diameters of tubes which are desired. Flat sheets of collagen can successfully be made by de-watering a dispersion on filter paper in a Buechner funnel. The process simply involves creating a partial vacuum on one side of the filter paper which draws the collagen solvent through the filter paper,
leaving only the collagen particles in a float sheet on the other side. Tubes could be made by rolling the sheet and gluing or suturing along one side. Unfortunately, the mere presence of an internal seam has been observed to cause thrombosis (Yannas 1977).

The idea of the filtration process for forming collagen articles is basically sound, and resulted in the crossflow filtration concept. Figure 8.1 illustrates the process with a cross-sectional view of the crossflow filtration mold. The pressurized dispersion flows through the cylindrical mold which is lined with filter paper. The high internal pressure drives the dispersion to the perimeter, forcing the dispersing medium through, and depositing the collagen particles on the filter paper. This forms a gel of collagen particles which can be strengthened to form a useful tube. This process is similar to ultrafiltration, but, in this case, the gel formation is viewed as desirable, whereas in ultrafiltration, gel formation is viewed as an efficiency reducing undesirable effect. The rate at which the gel forms is dependant upon the pressure gradient, and the resistance of the mold, filter paper and deposited particles to the filtrate. The crossflow is required to prevent the mold from clogging, to supply more material for filtration, and to allow the formation of a tube with smooth inner walls and a uniform thickness.

Filtration Theory

Filtration is a process which involves the separation of the two components of a mixture. A liquid mixture is composed
of the solvent and the solute, which is dissolved or suspended in the solvent. The solute could be solid particles, dissolved particles or another liquid with a larger molecular weight. The separation of the components of the mixture is achieved by creating a pressure difference across a filter membrane which is permeable only to the solvent. The placement of the mixture on one side of the membrane then results in concentration of the mixture on the high pressure side, while the low pressure side collects only the solvent.

The corrective transportation of the mixture is driven by the trans-membrane pressure, \( P \) \([\text{FL}^{-2} \text{ or } \text{ML}^{-1}\text{T}^{-2}]\). The flux of the solvent through the filter membrane, \( J \) \([\text{LT}^{-1}]\), is limited by the resistance to the flow, \( R \) \([\text{ML}^{-2}\text{T}^{-2}]\), and is governed by the relation:

\[
J = \frac{P}{R} 
\]

where \( R \) need not be constant (Copas 1973).

Experiments have shown that the resistance to the flow is not wholly due to the filtration membrane. The concentration of the solute is not uniform on the high pressure side of the membrane, this is referred to as "polarization" of the solute concentration. The solute concentration may reach a critical concentration where the mixture begins to act more like a solid than a free flowing liquid. This critical value of the solute concentration is called the gel concentration and is denoted by \( C_g \). The cross-flow filtration apparatus exploits the formation of this gel to create seamless tubes for use as vascular
prosthesis. This method of fabrication is not common, in fact, most researchers consider the formation of the gel layer to be a detrimental occurrence, as it slows the rate of separation of the components of the mixtures. The formation of the gel in the crossflow filtration mold yields a fragile tube which is then strengthened to become a useful product.

Figure 8.2 depicts the gel-polarization model used in the literature (Porter 1972). This model assumes that the resistance, $R$, is equal to the sum of the resistance of the filter membrane, $R_m$, and the resistance of the gel layer, $R_g$.

If there were no mechanism to transport the solute particles from the filter membrane the flux would continuously decrease, as the resistance increased, due to the gel layer build up. Experimentally, however, the flux is observed to reach a solid state value. The solute particles are transported away from the membrane by a variety of processes, including osmotic force, hydrodynamic shear and diffusion. In the case of stagnant filtration with no osmotic effects, diffusion is the dominant transport mechanism. Steady state results when the pressure driven connective transport of the solute to the filter membrane is balanced by the concentration gradient activated diffusive transport of the solute from the filter membrane. The mass flux balance of the steady state situation is given by:

$$JC = D \frac{dC}{dx}$$

(8.2)

Where $C$ is the concentration of the solute, $D$ is the diffusivity of the solute in the solvent and $dC/dx$ is the
FIGURE 82 GEL-POLARIZED MODEL
concentration gradient.

The dynamic gel layer is assumed to have a constant concentration, $C_g$. The thickness of the gel layer varies to produce the steady state resistance and, therefore, the steady state flux. The boundary conditions of the concentration are known; $C_B$ is the bulk stream concentration, and the concentration at the membrane surface has an upper limit of $C_g$. Integrating equation 8.2 yields:

$$J = \frac{D}{\delta} \ln \frac{C_g}{C_B}$$

where $\delta$ is the boundary layer thickness of the concentration polarized region.

In spite of the fact that the above model is derived for the stagnant case, experimental data correlate well with the theory for macromolecular solutions. The data for colloidal dispersions, however, often show steady state fluxes two orders of magnitude larger than predicted by this theory (Porter 1972). This indicates that the shear caused by the flow of the solution next to the gel layer is an important mechanism for the transportation of the solute from the gel layer.

The concentration of the gel is probably not constant throughout its width, but varies from the concentration at the inner surface of the gel layer to a maximum at the membrane surface. Associated with each gel concentration is a specific shear strength. If the material is more compacted (a higher gel concentration due to a larger local pressure gradient)
then the shear strength is greater. The flow of the solution past the inner surface of the gel layer exerts a shear force on the gel, removing all layers of the gel with shear strengths less than the shear force. The action of the shear force is to decrease the gel layer thickness and, therefore, increase the expected flux. Denoting the quantity of material removed by the shear force mechanism as $S$, results in a modification of equation 8.2:

$$JC = S + D \frac{dc}{dx}$$

(8.4)

The steady state flux must now be large enough to replace solute removed by both shear forces and diffusion.

Another experimental observation is that if the transmembrane pressure exceeds a transitional pressure, then the flux remains constant with respect to pressure. The significance of this finding is that above the transitional pressure, the resistance to flux flow (and hence gel layer thickness) is increased proportionally with pressure.

The filtration theory predicts that the gel layer formation will be enhanced if the shear force due to the cross-flow is kept at a minimum. The flow rate should, therefore, be kept as low as possible, but some flow is always necessary to prevent complete clogging of the mold. If the concentration activated diffusion transportation mechanism is significant, then a bulk stream concentration close to the gel formation concentration will limit back transport of particles from the gel. Also, operation at pressures above the transition pressure
should allow a linear increase in gel thickness and mass with increasing pressure.

Design of the Crossflow Filtration Apparatus

An experimental crossflow filtration apparatus was built, in this laboratory, by Sieverding (1976) and tested by Frank (1977). This background work showed that the crossflow filtration method for manufacturing tubes is feasible, but the experimental apparatus had many deficiencies.

A schematic representation of the experimental apparatus is shown in Figure 8.3. A reservoir, containing the bulk of the collagen dispersion, was pressurized by compressed air and stirred with a small magnetic stirring bar. The dispersion was channeled through a water cooled jacket, the crossflow filtration mold and returned to the reservoir by a pump. The filtrate passing through the mold was collected by a pan placed under the mold. The equipment auxiliary to the mold had many problems. The reservoir was a very leaky pressure vessel which lost more than 5psi over 30 minutes from an initial pressure of 40 psi. The valves and tubing used for the apparatus were only rated for 25 psi in spite of the fact that the apparatus was used at pressures of up to 40 psi. The tie rods which held the ends of the pressurized reservoir together were threaded into only plexiglas on one end. The pump which was used to recirculate the dispersion, heated up rapidly under the strain of pumping the fairly viscous dispersion at pressures well above its rated 15psi. This necessitated the insertion
FIGURE 8.3 EXPERIMENTAL CROSSFLOW FILTRATION APPARATUS
of a water cooled jacket to prevent the denaturing of the collagen flowing through the pump. The flap type valves of the pump often became clogged with collagen particles, occasionally stalling the pump and resulting in a very irregular flow rate.

The mold of the experimental apparatus was difficult to assemble and disassemble. The filter paper was glued to the mold at each end to help prevent the dispersion from flowing behind the filter paper. Prior to making a tube it was necessary to dry the filter paper in the mold to allow it to maintain a circular shape. The filter paper was also used, with limited success, as a gasket to prevent leakage from between the mold halves. The seals at the mold ends were made in such a way that if the mold halves were well sealed then the seals at the mold ends would necessarily be loose. The end seals were therefore copiously wrapped with teflon tape as a stopgap measure. The preparation of the mold for making a tube was a very laborious procedure which was often repeated several times before no leakage of the dispersion into the filtrate was evident. Removing the mold from the apparatus after pressurizing, was a very messy procedure which could result in contamination or loss of large quantities of the dispersion. Partly because of such difficulties, the total time required to produce 1 tube was about 12 hours.

The tubes which were formed by the mold had several defects, including two seams as shown in Figure 8.4(a). The seams
FILTER PAPER IN MOLD:

(a) TWO PIECES OF THICK FILTER PAPER

(b) ONE PIECE OF THICK FILTER PAPER

(c) ONE PIECE OF THIN FILTER PAPER

TUBE:

TWO SEAMS PRESENT ON BOTH THE INSIDE AND OUTSIDE OF THE TUBE

ONE SEAM PRESENT ON BOTH THE INSIDE AND OUTSIDE OF THE TUBE

HARDLY DETECTABLE SEAM ON OUTSIDE, NO SEAM ON INSIDE

FIGURE 8.4 THE EFFECT OF THE FILTER PAPER GEOMETRY UPON THE FINISHED TUBES
result from the groove formed by the radius of curvature of the relatively thick filter paper as it rounded the corner of each mold half. The wall thickness of end of the tube was formed at the entrance of the mold was consistently thinner than the wall thickness at the mold's exit. Also, the type of filter paper used in this apparatus was so difficult to remove from the tube that many fragments of the filter paper would remain on the completed tube. Furthermore, the actual filtration time required for forming these imperfect tubes was over 4 hours, due to the filter paper's high resistance and the low pressure capability of the apparatus as a whole.

Two other problems with the experimental apparatus were the inability to measure the flux and the time varying concentration of the collagen dispersion. The filtrate dripped out of the mold very slowly, so slowly, in fact, that most of the filtrate evaporated before it could reach the collection pan. This is a drawback, since an accurate knowledge of the flux rate through the mold is needed to determine the stage of formation of the tube. The continuous loss of the filtrate through the mold allows the concentration of the dispersion, within the apparatus, to gradually increase. No new dispersing medium could be added to the dispersion to counteract this time varying concentration without depressurizing the entire apparatus.

A prototype crossflow filtration apparatus was designed and built by the author to circumvent the short comings posed
by the experimental apparatus. The pressurized reservoirs were redesigned with O-ring seals, the mold was designed for ease of use and a new pump was purchased. Figure 8.5 shows a schematic drawing of the prototype apparatus. Also evident on this drawing are the addition of the auxiliary reservoir and vapour trap, and the deletion of the water cooled jacket.

The most obvious addition to the apparatus is the auxiliary reservoir, above the main reservoir in Figure 8.5. This reservoir is designed to hold additional dispersing medium which can be added to the main reservoir to replace the filtrate, while the apparatus is pressurized. The auxiliary reservoir can be depressurized, while the remainder of the system is pressurized, by closing the regulating valve and the lower toggle valve (see Figure 8.6). The reserve dispersing medium is taken in through the toggle fluid input valve, while the upper toggle vent valve is open. Toggle valves are used at these locations, since only fully opened or closed positions are required. The dispersing medium can be dispersed to the main reservoir by pressurizing the auxiliary reservoir, opening the lower toggle vent valve and adjusting the regulating valve to allow the desired flow of dispersing medium.

The main reservoir and mixing chamber incorporates several new features which are shown in detail in Figures 8.8 to 8.11. The diameter of the reservoir has been increased to allow a larger magnetic stirring bar to be used. Subsequently, the
FIGURE 8.5 PROTOTYPE CROSSFLOW FILTRATION APPARATUS
FIGURE 8.6 ISOMETRIC VIEW OF THE MAIN AND AUXILIARY PRESSURE VESSELS.
larger vortex assures uniform mixing when the dispersing medium from the auxiliary reservoir is added. The tie rods connecting the ends of pressure vessel have been welded to a flat plate on the bottom to avoid threading the plexiglas and to allow the thickness of the lower pressure vessel end to be kept to a minimum. This minimal thickness, in conjunction with the nonmagnetic tie rod construction material, brass, permits the greatest magnetic coupling from the stirring plate to the magnetic stirring bar. The plug shutoff valve at the exit, when opened, allows the dispersion to flow through the valve in a straight unobstructed path. When this valve is closed, it allows the removal of the mold without depressurizing the pressure vessels, or loosing or contaminating the dispersion. Plexiglas was used to construct both pressure vessels in order to allow observation of the dispersion and to allow easy cleaning.

The nominal wall thickness of the 3.5 inch (outer diameter) cast plexiglas cylinders is 0.25 inches. In fact, the inner and outer diameters were not concentric and, therefore, the wall thickness was machined to a uniform 0.215 inch thickness. This thickness and an effective inner diameter of 3.07 inches are used for the pressure calculations.

The main and auxiliary pressure vessels were designed to operate safely at pressures of up to 225psi, but the routine maximum pressure used was only 100psi due to the limitation of the recirculation pump. The strength of the threaded joint on each tie rod is the factor which limits the maximum safe
pressure of the pressure vessels. The ultimate tensile strength of brass ranges from 40,000 to 120,000 psi. The maximum safe load for a 7/16" diameter American Standard Bolt made from material with an ultimate tensile strength of 40,000 psi is 415 pounds (Baumeister 1978). The force exerted on one end of the pressure vessel is the internal pressure times the area, \( F_{\text{end}} = P \times 0.25 \times \pi \times (3.07)^2 = 7.40 \times P \). The maximum safe pressure which can be supported by the tie rods is, \( P_{\text{max}} = 4 \times 415/7.40 = 224.3\text{psi} \). This is based on the minimum ultimate tensile strength given for brass, so that if the brass used is of a stronger type, the maximum safe pressure may be up to three times higher.

The accepted method for determining the permissible load on a fillet weld is to assume that the force is transmitted by shear stress across the throat of the weld. In this case the area transmitting the shear force is the perimeter of the \( \frac{7}{16} \) diameter tie rod times the thickness of the \( \frac{1}{8} \) inch plate welded on the bottom. Therefore, the shear stress is:

\[
\sigma_s = 0.25(P \times 7.40)/[\pi \times 7/16 \times 0.125] = 10.77xP.
\]

The resulting shear stress on the weld which results from applying a pressure of 225psi (100psi) to the pressure vessel is 2,423psi (1077psi), well below the 24,000 to 34,000psi shear strength of brass (Parker 1967).

The walls of the pressure vessels are made from polymethyl methacrylate, which has the following properties: ultimate tensile strength, 8700 to 11000psi; tensile modulus
350000 to 450000 psi; and an elongation at break of 3 to 6% (Harper 1975). A poisson's ratio of 0.33 is assumed. The pressure vessels are best modelled as a cylinder with fixed ends and an internal pressure, P. The equations of stress and deflection for this situation are (Saada 1974):

\[
\sigma_{rr} = \frac{a^2 P (r^2 - b^2)}{r^2 (b^2 - a^2)}
\]
\[
\sigma_{\theta\theta} = \frac{a^2 P (r^2 - b^2)}{r^2 (b^2 - a^2)}
\]
\[
\sigma_{zz} = \frac{2va^2 P}{(b^2 - a^2)}
\]
\[
U_r = \frac{(v+1) a^2 r P}{E (b^2 - a^2)} \left(1 - 2v + \frac{b^2}{r^2}\right)
\]

where \(\sigma_{rr}\) is the radial stress, \(\sigma_{\theta\theta}\) is the hoop stress, \(\sigma_{zz}\) is the longitudinal stress and \(U_r\) is the radial displacement. The other terms used are: \(a\) for the inner radius, \(b\) for the outer radius, \(r\) for the radius at which the stress or strain is computed, \(E\) for the elastic modulus and \(v\) for Poisson's ratio. The maximum values obtained for these stresses and the deflection are found at the inner radius. The maximum values with an applied pressure of 225psi (100psi) are:

\[
\sigma_{rr}(\text{max}) = -225\text{psi} \quad (-100\text{psi})
\]
\[
\sigma_{\theta\theta}(\text{max}) = 1,726\text{psi} \quad (767\text{psi})
\]
\[
\sigma_{zz}(\text{max}) = 495\text{psi} \quad (220\text{psi})
\]
\[
U_r(\text{max}) = 0.0063\text{in.} \quad (0.0028\text{in.})
\]

The stresses are clearly well below the ultimate tensile strength of the plexiglas. In fact, the burst strength of the plexiglas pressure vessel wall should be in excess of
1100psi. The leakage from the pressure vessels is quite low; for example, the auxiliary pressure vessel lost only $1\frac{1}{2}$psi over 24 hours when initially pressurized to 50psi.

A variety of valves, fittings and lines were also required to meet the pressure requirements. The recirculation lines are made from 3/8 inch outer diameter impolene polyallomer tubing (Gould, Inc., Chicago, Illinois) which has good chemical resistance to acids. It can also be repeatedly autoclaved, has a burst pressure of 900psi and a maximum working pressure of 300psi. Swagelok fittings (Crawford Fitting Company, Solon, Ohio) are used for all fittings in the apparatus and are claimed to contain any pressure, without leakage, up to the burst point of the tubing. The Whitey toggle valves (Whitey Co., Cleveland, Ohio) used for most valves on the apparatus, are made from 316 stainless steel and have a pressure rating of 300psi. The stainless steel Whitey regulating valve found between the main and auxiliary pressure vessels has a pressure rating of 3000psi. The MR10 Series air pressure regulator (Circle Seal Corp., Anaheim, California) used to pressurize the apparatus is designed for dead end or low flow applications at pressures up to 150psi. The hose connecting the regulator to the apparatus is standard air brake hose with a maximum operating pressure of 500psi.

The recirculation pump is the pressure limiting component in the apparatus. An extensive review of many pump designs
by many manufacturers showed that very few suitable pumps are available. The desired specifications for the single channel metering pump are the ability to pump the viscous dispersion of 60 mesh sized collagen particles, without clogging, at flow rates of 1 to 15 ml/min and at pressures of up to at least 100psi. The viscosity of the collagen is difficult to determine as it exhibits decreasing viscosity with time, at a constant shear rate, and decreasing viscosity with an increasing shear rate. The range of the viscosity for the collagen dispersions at low shear rates was determined by Stein (1978) to be in the 10 to 100 poise range. A viscosity this high is often too great for most pumps. Also, since there is no pressure drop across the pump, a pump design which does not permit overdelivery is preferable.

The Fluid Metering, Inc. (Oyster Bay, New York) pump model #RP-SY-ISSY met all of the above specifications. Its valveless rotating reciprocating piston design assured free flowing of the dispersion. The positive volume displacement and synchronous motor assure accurately metered flow with no possibility of overdelivery at flow rates of up to 19.4 ml/min. The pump does not heat the fluid which it pumps, thereby eliminating the need for the water cooled jacket of the previous apparatus. The pressure limit of the pump, however, limits the maximum operating pressure of the apparatus to 100psi. As mentioned before, the pressure vessels and other parts of the apparatus are designed to operate safely at pressures of up to 225psi.
The heart of the prototype crossflow filtration apparatus, the mold, is shown in an exploded view in Figure 8.7. Figures 8.12 to 8.15 are detailed drawings of the mold which has been designed for ease of use and cleaning, while producing seamless tubes. The change to a 3.0um pore sized polycarbonate filter membrane (Bio Rad Laboratories, Richmond, California) is important for several reasons. First, the thickness of the polycarbonate filter membrane is 10um, only 8% of the thickness of the cellulosic filter membranes used previously. Since this thickness is also only a small fraction of the wall thickness of the vascular prostheses, the filter paper configuration shown in Figure 8.4(c) can be used. The overlap of the filter paper causes a slight seam on the outer surface, but as the collagen particles are deposited on the inner surface, the discontinuity of the overlap is gradually smoothed. This prevents the formation of a seam on the inner surface of the completed vascular prosthesis. This configuration is also superior to that shown in Figure 8.4(a) or (b), as the filter paper does not interfere with the seal between mold halves.

A second advantage of the polycarbonate filter paper is its low resistance to the solvent flux. This filter paper permits the passage of water at a rate of 2,000ml/min/cm² with a pressure difference of 70mm Hg; a much higher rate than that found with the cellulosic filter paper. This reduced resistance of the filter membrane permits essentially
all of the resistance to the steady state flux to be due to the gel layer. The larger gel resistance, of course, implies a thicker wall for the vascular prosthesis. A problem encountered with the cellulosic filter paper is the mechanical interlocking of the finished vascular prosthesis with the pores in the filter paper. The very smooth surface of the polycarbonate filter membrane alleviates this problem, as it allows very easy separation of the filter paper from the finished prosthesis.

The filter paper is securely held against the inner surface of the mold by the cylindrical section of the inner end cap, preventing leakage of the dispersion behind the filter paper (see Figure 8.15). The wall thickness of the cylindrical section is 0.008 inches and provides stagnation regions next to the filter paper at the entrance of the mold. This prevents the shearing off of the gel at the mold entrance which was believed to be responsible for the diminished wall thickness of the vascular prosthesis at the mold entrance in the previous apparatus.

The mold was designed to permit securing of the seals between the mold halves independently from the face seals at the mold ends. The development of the special O-ring seal (Figure 8.12) assured seal continuity and prevented leakage. Hose clamps were found to be the most convenient method for applying pressure on the seals between the mold halves, while the mold end caps connected by tie rods were used to secure the face seals.
The Swagelok tube fitting in each mold end butts directly with the inner end cap. It has the same inner diameter as the inner end cap to cause as little flow disturbance as possible. The counterbore in the mold end which accepts the assembled mold halves is offset from the center line. This permits the accommodation of the hose clamps within the vapour trap, using as little volume as possible.

The vapour trap about the mold creates a local saturated atmosphere which prevents the evaporation of the flux droplets. The vapour trap also acts as a droplet collector concentrating the flux for collection in a graduated cylinder. The addition of the vapour trap allows accurate measurement of the flux to determine when the steady state flux is reached.

The incorporation of the new design features and accurate machining of the components has resulted in a more reliable prototype apparatus. Several features make the apparatus easier to use and extend the useable range of the operating parameters, thus reducing the time required to make each vascular prosthesis.

**Procedure for Manufacturing Vascular Prostheses**

Prior to pressurizing the prototype apparatus to make a vascular prosthesis, several preliminary procedures are necessary. The dispersion must be made and placed in the main pressure vessel. The acetic acid should be placed in the auxiliary pressure vessel and the mold, with the filter paper in place, must be inserted into the recirculation line.
DRILL, 4 HOLES

PART 'A'
HALF SIZE

PART 'B'
HALF SIZE

FIGURE 8.8 AUXILIARY PRESSURE VESSEL ENDS
FIGURE 8.9  MAIN PRESSURE VESSEL ENDS
FIGURE 8.10 PRESSURE VESSEL WALLS AND O-RING SEAL
**UNF-2A**

10.4

**FILLET WELD**

ALL AROUND

0.13

2.25

60.62

**ALL ROUNDS 2.25 APPROX.**

0.05

---

**20UNF-2A**

10.0

0.13

2.25

---

**BRASS TIE ROD 'H'**

4 REQD

**ALUMINUM SUPPORT 'J'**

2 REQD

**ALUMINUM SUPPORT 'K'**

2 REQD

---

**ADDITIONAL PARTS REQUIRED:**

- 0.50 x 1.25 x 0.083 PLAIN WASHERS 12 REQD
- 7/16-20UNF-2B HEXAGONAL NUTS 12 REQD
- 3" DIA x 0.103 CROSS-SECTION DIA O-RINGS 4 REQD

---

**ALL DIMENSIONS IN INCHES**

**ALL DRAWINGS HALF SIZE**

---

**FIGURE 8.11 TIE RODS AND SUPPORTS FOR PRESSURE VESSELS**
FIGURE 8.12 MOLD & O-RING SEAL

ALL DIMENSIONS IN INCHES
FIGURE 8.13  MOLD ENDS & TIE RODS
ADDITIONAL PARTS REQUIRED:

- \( \frac{3}{8} \) OD TUBE TO \( \frac{1}{8} \) (MALE) SWAGELOK FITTINGS 2 REQD TO MATE WITH MOLD ENDS
- \( \frac{3}{16} \) OD x 4 POLYETHYLENE TUBE 1 REQD DRAIN TUBE FOR VAPOUR TRAP
- \( \frac{3}{4} \times 11 \frac{3}{4} \) 3\( \mu \)m PORE SIZE POLYCARBONATE FILTER MEMBRANE 1 REQD PLACED INSIDE MOLD HALVES
- \( \frac{1}{8} \) DIA HOSE CLAMPS 4 REQD TO CLAMP MOLD HALVES TOGETHER
- 1.614 ID, 0.070 X-SECT DIA O-RINGS 2 REQD SEAL ON OUTSIDE OF MOLD ENDS
- 0.676 ID, 0.070 X-SECT DIA O-RINGS 2 REQD FACE SEAL ON INNER END CAPS
- 0.250 x 0.562 x 0.065 PLAIN WASHERS, DRILLED WITH F (0.257) DRILL 2 REQD FOR TIE RODS
- \( \frac{1}{4} \) -28 UNF-2B HEXAGONAL NUTS 2 REQD FOR TIE RODS

FIGURE 8.14 MOLD WALL & PARTS LIST
FIGURE 8.15 ASSEMBLED MOLD
At this point, the apparatus can be pressurized to form a vascular prosthesis.

The preparation of the mold begins by placing the inner end caps of the mold, 12 inches apart, on a 0.185 inch diameter aluminum rod. Figure 8.7 should be consulted to determine the relationship of the mold parts during assembly. The aluminum rod is simply used as an aid to placing the filter paper in the mold. Two pieces of 11 3/4" x 3/4" polycarbonate filter membrane are wrapped around the rod and over the cylindrical portion of the inner end caps. The second piece of filter membrane is centered over the seam of the first to prevent leakage. A little distilled water may help to keep the filter membranes around the rod. The O-ring seal which fits between the mold halves is stretched over each end cap. The rod, inner end caps, filter paper and O-ring seal can now be set onto the mold half which has the groove between the mold halves (Part L, Figure 8.12). Note which end of the mold half has a "1" engraved on it. While placing the filter paper in the mold, the distance between the inner end caps is slightly increased to allow the face seal between the inner end caps and mold half to seal properly. The second mold half is then placed on the other half, with the engraved "1" on its end placed over the end of the other mold half which has the engraved "1".

The four hose clamps are placed around the mold halves with a medium tightness. The aluminum rod can now be removed.
easily if the hose clamps are not too tight. The mold assembly can now be fitted into the mold ends. Note that it is not necessary to completely remove the nuts on the tie rods while inserting the mold assembly. Tightening of the tie rod nuts and the hose clamps now assures the integrity of the seals. The vapour trap slides over the mold ends to form the complete mold module for insertion into the recirculation line. This module is inserted by tightening the appropriate Smagelok fittings at either end of the mold to connect it with the recirculating line.

The reciprocating, rotating piston pump may leak, slightly, unless the seals around the pistons are flattened after each use and carefully reformed as they are put on the piston just prior to use. These seals should be put on the piston, the first one with the raised side toward the motor, and the second two seals with the raised side away from the motor. The usual flow rate of 9.7 ml/min is set by adjusting the micrometer to 0.500.

If the pressure vessels have been disassembled for cleaning they must be reassembled using Figure 8.6 for reference. The teflon coated stirring bar should be placed in the main pressure vessel during reassembly. The collagen dispersion (usually 400 ml) can be funneled into the main pressure vessel through the toggle input valve above the street tee in the recirculation line. The vent selector valve on the left should be opened (horizontal) and the plug shut off valve should be
closed. The stir plate can be started to mix the dispersion. The auxiliary pressure vessel is filled with a measured amount (usually 250 ml) of 0.05M acetic acid through the funnel above the toggle fluid input valve in the auxiliary pressure vessel. The toggle vent valve on the top of the auxiliary pressure vessel must be opened and the regulating valve on the bottom of the vessel must be closed.

The two toggle valves on top of the auxiliary pressure vessel and the toggle valve above the street tee in the recirculation line must be closed prior to pressurizing the system. The vent selector valve on the left of the main pressure vessel should be turned to the vertical position to connect the compressed air tank to the pressure vessels. The pump should be started, the plug shut off valve opened, and the air regulator adjusted to the desired pressure, usually 100psi. Initially the flux may be quite high and the operator should be prepared to immediately measure the collected filtrate in the graduated cylinder. Note that it will take approximately five minutes for the first drop of recirculated dispersion to appear at the top of the main pressure vessel.

Very seldom, the filtrate may appear milky in color. This probably indicates a tear in the filter membrane which means that the filter membrane must be replaced. The mold may be removed by first closing the plug shut off valve and loosening the Swagelok fittings. The pump may be turned off a minute later when the dispersion has been pumped from the
mold. The mold can then be removed from the recirculation line to replace the filter paper. The apparatus will remain pressurized and very little dispersion will be lost while the defective filter membrane is replaced.

The total volume of filtrate is collected in a 25 ml graduated cylinder and is measured at frequent intervals to allow the calculation of the flux. The time is set to zero when the first drop of filtrate reaches the graduated cylinder, although this may be 1 or 2 minutes after the pressurization. The measurements are initially recorded once per minute, then once every 2 1/2 minutes and finally at 5 minute intervals. The measurement intervals are adjusted so that at least 2 ml of filtrate are collected from the 10 inch mold during each interval. When the graduated cylinder is filled to the 25 ml mark a second graduated cylinder is used for the filtrate collection while the first is emptied. The flux is quite dependent upon the composition of the dispersion, especially with respect to the chondroitin-6-sulfate content. The operator must be ready to make rapid changes in his original measurement schedule.

Acetic acid from the auxiliary pressure vessel is added to the dispersion in the main pressure vessel, as the filtrate is collected. The toggle valves at the top of the auxiliary pressure vessel must be closed before opening the toggle vent valve between the pressure vessels. The regulating valve can be left opened to allow a steady flow of acetic acid or opened periodically to allow 10 to 30ml quantities of acetic acid into
the main pressure vessel. The acetic acid is replaced in a one to one ratio to the filtrate collected. The amount added to the main pressure vessel is determined by the volume calibration marks on the auxiliary pressure vessel.

Additional acetic acid may be added to the auxiliary pressure vessel without depressurizing the remainder of the apparatus. This is performed by closing the regulating valve and the inter-pressure vessel toggle vent valve, and opening the upper toggle valves. The additional acetic acid can be funneled into the auxiliary pressure vessel followed by restoration of the valves to their previous positions.

The filtration process is terminated by turning the air regulator off and opening (horizontal position) the selector valve on the left of the main pressure vessel. The plug shut off valve is closed, the Swagelok fitting next to valve is loosened and pump is allowed to empty the mold of dispersion before it is shut off. The mold module is then removed from the recirculation line and disassembled. The filter paper and gel are carefully removed from the mold for the next manufacturing step, usually either air drying or glutaraldehyde crosslinking.

Manufactured Vascular Prostheses

A large number of parameters affect the formation of the cross flow filtered vascular prostheses. The composition of the collagen composite dispersion greatly affects the viscosity and subsequent filtration characteristics. The variables of the
dispersion production are the pH and ionic strength of the dispersing medium, the collagen: dispersing medium ratio, the particle size of the collagen, and the chondroitin-6-sulfate: collagen ratio. The composition of the dispersion is not very flexible, as the non-thrombogenic properties of the material are strongly affected by the composition of the dispersion. The mechanical and physical properties of the vascular prosthesis can also be affected by the operating parameters of the cross-flow filtration apparatus. These parameters include the internal pressure of the apparatus, the flow rate of the dispersion through the mold, the fabrication time and the filter membrane characteristics. The experimental vascular prostheses which were manufactured varied four of the above parameters, pressure, percentage of collagen, percentage of chondroitin-6-sulfate and fabrication time.

The dispersions were all made by blending collagen in 0.05 M acetic acid, before adding the chondroitin-6-sulfate. The dispersions of the first six prostheses were made by adding the chondroitin-6-sulfate to the collagen dispersions a few minutes after forming the dispersion. The later dispersions were made by swelling the blended collagen for at least three days before the addition of the chondroitin-6-sulfate. Table 8.1 summarizes the values of the dispersion composition and apparatus pressure used to make the vascular prostheses. Following the formation of the tubular gel, the first seven tubes were air dried before crosslinking, while the last tubes were crosslinked in the wet state.
### Table 8.1 Manufactured Vascular Prostheses

<table>
<thead>
<tr>
<th>Tube #</th>
<th>Pressure (psi)</th>
<th>% Collagen</th>
<th>%CGS</th>
<th>Mold flux (ml/min)</th>
<th>Filtration Time (min)</th>
<th>Average Thickness (mils)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>35</td>
<td>3/4</td>
<td>10</td>
<td>--</td>
<td>--</td>
<td>240</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>3/4</td>
<td>10</td>
<td>1.8</td>
<td>1.08</td>
<td>60</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>3/4</td>
<td>20</td>
<td>45</td>
<td>3.73</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>3/4</td>
<td>8</td>
<td>0.23</td>
<td>0.25</td>
<td>210</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
<td>3/4</td>
<td>12</td>
<td>2.7</td>
<td>0.68</td>
<td>120</td>
</tr>
<tr>
<td>6</td>
<td>100</td>
<td>3/4</td>
<td>12</td>
<td>2.4</td>
<td>0.60</td>
<td>120</td>
</tr>
<tr>
<td>7</td>
<td>100</td>
<td>1/4</td>
<td>12</td>
<td>6.0</td>
<td>0.58</td>
<td>90</td>
</tr>
<tr>
<td>8</td>
<td>100</td>
<td>1/4</td>
<td>12</td>
<td>3.6</td>
<td>0.80</td>
<td>120</td>
</tr>
<tr>
<td>9</td>
<td>100</td>
<td>1/4</td>
<td>12</td>
<td>4.8</td>
<td>1.08</td>
<td>120</td>
</tr>
<tr>
<td>10</td>
<td>100</td>
<td>1/4</td>
<td>12</td>
<td>5.9</td>
<td>1.68</td>
<td>60</td>
</tr>
<tr>
<td>11</td>
<td>100</td>
<td>1/4</td>
<td>12</td>
<td>15</td>
<td>2.12</td>
<td>90</td>
</tr>
<tr>
<td>12</td>
<td>100</td>
<td>1/4</td>
<td>12</td>
<td>40</td>
<td>4.64</td>
<td>60</td>
</tr>
<tr>
<td>13</td>
<td>100</td>
<td>1/4</td>
<td>12</td>
<td>40</td>
<td>4.80</td>
<td>30</td>
</tr>
<tr>
<td>14</td>
<td>100</td>
<td>1/4</td>
<td>12</td>
<td>5.9</td>
<td>2.40</td>
<td>90</td>
</tr>
<tr>
<td>15</td>
<td>100</td>
<td>1/4</td>
<td>12</td>
<td>0.32</td>
<td>0.24</td>
<td>120</td>
</tr>
</tbody>
</table>
The flux through the mold is initially high and gradually decays to a steady state value. Figure 8.16 shows the total flux through the mold versus time for vascular prostheses made from 1/4% collagen-acetic acid, 12% chondroitin-6-sulfate dispersions. The pressure of the apparatus was maintained at 100 psi for each experiment. The initial flux ranged from 3.5 to 6 ml/min through the 6.5 square inches of the molds inner surface area. The final flux varied from less than 1 to almost 2.5 ml/min, in spite of the similar preparation of the dispersions. The final flux was generally reached in 50 to 100 minutes.

Figures 8.17 and 8.18 show that the chondroitin-6-sulfate content of the dispersion greatly affects the flux. Comparing a 10% chondroitin-6-sulfate dispersion with one containing 20%, shows that the initial flux is 25 times higher with the greater chondroitin-6-sulfate. Again the difference in chondroitin-6-sulfate content results in an order of magnitude difference in the flux. It was noted while handling the dispersions that 10% chondroitin-6-sulfate was a critical value. Above 10%, the dispersion was very fibrous, with clumps of fibers which could settle; below 10% the dispersion was very homogeneous and viscous similar to a pure collagen dispersion. For example, the flux of vascular prosthesis #15 (not shown) is practically identical to that of #4 (Figure 8.18), in spite of the fact that it contained no chondroitin-6-sulfate. These facts show that the flux through the mold is particularly sensitive to the concentration of the chondroitin-6-sulfate at concentrations above 10% and may explain the minor variations of flux found in Figure 8.16.
FIGURE 8.16 TOTAL FLUX THROUGH MOLD VERSUS TIME FOR VASCULAR PROSTHESES MADE FROM 1/4 % COLLAGEN-ACETIC ACID, 12% CHONDROITIN-6-SULFATE DISPERSION AT 100 PSI.
FIGURE 8.17  TOTAL FLUX THROUGH MOLD VERSUS TIME FOR VASCULAR PROSTHESES MADE AT 50 PSI.

3/4 % COLLAGEN, 20 % CHONDROITIN-6-SULFATE (TUBE #3)

3/4 % COLLAGEN, 10% CHONDROITIN-6-SULFATE (TUBE #2)
FIGURE 8.18  TOTAL FLUX VERUS TIME FOR VASCULAR PROSTHESES MADE AT 100 PSI, SHOWING THE EFFECT OF CHONDROITIN-6-SULFATE CONCENTRATION.

-335-
The concentration of the collagen, however, does not seem to play as large a role in determining the flux through the mold. Figure 8.19 shows that although the flux is initially higher for the smaller collagen concentration, the flux after the first ten minutes becomes identical.

Figure 8.20 shows the effects of two parameters upon the flux through the mold. One dispersion was used to make all three vascular prostheses. The dispersion was made in a water jacketed blender, instead of an ice jacketed blender like all of the other tubes. The result of the higher blending temperature is higher a flux throughout the filtration time of the vascular prostheses (compare with Figure 8.16). This effect was also noted by Stein (1978) who showed that dispersions blended in a water jacketed blender had a lower viscosity than dispersions made in an ice jacketed blender (viscosity measurements made at the same temperature). The prostheses were made in the order #11, #12, and #13. The concentration of the dispersion was maintained at 1/4% collagen by adding acetic acid from the auxiliary pressure vessel. The flux of each successive experiment is greater than the previous one, indicating that the particles which maintained a high resistance to the flux are deposited on the filter first.

The time of filtration increases the average wall thickness of the vascular prostheses. The tubes made from the 1/4% collagen-acetic, 12% chondroitin-6-sulfate dispersions in Figure 8.16 show this trend. The wall thickness of prostheses
FIGURE 8.19 FLUX THROUGH MOLD FOR VASCULAR PROSTHESES AT 100 PSI FROM 1/4% AND 3/4% COLLAGEN-ACETIC ACID, 12% CHONDROITIN-6-SULFATE DISPERSIONS. NOTE THAT THE FINAL FLUX IS NOT AFFECTED BY THE COLLAGEN CONCENTRATION.
FIGURE 8.20 TOTAL FLUX THROUGH MOLD FOR VASCULAR PROSTHESSES MADE AT 100 PSI, 1/4% COLLAGEN, 12% CHONDROITIN-6-SULFATE.
made after 1/2 hour or 1 hour average: 1.20 mils (#12), 1.15 mils (#2) and 2.0 mils (#10). The wall thickness increased, on the average, after 90 minutes filtration (1.4 mils, #11; 1.5 mils, #7). After two hours of filtration, the thicknesses of the prostheses are 3.0 mils (#9) and 3.25 (#8). This thickness is similar to the thickness measured on a 4 hour filtered prosthesis, 3.35 mils (#1). The thicknesses measured along each tube are recorded in chapter IX, and do not seem to vary in any significant way.

The thickness of the prostheses with 8% and 0% chondroitin-6-sulfate, #4 and #10, showed wall thicknesses which were much thicker (7.4 mils and 12.7 mils) than the other prostheses. This increased wall thickness is due to the gel-like nature of these dispersions, which does not compact as easily.

In summary, the ratio of chondroitin-6-sulfate to collagen was found to have a great effect upon the fabrication of tubes. A low concentration resulted in a low flux and relatively thick walls. A higher concentration resulted in an increased flux and thinner walls. The concentration of the collagen, however, affected the initial flux only. The wall thickness of the vascular prostheses increased with filtration time, up to about 2 hours, when the steady state value was reached.
CHAPTER IX

Mechanical Testing

Introduction

Two mechanical tests, the determination of the ultimate tensile strength and the determination of the crosslink density, are of primary interest. Measurement of the wall thicknesses of the vascular prostheses are required as part of the above tests but also yield direct information concerning the cross flow filtration manufacturing method.

The tensile tests are performed to see if the vascular prostheses meet the design specifications for sustaining the internal pressure and holding sutures. A tear test could also be used to determine the suturability of the material. However, Greensmith (1960) has shown that the tear strengths of rubber-like materials are related to the fracture energy, which equals the area under the stress-strain curve of the tensile strength test. The ultimate tensile strength and elongation at break are, therefore, good indicators of the suturability of the material, eliminating the need for a separate tear test. The crosslink density is of interest, as the degree of crosslinking indicates the biological longevity of the collagen materials.

Thickness Measurement

The measurement of the thickness must be performed accurately and without deforming the material greatly. A standard
micrometer exerts a force in excess of 2000 gms when tightened until a click is heard and the handle turns without moving the spindle. The click feature is designed to apply a constant force to each specimen which is measured. However, since the micrometer is designed to measure metal specimens, the force applied to the 1/4 inch diameter anvil is far too great.

The Mitutoyo, Inc. (New York, New York) Low Pressure Micrometer (model #227-111) is designed to apply a much smaller force to specimens. As purchased, it applies 200 gms to its 1/4 inch diameter anvil, still too large for the collagen specimens. The design incorporates a moveable anvil which is supported by parallel cantilevered beams. The force increases with the deflection of the beams and a needle indicates the deflection at which the readings are to be taken. This device was modified by carefully disassembling the cantilever mechanism to file down all points which caused hysteresis by rubbing. The needle was rotated to indicate a smaller deflection and hence a smaller force. The micrometer was recalibrated, using a table model Instron, to exert a force of only 50 grams. This device proved to be a rapid, accurate and consistent method for measuring the thicknesses.

Table 9.1 shows the wall thicknesses for several vascular prostheses at a number of points along their lengths. The measurements were taken by measuring the thicknesses of the two walls of the collapsed tubes with the low pressure micrometer
<table>
<thead>
<tr>
<th>Vascular Prosthesis Number</th>
<th>Average Thickness (mils)</th>
<th>Distance from the leading edge of the mold (inches)</th>
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<tr>
<td></td>
<td></td>
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</tr>
<tr>
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<tr>
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<td>3.25 4.3 3.85 4.0 4.7 4.8 5.2 5.15 4.35 4.85</td>
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<tr>
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<td>7.4</td>
<td>5.4 6.4 5.7 6.8 7.5 8.25 8.8 7.6 8.45 8.85</td>
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</tr>
<tr>
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<td>1.9</td>
<td>1.25 1.05 1.4 1.5 1.5 2.85 3.05 2.4 1.75 2.5</td>
</tr>
<tr>
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<td>12.7</td>
<td>17.2 16.15 10.85 10.25 11.15 11.65 13.3 13.4 13.15 9.65</td>
</tr>
</tbody>
</table>
Tensile Strength Testing

The ultimate tensile strength tests and crosslink density tests were performed on a table model Instron tensile tester (Instron Engineering Co., Canton, Massachusetts). The tubes were prepared for the tests by slitting the side with a scalpel and cutting into rectangular sections. During the test, each 1 1/2" x 1/4" specimen was gripped by a pair of special clamps designed to hold a soft membrane.

The clamped specimen was immersed in a large vacuum flask containing physiological saline (0.9 gms of NaCl per 100 ml of water) at pH 7. The temperature of the saline was maintained at 37°C by a temperature controller.

The stress-strain tests were performed using a cross-head speed of 0.2 inches/minute, and a specimen gauge length of 1.0 inch. The gauge length and minimum specimen width were measured using dial gauge calipers. The thickness of the wet specimen was measured with the low pressure micrometer described previously. Engineering stress and strain were used, that is, the original cross-sectional area of the unstressed specimen was used to divide the force to obtain the stress, and the strain was taken as the change in length divided by the gauge length.

Figure 9.1 shows the stress-strain curves for two vascular prostheses, numbers 1 and 8, as described in the preceding chapter.
FIGURE 9.1 STRESS VERSUS STRAIN FOR THE VASCULAR PROSTHESIS.
Number 1 was found to have an ultimate tensile strength of 421 psi, and elastic modulus at break of 6800 psi and elongation at break of 8.3%. Number 8 had values of 504 psi, 2700 and 27% in one test and 529, 3300 and 24% in another test. A crosslinked membrane made from a 12% chondroitin-6-sulfate, collagen dispersion had an ultimate tensile strength of 607 psi, an elastic modulus at break of 4100 psi and an elongation at break of 8.3%. The values of 335 psi, 5500 psi and 6.9% characterized a highly crosslinked membrane made from a glutaraldehyde treated, 1/4% collagen dispersion.

Measurement of Crosslink Density

The kinetic theory of rubber elasticity is obeyed by collagen materials which have been thermally contracted. (Wiederhorn et al 1952 & 1955, Treloar 1958) This theory allows the prediction of the average molecular weight of chains between crosslinks, $M_c$, according to the equation:

$$f = \frac{V_2^{1/3} RT \rho}{M_c} \left( \alpha - \frac{1}{\alpha^2} \right)$$

In this equation, $f$ is the retractive force per unit area, $V_2$ is the volume fraction of the protein, $R$ is the universal gas constant, $T$ is absolute temperature, $\rho$ is the density of non-swollen fibers, and $\alpha$ is the ratio of the extended length to the original length.
The tests are performed by immersing the collagen specimen in physiological saline at 85°C for 5 minutes before testing. Following the denaturation of the collagen by the heat, the specimen is strained at 2.5 or 5% increments of elongation. The equilibrium stresses are recorded at each level of strain until fracture occurs. The procedure used is described in more detail by Yannas and Stasikelis (1978) and Stasikelis (1979).

The vascular prostheses were crosslinked for 24 hours in glutaraldehyde baths with concentrations of 0.25% and 0.5%. Co-workers in the Fibers and Polymers laboratory have prepared and tested materials which are similar in composition to the cross flow filtered vascular prostheses. Stein (1978) reported an $M_C$ value of 2300 for such materials, while Huang (1974) reported $M_C$ values of 1500 to 3800. The crosslink density of the vascular prostheses are expected to be in this range, as 1,500 represents a practical lower limit for $M_C$ in these materials.

Discussion

The average thickness of the vascular prostheses increases with the length of filtration time, as mentioned in the last chapter. However, the thickness measurements taken along each of the artificial blood vessels do not seem to show a clear cut trend. The thickness varies along the length of each prosthesis, usually in a somewhat irregular manner.
A few vascular prostheses indicate a lesser thickness at the entrance to the mold, but it does not seem to be a consistent observation. Visually, the prostheses appear very smooth and uniform, with no obvious seams or obvious wall thickness irregularities.

The tensile strengths correlate well with values found by co-workers in the Fibers and Polymers Laboratory (Huang 1974, Silver 1975). These tensile strength tests indicate that the vascular prostheses should be strong enough to withstand the physiological transmural pressures with a generous safety factor. For example, prosthesis number 8, sustained a tension of 3.7 lb/in, which is about five times that required to prevent bursting of a 0.2 inch diameter (.262 cm, radius) blood vessel (see Chapter IV, Table 4.1). This safety factor is desirable due to the unknown effect of the pulse fatigue upon the collagen materials. Due to the thinner walls of some prostheses, tensions as low as 0.8 lb/in were recorded. This is still adequate to sustain the pressure of a 3 mm diameter vascular prosthesis with a safety factor. Stein (1978) stressed a specimen at fairly high stresses for 60,000 cycles without breakage, before voluntarily discontinuing the test. However, the long term fatigue properties of the collagen vascular prostheses are currently unknown.
Prosthesis number 8 exhibited (Figure 9.1) a 25% elongation at break. This resulted from crosslinking this prosthesis before completely air-drying. The slightly less dense wall could be stretched much farther before failure, while still maintaining a high ultimate tensile strength. Prosthesis number 1, which was completely air dried before crosslinking, was much more rigid, exhibiting an elongation at break of only 8%. The degree of air-drying before crosslinking could, therefore be used to control the elongation at break and elastic modulus of the vascular prostheses. The greater elasticity of vascular prosthesis number 8 is desirable both to make the material easier to suture and to approximate more closely the characteristics of the natural blood vessels.

Cater (1963) showed that glutaraldehyde crosslinking of collagen resulted in a large number of stable crosslinks, with $M_c$ values as low as 3900. Carpentier (1969) found that glutaraldehyde treated collagen exhibits good implantation longevity and no antigenicity in clinical trials lasting over three years. It is felt that the collagen crossflow filtration vascular prostheses should exhibit the same promising characteristics.

From the mechanical point of view, therefore, the vascular prostheses appear to meet the design specifications. The strength is more than adequate to permit suturing and sustain the transmural pressure. The material should not
degrade within the body and the geometric uniformity should prevent the antagonizing of platelets. The coupling of these characteristics with a non-thrombogenic material should result in a successful vascular prosthesis.
CHAPTER X

Discussion of the Collagen Materials for Use as Vascular Prostheses

The material from which the vascular prostheses are made is of primary importance. The technique for processing the bovine hide collagen to create a non-thrombogenic material was found to involve three critical steps: immersion in acetic acid, treatment with glutaraldehyde and air-drying or dehydrothermal treatment. These steps were isolated as the critical ones by comparing the platelet response to the intermediate materials in the fabrication procedure.

The ultrastructures of many of the intermediate materials were also examined. The correlation between the observed ultrastructure and platelet response was not exactly as anticipated. Jaffe and Deykin (1974) and Brass and Bensusan (1974) reported that the quaternary structure of collagen was required for platelet aggregation. This was based on two observations. One was that monomeric collagen, which was not organized into the quaternary structure, required a long lag time before causing platelets to aggregate. The second was that native collagen, which is organized in a quaternary structure as evidenced by the transmission electron micrographic banding, aggregated platelets with a very short lag time. The explanation for this phenomena was believed to be, that the long lag time of the monomeric collagen was required to arrange the
monomers into a native quaternary structure. This banded structure would then cause the platelets to aggregate. The conclusion which was reached was that the quaternary structure was required to cause platelet aggregation.

In this work, all collagen structures which exhibited native banding, were found to cause platelet aggregation, as expected (ie. Samples 2.20:1 and 3.20:1). It was noted, though, that acid swollen collagen samples, such as Samples 4.54:0 and 4.57:0, which exhibited only very low level banding in a few fibrils, also strongly induced platelets to aggregate.

Acetic acid swollen bovine hide collagen fibrils which have received no further treatment do de-swell when subjected to a neutral pH (Sample 4.53:3). The time constant for this de-swelling procedure is not precisely known. Since blood plasma is at neutral pH, it could be argued that the platelet aggregation is subsequent to the de-swelling and reappearance of the native banding. However, some samples were acid swollen and stabilized with glutaraldehyde to prevent a tendency to de-swell or reform the native banding when subjected to a pH 7 medium (Samples 4.53:13 and 4.56:10). These samples, which exhibited no banding caused a strong platelet aggregation reaction. This implies that the quaternary structure is not a necessary requirement to cause platelet aggregation, and agrees with the findings of a recent paper by Muggli (1978).
It should be noted that the structures which were found to lack the native banding are not exactly the same as those described in the literature. The nonbanded monomeric collagen commonly described in the literature exhibits only the tertiary structure of the molecule and does not show any organization at a higher level. The bovine hide collagen specimens, however, which exhibited no banding were still organized into fibrils. These highly swollen fibrils were often 15 to 20 times their original widths, but were still organized in fibrils. The monomeric components of these fibrils were held loosely together, presumably by the unbroken physiological cross-links in the bovine hide collagen. This nonbanded structure reveals that there is a level of organization between the structures which are normally called tertiary and quaternary. It is this structure, which although nonbanded, does aggregate platelets.

From the point of view of this work, which is directed toward creating a non-thrombogenic material, it can be stated that nonbanded swollen collagen is not a sufficient requirement for producing a non-thrombogenic material. All samples which were found to be non-thrombogenic required an acid swelling step which virtually eliminated the banding. The nonbanded structure may, therefore, be a necessary but not a sufficient requirement to create a non-thrombogenic material from collagen.
The samples which were found to be non-thrombogenic were all processed using the critical steps of acid swelling, glutaraldehyde treatment and air-drying or dehydrothermal treatment. The effects of these three steps are as follows. The acetic acid swells the collagen to destroy the quaternary structure, creating the structure which is between the tertiary and quaternary structure, as described earlier. The glutaraldehyde is capable of crosslinking the side chains on the lysine and histidine amino acid groups. (Friedman 1977, pp 178,350), of which there are 32 and 2 residues per 1011 amino acid residues in collagen (Ramachandran and Reddi 1977). The air-drying procedure causes an insolubilization of the samples which is due to a poorly understood mechanism. However, the dehydrothermal treatment is believed to cause insolubilization of the collagen by creating inter-chain amide links (Yannas and Tobolsky 1967). The dehydrothermal treatment is really an extreme form of air-drying which involves heating the specimens in a high vacuum to remove most of the water. It is proposed that each of these three effects are necessary to create a non-thrombogenic material from bovine hide collagen. The implications are that the platelets may require interaction with the quaternary structure, lysine, histidine, amino groups or carboxyl groups of the collagen to initiate the aggregation reaction.

As noted in Chapter IV, choice of the material for
the vascular prostheses is very important. The material must meet the design specifications of biological compatibility, adequate strength and fatigue resistance.

The bovine hide collagen materials which are recommended for use in vascular prostheses follow two manufacturing procedures. One procedure is to allow the collagen to swell in acetic acid for at least 3 days followed by forming the tube, air-drying and crosslinking in a glutaraldehyde bath (Sample 4.53:2). The alternate procedure is to add glutaraldehyde to the swollen collagen dispersion, form the tube, air-dry and crosslink in a glutaraldehyde bath (Samples 4.53:12 and 4.56:5). Chondroitin-6-sulfate can also be added in this procedure, following the addition of the glutaraldehyde to the dispersion (Samples 4.53:17 and 4.56:9).

All of these materials are similar in ultimate tensile strengths and can meet the stresses encountered by small blood vessels. Additionally, the strength required for suturability is also exceeded. Of course, none of these materials exhibited any tendency to aggregate platelets. In conclusion, the vascular prostheses described in this thesis appear to hold promise, and should prove themselves in implantation studies.
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