Surface Properties Of The Arterial Wall And Their Relevance To Atherosclerosis

John Frederick Boyce
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THIS DISSERTATION HAS BEEN MICROFILMED EXACTLY AS RECEIVED
SURFACE PROPERTIES OF THE ARTERIAL WALL
AND THEIR RELEVANCE TO ATHEROSCLEROSIS

by

John Frederick Boyce
Department of Biophysics

Submitted in partial fulfillment
of the requirement for the degree of
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Faculty of Graduate Studies
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ABSTRACT

Adhesion of platelets to the arterial subendothelium exposed by injury to the endothelium, is thought to initiate atherosclerosis. Thermodynamically, adhesion will occur if it decreases the free energy of the system. My initial objective was to determine if changes in the thermodynamic properties of the arterial surface could be shown which supported a physical model of adhesion.

In this thesis, I have developed a contact angle technique to measure relative changes in the interfacial free energy of the arterial wall, before and after removal of the endothelium. Sections of rabbit aorta were tested with an equilibrium two phase system of 4% poly (ethylene glycol) (PEG)/4% dextran, in buffered physiological saline. The tissue was immersed in the PEG phase and droplets of the denser dextran phase were placed on its lumenal surface. After testing, the endothelium was removed with a saline jet, and the tissue was retested. Contact angles of the droplets were measured from photomicrographs. Angles of 86.0 ± 1.1° (SEM), n = 64 for the intact endothelium, and 20.0 ± 0.08° (SEM), n = 61 on the subendothelium, were measured. Since the physical behavior of blood is similar to the PEG phase, the subendothelium probably

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represents a high energy surface in vivo. Adhesion of platelets could reduce the free energy of the system, by covering this high energy surface.

Factors which cause endothelial injury may act chronically to cause desquamation. Since injury mechanisms must act at the vessel/blood interface my second objective was to detect changes of the endothelial surface before sudanophilic lesions appeared.

Rabbits fed a 2% cholesterol diet to induce atherosclerosis were divided into 3 experimental groups: continuous feeding, interrupted feeding, and controls. Areas proximal and distal to intercostal orifices, where no plaque had yet developed, were tested with the PEG/dextran system. Proximal areas on the diseased arteries, and both the proximal and distal locations in controls gave contact angles of approximately 90°; however those areas distal to orifices, where lesions eventually occur, yielded significantly different angles (p < 0.05), approximately 78°. This difference indicates a change in the glycocalyx, which could weaken the cell resistance to injury, and ultimately lead to desquamation, platelet adhesion and plaque formation.
To

Les and Ruth –

my parents
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"This is magic – I can tell by the feeling. Quick! ..... Next moment the luggage, the seat, the platform, and the station had completely vanished. The four children, holding hands and panting, found themselves standing in a woody place – such a woody place that branches were sticking into them and there was hardly room to move. They all rubbed their eyes and took a deep breath ....."

C.S. Lewis – Prince Caspian
1.1 Introduction

Seventy-six thousand or 45% of all the people that died in Canada in 1981, perished from some form of cardiovascular disease (Statistics Canada, 1981). A large proportion of this mortality may be directly or indirectly attributed to the disease known as atherosclerosis and its complications. Atherosclerosis is a specific pathological process that the World Health Organization in 1958 defined as a "variable combination of changes of the intima of the arteries consisting of an accumulation of lipids, complex carbohydrates, blood and blood products, fibrous tissue and calcium deposits, and associated with medial changes" (World Health Organization, 1958; quoted by Woolf, 1982).

It is a disease that is not new to mankind. Arterial lesions of well preserved Egyptian mummies were extensively investigated by Ruffer (1911) and more recently by Sandison (1962). Ruffer reported regularly finding calcified plaques in the aorta and its branches, and concluded that the disease was at least as common among the ancient Egyptians as it is in developed countries today.

The following section will examine our understanding of the pathology of atherosclerosis and how it has developed through the ages.
1.2 Historical Review of the Pathology of
Atherosclerosis\(^1\)

While educated physicians in the 16th and 17th
centuries were probably aware of the "ossified" lesions
which developed in arteries, no real progress in
understanding the pathology of atherosclerosis was made
until the 18th century. This might be attributable to
the prevailing view that the "ossification" of arteries
was not a disease but rather a natural phenomenon of
aging; perhaps a compensatory mechanism to keep the
arteries open as the heart gradually failed. Johann
Friedrich Crell of Helmstadt in 1740 was the first to
identify calcified plaque as a non-bony, gritty
substance, and postulated that it was derived from
hardened pus (Leibowitz, 1970; p. 76). Further, he took
the radical view that the development of lesions was
independent of age and that they could occur at any time
in a person's life. Albrecht von Haller of Berne
extended the views of Crell in 1755 by concluding that a
gradual, uninterrupted process caused the eventual
formation of hardened, bone-like plaque from the soft
material which he called atheroma. The most extensive

---

\(^1\) Most of the historical data and quotes of this
section were obtained from an excellent review by Long
(1933) in Cowdry's Arteriosclerosis. Other sources are
cited where appropriate.
work of the period on the lesions of atherosclerosis was written by Giovanni Battista Morgani in 1761. Morgani gave detailed and extensive descriptions of lesions with clinical correlations in his treatise: "On the Seats and Causes of Disease Investigated by Anatomy" (Leibowitz, 1970; pp. 77-80).

In the 19th century the development of chemical and histological techniques caused an evolution in vascular pathology from being essentially descriptive in nature, to a distinct science. Xavier Bechot of Paris in 1801 identified the intima as the site of "ossification" in early lesions. Although he considered the accumulation of calcified deposits as a natural feature of the aging process, he clinically related it to many of the symptoms formerly credited to a failing heart. In 1815 Joseph Hodgson of London published an extensive monograph entitled: "Treatise on the Diseases of Arteries and Veins". In this work Hodgson was responsible for arranging the first chemical analysis of calcified plaque to ascertain scientifically whether the hardened material was true bone in composition or not. Since no "carbonate of lime" was identified he concluded that the hardened atheromatous lesions were not derived from bone material. After much consideration Hodgson became convinced that the condition of "hardening of the arteries" was in fact a true disease, rather than a
consequence of aging because the extent of arterial degeneration was not proportional to the age of the subject.

The middle of the 19th century saw the two major theories of atherogenesis proposed. In one form or another, many features of these two original hypotheses persist today:

Karl von Rokitansky treated the subject of atheromatous deposition extensively in his "Manual of Pathological Anatomy" (1844). In his own words (quoted by Long, 1933) "The deposit cannot be regarded as an exudate from the inflammation of the artery. It is an endogenous product from the blood mass, and one derived preponderantly from the fibrin of the arterial blood. Its development is evidence of the existence of a particular blood crasis, which is intrinsically arterial". Thus von Rokitansky described a typical atheroma and postulated that it results from degeneration of blood proteins (notably fibrin) on the arterial intima. Rokitansky's theory is popularly referred to today as the "encrustation theory".

Rokitansky's views were strongly opposed by Rudolf Virchow a few years later in 1856 on the grounds that lesions found underneath the endothelium could not be derived from surface deposits. Virchow regarded the pus-like atheromatous material to be the result of an inflammation of the intima. The inflammation was
proposed to be caused by the mechanical "irritation" of the blood at the affected point, i.e. haemodynamic stress. The final result of this process was a fatty degeneration of the connective tissue of the intima and a softening of the internal ground substance. Because Virchow considered the early deposits to be an infiltration of lipids from the passing blood his theory became referred to as the "imbibition" hypothesis.

1.3 The Response to Injury Hypothesis of Atherogenesis

The two main theories of Rokitansky and Virchow have each undergone substantial modification to the present where they have been integrated into a single complex theory known as the "response to injury hypothesis". This theory proposes that atherogenesis results from a precise series of critical events involving the interactions of the various blood components with the arterial wall. The primary events leading to the development of an atherosclerotic plaque appear to be: (A) injury to the endothelial layer; (B) platelet adhesion and aggregation; (C) smooth muscle cell proliferation; (D) entry and accumulation of lipid; (E) fibrosis and development of thrombi; and finally (F) calcification and ulceration (Ross and Glomset, 1976).
1.3.1 **Endothelial Injury**

Hyperlipidemia, immunological disfunction, high shear stress, and other factors have been implicated as possible agents of endothelial injury (Ross and Harker, 1976; Minick, 1981; Glagov, 1971). These factors may disrupt the cell-cell relations of the endothelium or affect the adhesive contacts with the substrate, to permit haemodynamic forces to lift and detach endothelial cells from the arterial wall. A haemodynamic mechanism is favoured because there is a strong correlation between the regions of maximal shear stress determined from model studies (Wesolowski et al., 1965; Adamson and Roach, 1981) and those locations around branch points where atherosclerotic lesions tend to develop (Cornhill and Roach, 1974; Fry, 1973; Flaherty et al., 1972). Also experimental injury designed to mimic the mechanical injury believed to be produced by haemodynamic factors, can reproduce the full spectrum of early atherosclerotic lesions (Lee and Lee, 1975; Moore, 1981). Regardless of what the true agent of endothelial injury is however, the end result according to the hypothesis is endothelial desquamation and exposure of the subendothelial connective tissue matrix.
1.3.2 **Platelet Adhesion and Aggregation**

While the intact arterial wall is normally thromboresistant, exposure of the subendothelial connective tissue immediately causes platelets to adhere at the site of injury (Groves et al., 1979; Stemmerman, 1973). The mechanism responsible for this platelet "sticking" remains unknown. Following adhesion, the platelets interact with the fibrillar collagen present in the subendothelium and release materials which cause the aggregation or "clotting" of other platelets at this location (Mustard and Packham, 1970; Weiss, 1975).

1.3.3 **Smooth Muscle Cell Proliferation**

Contained within the granules released by the activated platelets is a substance identified by Ross et al. (1974) simply as: "platelet derived growth factor" (PDGF), which has been shown to stimulate the growth of fibroblasts and smooth muscle cells (Kohler and Lipton, 1974; Rutherford and Ross, 1976). The smooth muscle cells, normally resident in the medial layer, respond to the secreted platelet stimulant (and possibly other plasma constituents such as hormones and lipoproteins) by migrating into the intimal region where they proliferate (Ross and Glomset, 1973). This causes a small focal enavagitation of the arterial wall into the vessel lumen, which enlarges as the smooth muscle cells
produce connective tissue (types I and II collagen, elastin and proteoglycans) (Fisher-Dzoga et al., 1973), and as lipids accumulate.

1.3.4 Entry and Accumulation of Lipids

Disruption of the blood barrier by injury of the endothelium exposes the subendothelium to plasma lipoprotein deposition. Smith et al. (1968) have shown that the deposit is primarily cholesteryl oleate intracellularly and free cholesterol with cholesteryl linoleate extracellularly in the connective tissue matrix (Smith, 1974). This difference between the intracellular and extracellular cholesterol esters implies that cells of the arterial wall have an active role in accumulating lipid (Small, 1977). Brown and Goldstein (1976) have suggested that transport of low-density lipoprotein into the cell is mediated by surface receptors on arterial smooth muscle cells. In a recent study, Boyce et al. (1980)² have shown that lesion surfaces are significantly more hydrophobic, and hence more attractive as sites of continued lipid deposition than undiseased or non-plaque areas. Elastin has an important role in binding extracellular lipid in the

² The cited work was completed as a year IV undergraduate project and is not included as part of this thesis.
arterial wall and there is a substantial increase in the amount of this material bound by elastin in atherosclerotic lesions (Kramsch and Hollander, 1973). While the continued deposition and transport of lipids certainly contributes to the size of the lesion, there is evidence that lipoproteins may stimulate the growth and proliferation of smooth muscle cells to further add to the mass (Myasikov and Block, 1965). Ross and Harker (1976) pointed out that the lipid may simply act as nutrients for growing smooth muscle cells which are actively producing new membrane material.

1.3.5 **Fibrosis and Thrombosis**

Smooth muscle cells can synthesize all three of the characteristic connective tissue matrix components: collagen (Ross and Klebanoff, 1971), elastin (Narayanan et al., 1976) and proteoglycans (Wight and Ross, 1975). Of the three, type I collagen is the major component contributing to the growth of the atherosclerotic plaque, although type III collagen predominates in the normal aortic tunica media (McCullagh and Balian, 1975). In contrast, the amount of elastin in atherosclerotic plaques appears to be lower than in normal arteries (Kramsch et al., 1971), and the elastin may have some role in binding lipid within the arterial wall as stated earlier. Urry (1974), noting that elastin binds calcium, has suggested that the elastin fibres are the principal
site of calcification in the arterial wall. The glycosaminoglycans may play a major role in the binding and transport of lipoproteins because complexes of these components have been identified in fatty streaks (Srinivasan et al., 1972) and fibrous plaques (Srinivasan, 1979). Most fibrous atherosclerotic plaques progress very slowly, but in the cases where rapid progression occurs a thrombotic mechanism may be responsible (Fuster and Cheseboro, 1982). Thrombi composed of fibrin, platelets, leucocytes and red cells have been identified on the surface of atherosclerotic plaques (Warren et al., 1978). Within days re-endothelialization of the surface begins, and after ten days the thrombus material has become incorporated in the vessel wall (Woolf, 1981). The thrombus is subsequently converted to fibrous tissue (Haust, 1981), a process which may be responsible for the accelerated rate of atherosclerosis observed in some patients (Fuster and Cheseboro, 1982; Chandler et al. 1974).

1.3.6 Calcification and Ulceration

While calcification of arteries occurs normally with age, its incidence is unusually high in lesions. This is the "ossification" of arteries which the early pathologists referred to in the first published works on the subject. The calcium deposits are primarily associated with the elastin fibres which become
fragmented as the age of the plaque increases (Kramsch and Hollander, 1973). The degeneration of the elastin elements causes ulceration of the lesion surface and eventual weakening of the arterial wall (Yu, 1974) probably leading to aneurysm formation.

1.4 Objectives and Rationale for this Study

Several features of the response to injury hypothesis focus on phenomena occurring at the surface of the arterial wall. Perhaps the most conspicuous surface event is the adhesion of platelets to the subendothelium after the endothelium has been injured and removed. No less important however, is the fact that the various injury mechanisms, whether they be mechanical, chemical or immunological in nature, also act on the vessel surface. The surface of the artery is where the lipoprotein deposition occurs which ultimately leads to lipid accumulation within the vessel wall.

Therefore, in terms of atherogenesis, the arterial surface appears to be important. Unfortunately, studies which investigate the specific role of the arterial surface in atherogenesis from a physical perspective, cannot be found in the literature. The main objective of this thesis is to conduct such a study.

I will concern myself with the two main questions:
(1) Why do platelets not stick to the healthy endothelial surface yet adhere rapidly to the underlayer which is exposed by endothelial injury? and (2) Are the surface properties of the artery affected by the onset of atherosclerosis?

To attempt to answer these questions it is necessary to first adapt thermodynamic methods already used in physical systems to study surfaces.

1.5 Thermodynamics of Surfaces

Because this thesis deals with the chemistry of biological interfaces, I do not feel comfortable treating the junction between two bulk phases simply as a two-dimensional plane having no thickness, as Gibbs does (Gibbs, 1878). The very nature of the cell surface with its "dangling" membrane proteins makes a definition of a sharp dividing surface virtually impossible. For this reason I have adopted Guggenheim's approach (Guggenheim, 1940), which treats the interfacial region as a separate phase having a finite thickness and area. This "interphase" can be described with all the same thermodynamic variables used to characterize regular bulk phases except that the thickness parameter cannot be varied at will by the experimenter. Hence, the interphase is usually termed a 2-dimensional phase.
Consider a system consisting of 2 bulk phases A and B separated by a surface phase S. Any change in the internal energy of the system (U) may be accounted for by the exchange of heat and the performance of work, and is expressed by the First Law of Thermodynamics:

\[ dU = q + w \]  \hspace{1cm} [1 - 1]

where \( q \) is the quantity of heat taken in and \( w \) is the work done on the system. Substituting the definition:

\[ q_{\text{reversible}} = TdS \]  \hspace{1cm} [1 - 2]

into equation [1 - 1] gives:

\[ dU = TdS + w \]  \hspace{1cm} [1 - 3]

where \( T \) is the absolute temperature and \( S \) is the entropy of the system.

Work is done on the system when the volume of the system changes against an external pressure. Thus

\[ w_{\text{displacement}} = -pdV \]  \hspace{1cm} [1 - 4]

When \( dV \) is positive, the work done is negative and the internal energy of the system decreases. Conversely with
a negative change in \( dV \), i.e. a compression, work on the system is positive and hence the internal energy increases.

Since a small drop of liquid spontaneously contracts to form a sphere (the minimum surface to volume ratio) we know that there is energy associated with the surface and work must be done to increase it. Representing the work per unit area that must be performed by \( \gamma \), and the change in the area of the interphase by \( dA \) we have:

\[
W_{\text{surface}} = \gamma dA \quad [1-5]
\]

Thus including [1 - 4] and [1 - 5] as the total work in [1 - 3] gives:

\[
dU = TdS - PdV + \gamma dA \quad [1-6]
\]

for any system containing an interface.

Using standard definitions for the enthalpy \( (H) \), Helmholtz \( (F) \) and Gibbs functions \( (G) \):

\[
H = U + PV
\]

\[
F = U - TS
\]

\[
G = H - TS
\]

we can write for the differentials:

\[
dH = dU + PdV + VdP \quad [1-7]
\]

\[
dF = dU - TdS - SdT \quad [1-8]
\]

\[
dG = dH - TdS - SdT \quad [1-9]
\]

Substituting \( dU \) and \( dH \) in [1 - 7], [1 - 8] and [1 - 9]:

\[
\int dU = \int TdS - \int PdV + \int \gamma dA
\]

\[
\int dF = \int dU - \int TdS - \int SdT
\]

\[
\int dG = \int dH - \int TdS - \int SdT
\]
\[ dH = TdS + VdP + \gamma dA \]  \[ 1 - 10 \]
\[ dF = -SdT - PdV + \gamma dA \]  \[ 1 - 11 \]
\[ dG = -SdT + VdP + \gamma dA \]  \[ 1 - 12 \]

For systems, which have changeable chemical compositions, the Gibbs and Helmholtz functions become:

\[ dG = -SdT + VdP + \gamma dA + \sum_i \mu_i dn_i \]  \[ 1 - 13 \]
\[ dF = -SdT - PdV + \gamma dA + \sum_i \mu_i dn_i \]  \[ 1 - 14 \]

where \( \mu_i \) is the chemical potential and \( n \) is the number of moles of species \( i \). Therefore from equations \[ 1 - 13 \] and \[ 1 - 14 \] we may define:

\[ \gamma = \left( \frac{\partial G}{\partial n} \right)_T, P, n_i \]
\[ n = \left( \frac{\partial F}{\partial A} \right)_T, V, n_i \]

\[ 1 - 15 \]

The Gibbs and the Helmholtz functions (or free energies) are preferred for describing chemical and physical systems because they yield work terms when used under the appropriate conditions. For example at constant temperature the change in the Helmholtz function yields the maximum work attainable from the system:

\[ dF = dU - TdS = -PdV + \gamma dA \]
\[ dF = \omega_{\text{max}} \]  \[ 1 - 16 \]

For bulk phases the change in the Gibbs free energy at constant \( T \) and \( P \) was designed to give the net or "useful" work available from the system, i.e. the work in excess of the work of displacement (volume change). However, when a system which includes an interface is considered, the change in the Gibbs function is not
equal to the net work because it includes the $\gamma dA$ term which cannot be used to do "useful work" (Jaycock and Parfitt, 1981):

$$dG = dU - TdS + PdV = w_{max} + PdV \text{ (const. } T,P)$$

$$\therefore dG \neq w_{net} \quad [1 - 17]$$

because $\gamma dA$ is still included in the work term.

To circumvent this difficulty with the Gibbs function, Guggenheim (1967) redefined the enthalpy, $H$ and hence the Gibbs function, to give a new function which yields the net work for a system independent of the presence or absence of an interface. Such a treatment has yet to be widely adopted. Instead, the question of whether interfaces should be described with the ordinary Gibbs, or Helmholtz functions, remains controversial. Good (1977) claims that it does not matter, since according to Gibbs' classical treatment, the volume of the interface is zero. However, since Guggenheim's "interphase" approach treats the interface as having a finite thickness and volume, Good's statement may be questioned.

For this thesis I will adopt the use of the Helmholtz free energy $F$, as does Johnson (1959) in his thermodynamic treatment of a solid/liquid/vapour system. Johnson uses $F$ because the variables: $P$, $V$, $\gamma$ and $A$ must be considered explicitly when using it. For this reason his approach will probably be the least confusing to the reader.
Much of the material presented in this thesis has come from three papers which have been previously published, or submitted for publication. When pertinent, I have noted the bibliographic details in a footnote at the appropriate place in the text. I have already referred to a fourth published paper (Boyce et al., 1980) on relevant material which is not included in the thesis. Since all of these publications have been co-authored with other individuals, I will try to note explicitly, those contributions which were not primarily mine.
CHAPTER 2

METHODS OF STUDYING SURFACE PROPERTIES OF SOLIDS
AND APPLICATIONS TO BIOLOGICAL MATERIALS

"That meant that I came to have a fair idea
what sort of things might be in the box. By
various tests I narrowed down the
possibilities. I had to get to know some
—well, some devilishly queer people, and go
through some very disagreeable experiences.
That was what turned my head grey. One
doesn't become a magician for nothing."

C.S. Lewis — The Magician's Nephew
2.1 Introduction

Building a house with nothing but your bare hands is impossible, but the task becomes trivial when one has the proper tools. This chapter is a comprehensive look at the tools required to investigate the surfaces of biological materials. In the broadest sense it is a methods chapter, devoted to familiarizing the reader with the background, development and specific applications of the techniques used in the investigations I will discuss later.

The first section introduces the contact angle as our investigative tool, and reviews its theoretical foundations. The development and evolution of the contact angle method is discussed. In the second section, I examine the advantages and disadvantages of different systems for testing biological surfaces. The section following this, introduces a new method of calculating contact angles and interfacial tensions. Here the method is explored, and tested. Finally, the chapter ends with the details of an attempt to calibrate the system I have chosen for my experimental studies.

2.2 Contact Angles and Wetting

If a liquid droplet is placed on a smooth, homogeneous, solid surface one of three things may happen:
FIGURE 2 - 1

The work derivation of Young's Equation; $\theta$ is the contact angle and $dA$ is an incremental change in the area of the contact region.
Bulk Fluid

$dA \cdot \cos \theta$

$\theta$

$dA$

Liquid

Solid
(1) It may spread completely in a film over the surface. This is known as complete wetting.

(2) It may bead up, making only point contact with the solid surface. This is referred to as non-wetting.

(3) It may do something in between and form an angle of contact at the junction of the three phases on the solid surface.

If we take such a drop and deform it so the contact region is changed by an increment of area \( dA \), (Figure 2-1) we increase the solid/liquid and liquid/bulk fluid interfaces. Therefore we may write for the total change in free energy:

\[
dF = -\gamma_{SB} dA + \gamma_{SL} dA + \gamma_{LB} \cos \theta dA \quad [2 - 1]
\]

where: \( dA \) is the change in interfacial area and \( \theta \) is the contact angle. \( S \), \( L \) and \( B \) represent the solid, liquid and bulk phases respectively. However for a system in equilibrium the net change in energy produced by a small shift in the position of the interface must be zero, therefore

\[
-\gamma_{SB} dA + \gamma_{SL} dA + \gamma_{LB} \cos \theta dA = 0 \quad [2 - 2]
\]

or

\[
\gamma_{SB} - \gamma_{SL} = \gamma_{LB} \cos \theta \quad [2 - 3]
\]

---

1 Since the bulk fluid phase is usually a vapour, most authors use the symbol \( V \). I will use \( B \) here to represent the general case where the bulk fluid phase may be liquid or vapour.
Since equation [2 - 3] was first expressed qualitatively as an equilibrium of forces by Thomas Young in 1805, it has become known as Young's equation. More rigorous thermodynamic derivations of Young's equation are given by Johnson (1959) and Boruvka and Neumann (1977).

Young's equation is deceptively simple. Although it establishes a relation between the two solid interfacial free energies, neither of these individual parameters can be conveniently or reliably measured. Unfortunately Young's equation is also the only general relation connecting interfacial free energies, no other theoretical or general equations are known. Therefore, since two unknown quantities are involved, one equation is insufficient to determine the solid interfacial free energies; the best we can do is determine the difference: $\gamma_{SB} - \gamma_{SL}$.

2.2.1 Critical Surface Tension of Wetting: Zisman's Approach

The first advance towards circumventing the difficulties of Young's equation was made when William Zisman and colleagues introduced the concept of critical surface tension of wetting. Zisman found that for a given low energy (non-metallic) solid tested with a homologous series of liquids (e.g. n-alkanes) a linear relation existed between $\cos \theta$ and the surface tension
(γ_{LB}) of the liquids. The critical surface tension of wetting, γ_c was defined as the extrapolated value for γ_{LB} where cos θ = 1.0 (Fox and Zisman, 1952a and b). The basis of this method is the assumption that as θ approaches zero γ_{SL} also approaches zero in a smooth and orderly fashion (Good and Girifalco, 1960; Fox and Zisman, 1952b). Thus at cos θ = 1.0, γ_{SB} = γ_{LB} = γ_c. γ_c is a very useful parameter because it permits the surface properties of various solids to be compared quantitatively. Zisman however does caution about using γ_c to define γ_{SB} because he notes that γ_c can vary between liquid types for the same solid substrate.

Unfortunately the linear nature of the cos θ vs γ_{LB} plot seems to depend on liquids where the intermolecular forces are primarily van der Waals in nature. Polar liquids and solids in which hydrogen bonding is the predominant interaction cannot be described as a linear relationship of cos θ vs γ_{LB} (Zisman, 1964). Good and Girifalco (1960) were able to extend Zisman's approach to a wider range of data and obtain a straight line by plotting cos θ vs (γ_{LB})^{-1/2}, but the data scatter was still substantial. The scatter of the data arises from the difficulties of accurate contact angle measurement and contact angle hysteresis.

Although a major advance in characterizing the surface properties of solids, Zisman's method has several limitations:
1) $\gamma_c$ is an empirical parameter derived without a sound thermodynamic or statistical mechanical basis (Zisman, 1960),

2) $\gamma_c$ is only a valid estimator of $\gamma^{SB}$ for a limited number of hydrophobic liquids and solids and

3) $\gamma_c$ is tedious and inconvenient to determine experimentally.

2.2.2 Neumann's Equation of State

Major progress in solving Young's equation was made in 1974 when Ward and Neumann provided a thermodynamic argument for the existence of an equation of state relating $\gamma^{SL}$ to $\gamma^{SB}$ and $\gamma^{LB}$. Ward and Neumann (1974) began their argument by using the classical Gibbsian definition of the surface free energy. For the general case:

$$\gamma = u - Ts - \sum_{i=2}^{r} \gamma_i u_i [2-4]$$

where $u$ and $s$ are the internal energy and entropy per unit surface area. The adsorption of component $i$ at the surface is represented by $\gamma_i$. Since the solid component, i.e. component 1, is assumed to be completely insoluble, the summation is from the second to $r$th component of the system.

From the general equation [2-4] differentials may be derived to reveal the independent variables affecting each interface of the system. For the solid/bulk fluid interface:
\[ d\gamma_{SB} = -s_{(1)}^{SB} dT - \sum_{i=2}^{r} r_{i(1)}^{SB} d\mu_i \]  

Similarly, for the solid/liquid interface:
\[ d\gamma_{SL} = -s_{(1)}^{SL} dT - \sum_{i=2}^{r} r_{i(1)}^{SL} d\mu_i \]  

Following Gibbs' convention, the subscript (1) indicates that the position of the dividing surface is chosen so that there is no adsorption of the solid component, i.e. component 1.\(^2\)

For the liquid/bulk fluid interfacial tension, the position of the dividing surface is chosen to be the surface of tension. This definition could not be used previously because for any solid in a state of strain,

\(^2\) Because of its simplicity I have chosen to present Ward and Neumann's derivation which treats the interface as having no thickness and hence zero volume. If Guggenheim's approach were used another term in the defining equation for \(\gamma\) would be necessary to account for the volume and the pressure gradient within the interfacial region. The end result of Guggenheim's treatment would be three defining equations of three independent variables. To be analogous with Ward and Neumann's approach (i.e. \(n\) independent variables and \(n + 1\) equations) a fourth relation would be necessary which gives the dependence of \(\gamma\) on the two principal radii of curvature for different values of \(r\) and all \(\mu_i\). The derivation of such an equation is not trivial (see Guggenheim, (1940) for additional discussion).
\( \gamma_{LB} \) cannot be interpreted as a compressive force or "tension" in the plane of the surface (Johnson, 1959). Therefore:

\[
d \gamma^{LB} = -s^{LB} dT - \sum_{i=2}^{x} r_{i}^{LB} d\mu_{i} \quad [2-7]
\]

Ward and Neumann applied these equations to a system of three phases (solid, liquid and bulk fluid) and two components, where the liquid phase and the bulk fluid (in this case a vapour) consists of the same pure component. Equations: [2-5], [2-6] and [2-7] then become:

\[
\begin{align*}
    d \gamma^{SB} = & -s^{(1)}^{SB} dT - r_{2(1)}^{SB} d\mu_{2} \quad [2-8] \\
    d \gamma^{SL} = & -s^{(1)}^{SL} dT - r_{2(1)}^{SL} d\mu_{2} \quad [2-9] \\
    d \gamma^{LB} = & -s^{LV} dT - r_{2}^{LV} d\mu_{2} \quad [2-10]
\end{align*}
\]

The three equations above are functions of only two independent variables: \( \gamma \) and \( \mu_{2} \). Therefore, one of the above equations must be expressible in terms of the other two and the relation:

\[
\gamma^{SL} = f(\gamma^{SB}, \gamma^{LB})
\]

must exist for such a system. Neumann called this relation an equation of state. Together with Young's equation it allows \( \gamma^{SL} \) and \( \gamma^{SB} \) to be individually determined from measurements of \( \gamma^{LB} \) and \( \cos \theta \). The details of the empirical derivation of the equation of state appear in Neumann's next paper (Neumann et al., 1974).
Neumann observed that contact angle data plotted as $\gamma_{LB} \cos \theta$ vs $\gamma_{LB}$ resulted in a smooth curve which could be fitted with a second order polynomial equation. He used the intersection of this curve and the line of equality produced when $\theta = 0$ (since $\gamma_{LB} = \gamma_{LB} \cos \theta$ when $\cos \theta = 1$), to define $\gamma_{SB}$ for the solid. To define $\gamma_{SB}$ in this way, Neumann made the assumption that $\gamma_{SL} = 0$ when $\theta = 0$ for any fluid on an ideal solid (i.e. a solid that is smooth and homogeneous, and produces no contact angle hysteresis). Since $\gamma_{SB}$ is a constant for any homogeneous surface, Neumann could define $\gamma_{SL}$ as a function of $\gamma_{LB}$ and $\cos \theta$, using Young's equation and various contact angle data. With values of $\gamma_{SL}$ and $\gamma_{SB}$ which he determined for a series of eight solids and various liquids, Neumann was able to determine a generalized expression for Good's interaction parameter ($\phi$) which was a function of $\gamma_{SL}$ only. The definition of $\phi$ is:

$$\phi = \frac{\gamma_{SB} + \gamma_{LB} - \gamma_{SL}}{2 (\gamma_{SB} \gamma_{LB})^{1/2}}$$

[2 - 11]

Setting the derived relation, $\phi = \phi (\gamma_{SL})$ equal to the defining equation for the interaction parameter and substituting for $\gamma_{SL}$ in Young's equation gives the final result:

\[\text{---------}\]

3 Note that the definition of $\phi$ is incorrect in Neumann's original paper.
\[(\gamma_{SB})^3 - (\gamma_{SB})^2 \left[ \frac{4}{a} + 2\gamma_{LB} \cos \theta \right] + \gamma_{SB} \left[ \frac{\gamma_{LB} \cos \theta + 2}{a} \right]^2 \]
\[= \gamma_{LB} \left( 1 + \cos \theta \right)^2 \]

where \(a\) is a constant equal to the average slope of lines plotted (\(\phi\) vs \(\gamma_{SL}\)) for all 8 solids; \(a = 0.015\) \(m^2 \cdot mJ^{-1}\).

Although Neumann's equation of state was derived for a three phase-two component system, it has been successfully applied to a three phase-three component system. Schürch and McIver (1981) were able to test the equation of state using an oil droplet/water system on an adjustable monolayer substrate. They found that values of \(\gamma_{SB}\) calculated with the equation of state were generally within 1% of the experimentally determined results.

2.3 Applications to Blood Vessel Surfaces

2.3.1 Air/Liquid Systems

The first attempt to analyze the surface properties of the blood vessel wall using contact angles was made in 1970 by Bäuer, Dutton and Gott who were interested in duplicating the thromboresistance of arteries in synthetic materials. They formed air bubbles on the venous wall within various hydrophobic and hydrophilic fluids, and measured contact angles. Several aspects of their experimental design can be criticized:
1) The normal bulk fluid, blood, was replaced by a variety of different fluids (e.g.: methyl iodide, 1-bromonaphthalene, isopropylbiphenyl etc.); almost all of these would provide a hazardous environment for living cells.

2) Air bubbles were used as the test phase, which implies the measurement of an endothelium/air interface. This an artificial situation for the vessel wall and it exposes the endothelial surface proteins to possible denaturation at the test locations.

3) The large surface tension of the air/bulk-liquid interface produced high contact angles which are difficult to measure accurately especially for small drops or bubbles.\(^4\)

The substance of the last criticism becomes apparent when one calculates (using Neumann's equation of state) the free energy of the endothelium/air interface. This should remain the same in each test. According to Baier's data and Neumann's equation,

\[ \text{------------------} \]

\(^4\) The small angles reported in the Baier, Dutton and Gott paper have been given according to the convention of treating the vapour phase as the bulk phase and the liquid as the drop phase. They are the complements of the angles referred to above.
surface free energies (i.e. $\gamma_{SB}$) for the same interface vary from 0.002 to 11.95 mJ·m$^{-2}$, a range of over 4 orders of magnitude.

2.3.2 Oil/Saline System

About ten years after Baier's study, Boyce, Schurch and McIver (1981) sought to avoid some of the criticism of his approach by estimating arterial surface properties with an oil/saline system. Drops of a variety of immiscible fluids, all denser than water, with interfacial tensions between 7.5 and 24.0 mJ·m$^{-2}$, were placed on the lumenal surface of rabbit arteries immersed in buffered physiological saline. Calibration experiments indicated that Neumann's equation of state could be used to calculate the saline/arterial wall interfacial free energies from contact angle measurements since the interfacial tensions were relatively high.

The oil/saline system has several advantages over the original methods of Baier and coworkers (1970): The arterial tissue is completely immersed in buffered physiological saline, rather than in an organic bulk-fluid. Because cells in the presence of saline maintain metabolic activity, the test will be on a living cell surface. The method also minimizes the potential for denaturation of the cell surface proteins. Use of the equation of state permits the calculation of standard
free energy changes, which, in turn, allows biological tissues tested with different fluids to be compared quantitatively. Finally, the large difference between the indices of refraction of oil and water causes the droplets to be easily seen and photographed.

Nonetheless, the oil/saline system is not without its rather significant short-comings: On arterial surfaces, oil droplets always yield contact angles in excess of 140°. An explanation of this observation may be obtained from a consideration of Young's equation:

\[ \gamma_{SB} - \gamma_{SL} = \gamma_{LB} \cos \theta \quad [2-3] \]

Because the interfacial tension between the oil droplet and the water bulk phase is great, the contact angle of the drop on the arterial surface will be large, i.e. greater than 120°. Thus, the \( \gamma_{LB} \cos \theta \) term will be negative and its magnitude will increase as the contact angle approaches 180°. This means, according to Young's equation, that \( \gamma_{SL} \) must be substantially larger than \( \gamma_{SB} \) at higher contact angles. \( \gamma_{SB} \) will always be small compared to \( \gamma_{SL} \) because the hydrated arterial surface is chemically similar to the water bulk phase. Conversely, \( \gamma_{SB} \) is large because the oil droplet is chemically different from the arterial tissue. Therefore, when oil is used as a test droplet, small changes in \( \gamma_{SB} \) (which
FIGURE 2 - 2

Plot of the substrate/bulk fluid interfacial energy ($\gamma^{SB}$) (calculated with Neumann's equation of state) vs contact angle ($\theta$) for a hypothetical liquid. Note the rapid decline of $\gamma^{SB}$ with contact angles greater than 120°.
Surface Tension
of Liquid: 14.2 mJ·m²

Interfacial Free Energy $\gamma_{SB}$ (mJ·m²)

Contact Angle: $\theta$ (degrees)
may be significant pathologically) cannot be observed because they will always be insignificant compared to the magnitude of $\gamma_{SL}$.

Materials which give high contact angles on test surfaces are undesirable because:
1. they are insensitive to small, but significant changes in the substrate surface properties.
2. because of the large curvature near the three-phase contact line, the contact angle is difficult to measure accurately.
3. errors in contact angle measurement have the greatest effect on the calculated $\gamma_{SB}$ term when contact angles are above about $140^\circ$. This is illustrated by a hypothetical plot of $\gamma_{SB}$ (calculated with Neumann's equation of state) vs contact angle in Figure 2-2. Because the slope of the curve increases rapidly as $\theta$ approaches $180^\circ$, even a small inaccuracy in the contact angle measurement produces a huge error in the calculated value of $\gamma_{SB}$.

Because of the above problems with the oil/saline test system it was abandoned in favour of a more sensitive experimental approach.

2.3.3 Aqueous Polymer Systems
The tendency for mixtures of water-soluble polymers to separate into distinct phases was first reported in 1896 by Beijerinck (quoted by Albertsson,
1971). Beijerinck observed that mixing aqueous solutions of gelatin and agar produced a turbid mixture which eventually equilibrated to form two clear liquid layers. Most of the agar was found to be in the bottom phase while the top phase contained most of the gelatin. This phenomenon was rediscovered and extensively investigated by Per-Åke Albertsson in 1960 for a wide variety of different polymer combinations. An important feature of these systems is that the interfacial tensions between the equilibrated bulk phases are unusually low and very stable, generally between $1 \times 10^{-4}$ and $0.1 \text{ mJ} \cdot \text{m}^{-2}$. The low interfacial tensions are most probably attributable to the large proportion of water in both phases; for most systems, water forms greater than 90% of the total composition.

The high water content of the aqueous polymer systems makes them especially useful for work with biological materials. The solutions can be made isotonic and buffered to sustain cellular homeostasis and prevent protein denaturation (Little and Robinson, 1967). Further, Albertsson claims that the polymers may actually have a protective effect by acting to stabilize proteins in solution (Albertsson, 1971: p. 144).

While the interfacial tension is fixed for a particular test fluid in the oil/saline system, it may be easily adjusted in the aqueous system by varying the concentration of the constituent polymers (Figure 2-4).
The ability to adjust easily the interfacial tension of a system, is a substantial advantage because it allows the researcher to "tune" a test system to match the surface properties of the biological material being investigated. Albertsson (1971) p. 145 points out that the very low interfacial tensions of the polymer system provide a safe environment for fragile cell particles such as chloroplasts, which are normally destroyed by the large surface forces of conventional oil/water two phase systems.

Albertsson pioneered the use of two phase aqueous polymer systems for separating proteins and cell particles. Schürch, Gerson and McIver (1981) recognized the many biological advantages of these polymer systems and adapted their use to the contact angle method for estimating cellular surface properties. Aqueous polymer systems of polyethylene glycol (PEG) and dextran were chosen in which the denser dextran phase formed the test droplet and the lighter PEG phase provided the bulk bathing medium. Various concentrations of polymers (and hence different interfacial tensions) were used to construct a Good-Girifalco plot of \( \cos \theta \) vs \( \gamma^{-1/2} \) for layers of human red cells and pig pulmonary macrophages. By extrapolation back to \( \cos \theta = 1.0 \), an estimate of the critical interfacial of spreading, \( \gamma_c \) for the red cell surface was obtained.
The authors acknowledge a significant limitation to the above approach: To produce test droplets of different interfacial tensions as is required for a Good-Girifalco or Zisman style plot, the phase composition of the PEG/dextran system must be altered. A change in the droplet/bulk fluid interfacial tension ($\gamma^{LB}$) will normally alter the droplet contact angle, but since the composition of the PEG/dextran mixture has changed, the solid/bulk fluid interfacial free energy ($\gamma^{SB}$) will also be modified. Hence $\gamma^{SB}$ is not a constant as it is in a conventional Good-Girifalco or Zisman plot. A critical interfacial tension of spreading can be determined by extrapolating to $\cos \theta = 1.0$ but the composition of the PEG bulk fluid for $\gamma_c$ is not obvious. The polymer concentration in the bathing fluid may however be estimated from the tie line corresponding to $\gamma_c$ in the aqueous polymer phase diagram. Unfortunately another surface will have a different $\gamma_c$ referenced back to a different bulk phase composition, so this approach may have limited usefulness for comparing the surface properties of various substrates.

Such approaches are worth trying because Neumann's equation of state does not appear to be valid for such low interfacial tensions as the aqueous polymer
system provides. Such a result might have been predicted on general, theoretical grounds. In his derivation of the equation of state, Neumann assumed that the interaction parameter, $\phi$ varied substantially with droplet/bulk fluid interfacial tension ($\gamma_{LB}$). Since it may be that at low interfacial tensions, or where there is much water in both phases, $\phi$ does not vary sufficiently with $\gamma_{LB}$ to make this assumption valid, the equation of state is no longer applicable.

In summary, aqueous polymer systems seem to provide the best approach developed yet for investigating the surface properties of biological materials. These systems provide a safe environment for the study of living cells while their low interfacial tensions give them sufficient sensitivity to show subtle changes on cellular surfaces. The only disadvantage

---

5 For a dextran droplet within a PEG bulk phase (4%/4%) resting on a compressed DPPC monolayer ($\theta = 165^\circ$, $\gamma_{LB} = 2.4 \times 10^{-3}$ mJ.m$^{-2}$), the equation of state predicts $\gamma_{SB}$ to be $7.0 \times 10^{-7}$ mJ.m$^{-2}$. However shape analysis of the same monolayer substrate gives $\gamma_{SB}$ as 0.2 mJ.m$^{-2}$. The interfacial free energy predicted by the equation of state in this situation is obviously incorrect when compared with the experimentally determined value.
associated with their use is that a method to determine standard free energies from contact angle measurements has not yet been found.

The application of the aqueous polymer technique for the measurement of arterial surface properties presents a number of technical problems. Perhaps foremost among these difficulties is the determination of the interfacial tension of the test droplet, and the accurate measurement of the contact angle. Because the surface forces at aqueous polymer interfaces are of the order of $10^{-3}$ mJ·m$^{-2}$, many of the methods used to measure larger surface tensions (such as the nüoy ring method, or the Wilhelmy plate) cannot be used. The next section will introduce and test a newly developed method for determining low interfacial tensions and contact angles.

2.4 The Axisymmetric Drop Technique for Determining Contact Angles and Interfacial Tensions$^6$

2.4.1 Introduction

The existing methods for determining low

interfacial tensions employ mathematical solutions to
the Laplace equation:

\[ \Delta P = \gamma \left( \frac{1}{R_1} + \frac{1}{R_2} \right) \]

[2 - 13]

where \( \Delta P \) is the pressure difference across the
interface, and \( R_1 \) and \( R_2 \) are the principle radii of
curvature. Bashforth and Adams (1892) gave the first
numerical solutions to the differential equation for
sessile drops within a limited range of sizes and
shapes. The range of application was extended by Padday
(1971) and improved by Hartland and Hartley (1976) while
Fordham (1948) and others (Stauffer, 1965; Andreas et
al., 1938) developed similar solutions for the shapes of
pendant drops. All these approaches share the common
problem of having to locate critical points on the drop
profile, such as the maximum diameter and the height
from this point to the drop apex. Dorsey (1928) and
Dismukes (1959) discuss the substantial errors which may
result from slight inaccuracies in the location of these
points. Should the contact angle of a sessile drop be
less than 90\(^\circ\), no maximum diameter of the meridian curve
exists and further calculation is impossible.

Maze and Burnet (1969, 1971) attempted to
overcome many of these difficulties by using a non-
linear regression procedure to fit a calculated curve to
experimental points measured on the profile of the
entire sessile drop. Their procedure had the advantage of being able to determine the interfacial tensions of sessile drops with contact angles less than 90°. Unfortunately, the algorithm is not general enough because the fitting procedure is strongly influenced by the drop shape and the location of the drop apex. The implementation of the method also requires a good initial estimate of the shape parameter, along with other input variables.

It was after communicating our difficulties with using such methods to Drs. Y. Rotenberg and A.W. Neumann, University of Toronto, that they set about developing a numerical scheme for solving the Laplace equation of capillarity (Rotenberg et al., 1983). Once an algorithm had been established, we collaborated with them to test the procedure and refine it as a simple method for determining interfacial tensions from the shapes of both pendant and sessile drops. The computational procedure developed by Dr. Rotenberg constructs an objective function which expresses the error between the physically observed and a theoretical Laplacian curve. This function is minimized using the method of incremental loading in conjunction with a Newton-Raphson iterative procedure. The main purpose of the work presented here was to test this new approach on real systems, to estimate its precision, and to determine the limitations of its application.
TABLE 2 - 1

TEST SYSTEMS

<table>
<thead>
<tr>
<th>Pendant</th>
<th>Sessile</th>
<th>( \theta &lt; 90^\circ )</th>
<th>( \theta &gt; 90^\circ )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface Tensions (High ( \gamma ))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-Methylnaphthalene</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Dodecane</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Interfacial Tension (Intermediate ( \gamma ))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl Acetate/Water</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Interfacial Tensions (Low &amp; Ultra-low ( \gamma ))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEG/Dextrans (Various compositions)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>SDS/Cholesterol</td>
<td>*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.4.2 Methods

2.4.2.1 Test Systems

A variety of different test systems which provided a wide range of surface (or interfacial) tensions and contact angles, were chosen to test the new method. The systems chosen and their characteristics are summarized in Table 2-1. For surface tensions at liquid/vapour interfaces photographs were made of pendant drops of reagent grade 1-methylnaphthalene (Fisher Scientific) and dodecane (Eastman), and of sessile drops of the same liquids on polytetrafluoroethylene (PTFE) or glass with a hydrophobic anti-migration coating, FC721 (3M, Inc.). A similar setup was used to determine the interfacial tension (liquid/liquid interface) between ethyl acetate (Fisher Scientific) and water, again using pendant and sessile drops.

To explore the low to ultra-low range of interfacial tensions, two different test systems were employed: 1) a dextran/PEG aqueous polymer system, and 2) a Sodium dodecylsulphate (SDS)/cholesterol surfactant-cosurfactant system.

1) The dextran (mol. wt: $2 \times 10^6$, Pharmacia) and PEG (mol. wt: $2 \times 10^4$, Fisher Scientific) were dissolved in 0.9% NaCl in compositions ranging between 3% and 13.6% (w/w) at 22°C. Droplets of the denser
dextran-rich phase (volume 0.1 – 5 μl) were formed on a clean glass plate immersed in the lighter, PEG-rich bulk phase.

2) Lucassen (1979) showed that extremely low-interfacial tensions can be produced by dissolving cholesterol in an oil test droplet and using S.D.S. as a cosurfactant in an aqueous bulk phase. For the experiments outlined here we used a mixture of dibutyl-deocyt1 phthalate (1:1 by volume) as the oil droplet, in a 0.005 M SDS aqueous bulk phase.

2.4.2.2 Testing Procedure

Systems of intermediate and high surface or interfacial tensions (see Table 2-1) were photographed through a horizontally mounted stereomicroscope. A Nikon optiphot microscope equipped with differential interference optics was used to enhance the visual contrast of the lower interfacial tension systems (i.e. PEG/dextran and SDS/cholesterol). The droplets were formed within a small glass-sided test chamber supported on a Leitz micromanipulator. Transillumination of the chamber was provided with by a fibre optic light source dispersed through a frosted glass plate. We used polarizing filters placed in the incident light path and on the microscope objective to reduce glare and to enhance the contrast of the drop image. Pendant drops were formed on squared tips of stainless steel
hypodermic needles of various diameters attached to an ultraprecision micrometer syringe. The micrometer syringe was mounted on a micromanipulator to permit easy positioning of the drop for photography. We determined the densities of the fluids by weighing a solid, stainless steel sphere in air and in the test liquid, with a precision analytical balance. (The volume of the sphere was determined by comparing its weight in air and its weight in distilled water and then the density of water from published tables was used to calculate the volume of water displaced). 7

2.4.2.3 Digitizing Procedure

All photographs were taken with a pipette of known diameter showing in the frame, and this served as an accurate scale reference. Drop profiles were digitized from enlarged photographs or tracings with a Talos 600 series digitizer having a resolution of approximately 0.003 cm. Thirty-five to 50 points were generated for each profile and these data along with the respective scale factor and density difference were used as input for the main program.

7 The photography of the various drop series and the density determinations were performed by Dr. Samuel Schürch.
### TABLE 2 - 2

**SUMMARY OF RESULTS**

<table>
<thead>
<tr>
<th>Test System and Literature Value with Reference</th>
<th>Interfacial or Surface Tension (mJ \cdot m^{-2})</th>
<th>Standard Error of the Mean (\pm 1.8%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Methylnaphthalene 38.7 @ 20°C (^a)</td>
<td>Pendant 38.6 ± 1.1  Sessile 38.2 ± 1.0</td>
<td>Combined 38.4 ± 0.7</td>
</tr>
<tr>
<td>Dodecane 25.4 ± 0.5 @ 20°C (^a)</td>
<td>n = 5</td>
<td>n = 5</td>
</tr>
<tr>
<td>Ethyl Acetate/Water 6.8 ± 0.1 @ 20°C (^b)</td>
<td>n = 5</td>
<td>n = 6</td>
</tr>
</tbody>
</table>

* \(\pm\) SEM

\(^a\) (Gent and Shultz, 1972)

\(^b\) (Donahue and Bartell, 1952)
FIGURE 2 - 3

Typical examples of photographs used to determine surface and interfacial tensions.

(a) 1-methylnaphthalene, contact angle: 90°.
(b) dodecane, contact angle: 65°.
(c) ethyl acetate/water, contact angle: 159°.

The capillary in each photograph is 1.05 mm in diameter and all drops are enlarged to the same scale. (From Boyce et al., 1984; reproduced with permission).
FIGURE 2. - 4

Functional relationship between polymer concentration and interfacial tension. Two units of standard error are contained within each circular data point. (From Boyce et al., 1984; reproduced with permission).
FIGURE 2 - 5

Illustrated extremes of interfacial tension with the PEG/dextran system.

(a) 4% PEG/dextran. Interfacial tension for this example $4.05 \times 10^{-3} \text{ mJ m}^{-2}$. The dextran droplet has a volume of 0.1 $\mu$l.

(b) 13.6% PEG/dextran. Interfacial tension: $2.91 \times 10^{-1} \text{ mJ m}^{-2}$. The volume of this drop is 4.56 $\mu$l.

Both photographs are enlarged to the same scale. (From Boyce et al., 1984; reproduced with permission).
2.4.3 Results

A summary of the results obtained for the methylnaphthalene, dodecane and ethyl acetate/water systems appears in Table 2-2. Corresponding examples of photographs used to determine these surface or interfacial tensions are shown in Figure 2-3. The photographs also indicate the utility of this approach for calculating surface tensions from sessile drops with contact angles above, as well as below, 90°.

Figure 2-4 shows the calculated interfacial tensions for the PEG/dextran system as a function of concentration. Representative photographs of dextran droplets of different concentrations are shown in Figure 2-5. These photographs emphasize the effect of the variations of interfacial tension on droplet size.

2.4.3.1 Estimates of Uncertainty

To estimate the uncertainty of the experimental technique, we chose to use the SDS/cholesterol system because low interfacial tensions minimize the effects of surface contamination; surface contamination is the bane of surface tension determination in higher energy test systems. Interfacial tensions were determined for ten sessile drops of various sizes and shapes. For each of the ten calculations the density and scaling factor were independently determined. The diameters of the hypodermic needles used as scale references were
measured to ±0.05 mm using a microscope and a calibrated reference slide. For every drop, we separately set up the microscope and readjusted the orientation of the illumination to simulate a new experimental situation. The mean interfacial tension of the ten independent determinations was \( (5.78 \pm 0.20 \text{ (SEM)}) \times 10^{-3} \text{ mJ} \cdot \text{m}^{-2} \). This represents a percent standard error of the mean of ±3.4%.\(^8\)

To estimate the uncertainty due to the digitizing procedure, the same droplet profile was digitized ten times with different numbers of coordinates each time. Between 20 and 60 points were used in each computation. The mean interfacial tension obtained was \( (5.76 \pm 0.11 \text{ (SEM)}) \times 10^{-3} \text{ mJ} \cdot \text{m}^{-2} \) or ±0.2% SEM.

2.4 Discussion

A comparison of literature values with the values determined for sessile and pendant drops (Table 2-2) indicates agreement within our estimated

\(^8\) I have defined the percent standard error of the mean as:

\[
\% \text{ SEM} = \frac{\text{SEM}}{\text{MEAN}} \times 100
\]
uncertainties. An estimate of the relative uncertainty for each system was obtained by combining together the pendant and sessile drop results, and calculating the percent standard error of the mean. The data presented in Table 2-2 indicate that as the surface or interfacial tension decreases, there is a corresponding decline in the relative uncertainty associated with its measurement. The probable reason for this decline in the relative error is surface contamination. Higher energy interfaces quickly tend to accumulate contaminants which will reduce the surface tension and thus provide a substantially lower free energy for the system. However, interfaces of lower energy are less prone to contaminant accumulation because the system's free energy is already low and near its minimum; hence the contaminants have a diminished effect. Therefore, a contaminant's effects will always be greatest for the determination of large surface or interfacial tensions.

Our estimate of experimental uncertainty of $\pm 3.4\%$ SEM is larger than the values reported in Table 2-2 because we sought to estimate the error for a "worst case" situation, where every run was set up independently of the other nine runs. For each case, we have included in the calculation of the interfacial tension the variations in both the measured density and the measured scale factor. The results in Table 2-2 were obtained differently. The mean value of the density as
well as the mean value of the scale factor were calculated first, and then used in the analysis of the drop profiles. The estimated uncertainty due solely to the tracing/enlarging and digitizing procedure is substantially less, ± 0.2% SEM. This too is probably an overestimate because the number of digitized points used in the calculations was deliberately varied from a minimum of 20 to a maximum of 60. Increasing the number of data points taken from the drop profile increases the precision of the curve fitting procedure, but at the expense of the computing time required to obtain a solution. From my experience, 30 to 40 datum pairs provide a good compromise between precision and computing time.

As I discussed earlier in this chapter, the main disadvantage of using the aqueous polymer system is that standard solid/bulk fluid interfacial free energies cannot be obtained; Neumann's equation of state does not apply for this system, and a Good-Girifalco plot is impractical because the composition of the bulk fluid phase and hence \( \gamma_{SB} \) is not kept constant throughout the drop series. We do know, however, that in some situations the equation of state can be used for liquid/liquid systems if the interfacial tension of the drop/bulk fluid phase is sufficiently high (Schürch and McIver, 1981). After satisfying myself that Rotenberg's new axisymmetric drop technique was a dependable means
of calculating low interfacial tensions and contact angles, I devised a means of using it as a tool with Neumann's equation of state, for calibrating the PEG/dextran system. 9

2.5 Calibration of the PEG/Dextran System

2.5.1 Introduction and Experimental Design

In an earlier study we established that the equation of state gave valid results for several oil/water systems (Boyce et al., 1980). I planned to use this equation with an oil droplet to calculate the interfacial free energy of a reference substrate under PEG (γSB), and relate this to the contact angle of a dextran droplet resting on the same surface in the same bulk phase. By using a series of different surfaces, I hoped to construct a calibration curve of contact angle (of the dextran droplet) vs interfacial free energy of the substrate, γSB (calculated from an oil droplet using the equation of state).

The key to making the approach work is finding an oil-based test droplet which is sensitive enough to partially spread on the same surface as a dextran

9 By "calibration" in this context I mean: experimentally relate the dextran contact angle with the standard interfacial free energy of the substrate on which it rests, i.e. γSB.
droplet, and yet have a large enough interfacial tension \( \gamma^{LB} \) to give valid results with the equation of state. I assumed that the lower limit of droplet interfacial tension for which the equation of state applies, was in the order of 1 mJ \( \cdot \) m\(^{-2} \), since this is about as low an interfacial tension as one finds with an oil/water system (e.g. for water/butanol, \( \gamma^{LB} = 1.8 \) mJ \( \cdot \) m\(^{-2} \)). This assumption would of course have to be checked should the experiments prove to be successful. Butanol itself is not a very good candidate as a test fluid because it is partially soluble in water and does not form a very stable interface. A better system appeared to be an oil droplet with an insoluble surfactant which reduces the interfacial tension to approximately 1 mJ \( \cdot \) m\(^{-2} \).

Since the dextran droplet is composed mostly of water, the droplet, when spreading, is probably responding to the large water component of the cell surface. Therefore, a suitable reference substrate will require a hydrophilic character to cause the dextran droplet to spread. Ideally, one should be able to alter the hydration content of the reference substrate to adjust the extent of spreading (i.e. contact angle) of the dextran drop. After considering various substances it became apparent that a gel-like material would provide the best reference substrate because it has a very high water content which can be easily adjusted to modify its surface properties.
2.5.2 Methods

As substrate materials, because of their convenience and availability, I chose to use agar (negatively charged), agarose (uncharged) and commercial gelatin. Plates of 2% (w/w) agar and agarose in 0.9% NaCl solution were poured. Flat plates of glass and polystyrene were placed on the surface of the gel material while it was still warm and liquid. By doing this I hoped to produce a difference in the surface structure of the gel by letting it solidify against chemically different materials e.g. the hydrophilic glass and the hydrophobic polystyrene. Since the extent of surface hydration is critical for the spreading of the dextran droplet, I did not want to risk dehydrating the gel surface by letting it solidify at an air interface.

Therefore, the initial test group consisted of four different substrates: agar solidified against glass, agar against polystyrene, agarose against glass and agarose against polystyrene. After the plates had solidified and cooled overnight, sections of each gel were cut out and placed on a pivoting support within a small viewing chamber. A two phase system of 4% (w/w) PEG (mol. wt: 20,000; Fisher Scientific)/4% (w/w) dextran (mol. wt: 2,000,000) had been prepared earlier and allowed to equilibrate overnight at room temperature (21°C). After the two phases had separated, the viewing
chamber was filled with the PEG (top) phase, immersing the test substrate. Droplets of dextran solution and an oil mixture consisting of freon and decanol (1:10 by volume) with a dipalmitoyl phosphatidylcholine (DPPC) surfactant were placed randomly over the test substrate. Decanol was chosen because the hydroxide group would reduce the interfacial tension with water while the long hydrocarbon chain would strongly "anchor" the hydrocarbon chains of the surfactant molecule. Small amounts of freon were added to increase the density of the test droplet so that it would not float in the PEG solution. Since DPPC is an insoluble surfactant it can only accumulate at the oil/water interface. By providing an excess amount of DPPC in the freon/decanol mixture we ensured that the molecular surface density of the surfactant was constant for all test droplets and thus the interfacial tension was always the same. Whenever possible the dextran and oil droplets were photographed close together to ensure that both droplets rested on regions of identical surface properties. To avoid anomalous data which might result from substrate surface contamination, or heterogeneous surface structure, sections taken through the centre of the gel were also tested. A stainless steel needle of known dimension was used in each photograph as a scale reference.
Photographs of droplets taken on every substrate were enlarged and traced for digitizing. The axisymmetric drop procedure was used to determine interfacial tensions and contact angles from the digitized drop profiles of both dextran and oil droplets. For each oil droplet the equation of state was called as a subroutine in the main computer program, and the gel substrate/PEG interfacial free energy was calculated. The densities of the various phases were determined by using the steel sphere weight method described earlier.

Since the results for 2% agar and 2% agarose did not differ significantly, I decided to use only one gel material and concentrate instead on altering the gel composition. In order to vary the water component in the gel as widely as possible I chose to use agar in concentrations of 1/2 and 4% (w/w). One half percent is the lowest concentration of agar that produces a solid substrate, while concentrations greater than 4% agar will not completely dissolve to form a homogeneous gel.

To enhance the interaction between the two fluid phases (dextran and PEG) and the substrate, various amounts of polymer were dissolved in the liquid agar phase before the gel had set. The following combinations were tried: 2% agar - 2% PEG, 2% agar - 2% dextran, 3% agar - 1% PEG, 3% agar - 1% dextran, 3.5%
agar - 0.5% PEG and 3.5% agar - 0.5% dextran. All of these gels were allowed to solidify against a polystyrene interface.

A final series of test substrates was prepared from commercially available gelatin (Knox). The concentrations used were 1/2, 1 and 2% (w/w) in 0.9% NaCl solution.

2.5.3 Results

Not all the gels produced were suitable for experimental testing. The PEG and dextran seemed to dissolve completely with the agar in its hot liquid form, however upon cooling and solidification a rough granular surface resulted from most mixtures. The exceptions to this were the 3.5% agar - 0.5% PEG and 3.5% agar - 0.5% dextran combinations which formed smooth, homogeneous solids. Since roughness may produce substantial contact angle hysteresis, only the last named mixtures were used.

While contact angles of about 160 to 180° were frequently calculated for the oil-DPPC drop on various agar surfaces, a contact angle less than 180° was never found for the dextran droplet on any of the substrates tested. Despite altering the water composition of the agar substrate from 96 to 99.5% no decrease of the dextran contact angle was observed. Even when the substrate contained a substantial dextran component
(3.5% agar, 0.5% dextran) the contact angle of the dextran droplet did not fall below 180°. Similar results were recorded for all three gelatin substrates investigated.

2.5.4 **Discussion**

Since a range of contact angles for the dextran droplet was not obtained, I could not achieve my original objective of establishing a calibration curve for the PEG/dextran system. However, these results do assist our interpretation of substrate surface chemistry based on contact angle measurements. Specifically it now seems clear that surface hydration is not the only important factor which determines the spreading behaviour of a dextran droplet. For spreading to occur the surface structure of the substrate appears to be important, particularly as to how it affects the ability of polymers in the droplet and the substrate to interact.

Assuming constant substrate/bulk fluid surface properties, partial spreading of the droplet (θ < 180°) implies a decrease in the substrate/droplet interfacial free energy. This may be interpreted as an increased interaction between the two phases. Agar forms a gel because of the extensive crosslinking which occurs between the constituent polymers. The agar crosslinking could severely restrict the mobility of the agar
molecules as well as any other polymers which may be present in the gel, preventing them from interacting fully with the polymers of the drop phase. This might be the explanation for why the dextran droplet does not spread on a gel substrate despite both phases having very large water components. The glycocalyx of cell surfaces which the dextran droplet does spread on is not nearly so rigidly structured (Sherbet, 1978). On the cell surface, polymers in both phases would be more mobile and capable of greater interaction.

In this chapter I have attempted to familiarize the reader with some of the tools I will use to investigate the surface properties of arteries. By discussing the evolution of the various methods, and their strengths and weaknesses, I hoped to give some sense as to why I proceeded with a particular approach, in this case the PEG/dextran system. Although the attempt to calibrate the PEG/dextran system was unsuccessful, the conclusions arrived at will be valuable for interpreting some of the results obtained for arterial surfaces. I hope to have left the reader with an understanding of basic surface chemistry, so the logic of the interpretations and conclusions that I make in the forthcoming chapter, will be clearly apparent.
CHAPTER 3

EXPERIMENTS ON ARTERIAL TISSUE

"But when day came, with a sprinkle of rain, and he looked about him and saw on every side unknown woods, wild heaths, and blue mountains, he thought how large and strange the world was and felt frightened and small".

C.S. Lewis – Prince Caspian
3.1  Introduction to Chapter 3

In this chapter I will present and discuss the results of two separate investigations. Although different, both studies share common ground in that they examine the changing surface properties of the artery in the context of the initiation of atherosclerosis. In the first study I look for a thermodynamic explanation for platelet adhesion, an event which is a likely precursor to lesion development. The second study is concerned with changes at specific locations on the arterial surface known to be predisposed to atherosclerotic plaque development.

3.2.  Interfacial Free Energy of the Arterial Wall and Its Potential Role in Platelet Adhesion

3.2.1  Introduction

In the first chapter I discussed the important role that platelets may play in the initial stages of

\[1\]  Taken from:

atherosclerosis. Excellent reviews of this topic have been written by: Ross and Glomset, 1976; and Kottke and Subbiah, 1978.

Platelets normally do not stick to the healthy endothelial lining of arteries, but adhere rapidly when injury exposes the subendothelial material (Groves et al., 1979). Such adhesion, and subsequent platelet aggregation, promote development of lesions by inducing smooth muscle migration and thickening of the arterial wall (Ross and Glomset, 1976; Mustard et al., 1978). Much of the research to date has concentrated on platelet biology, specifically on those mechanisms responsible for activation after the first platelets have adhered to the wall (Barrier and Ellison, 1977; Schafer and Handin, 1979). However, few studies have appeared which investigate why platelets suddenly stick to the arterial wall in the first place. The key to answering this question may rest in examining—not the platelet—but, rather, changes in the arterial wall prior to platelet involvement.

From the thermodynamic point of view, adhesion of cells to other cells or to foreign substances is favoured if the change in the (Helmholtz) free energy for the process of attachment is negative; i.e., if the
total energy of the exposed surface is decreased by the adhesion (Neumann et al., 1979). This change is, per unit surface area

$$\Delta F_{\text{adh}} = \gamma_{CS} - \gamma_{CB} - \gamma_{SB} \quad [3-1]$$

where $\gamma_{CS}$ is the cell-substrate interfacial free energy, $\gamma_{CB}$ is the cell/bulk fluid interfacial free energy and $\gamma_{SB}$ is the substrate/bulk fluid interfacial free energy.

In this free energy balance, the substrate may be any solid surface or another cell. This adhesion of a cell to a substrate is favoured for relatively large cell/bulk fluid and substrate/bulk fluid interfacial free energies, or when the sum of the two free energies involving the liquid medium is greater than the cell/substrate interfacial free energy:

$$\left(\gamma_{CB} + \gamma_{SB}\right) > \gamma_{CS} \quad [3-2]$$

If we assume that $\gamma_{CB}$ is constant, then this relation indicates that cell adhesion is dependent upon the surface properties of the substrate. Whereas $\gamma_{CS}$ cannot be determined directly, it is possible to document changes in $\gamma_{SB}$ which are produced by an alteration of the surface substrate. Young's equation which was introduced in Chapter 2, is useful for this purpose:

$$\gamma_{SB} = \gamma_{SL} + \gamma_{LB} \cos \theta' \quad [3-3]$$

where $\theta$ is the angle of contact the test fluid droplet makes on the tissue surface, $\gamma$ is the interfacial free energy and the superscripts B, L and S denote the bulk fluid, the liquid, test droplet and the substrate,
respectively. Therefore by using Young's equation to estimate changes in $\gamma^{SB}$, and Relation [3 - 2], one can predict whether a newly exposed substrate surface may promote or inhibit cell adhesion.

We have used this method to compare the relative interfacial free energies of intact endothelium and exposed subendothelium. Such information can be used to assess the adhesiveness of arterial tissue for blood components such as platelets which may be important to the initiation of atherosclerosis.

3.2.2 Methods

The three techniques used in these investigations are:

(1) a contact angle method to determine changes in tissue interfacial free energy,

(2) a saline jet technique to remove the endothelium and

(3) an isotope-labeling experiment to test for dextran adsorption artefact.

3.2.2.1 Contact Angle Method: Sample Preparation and Test System

We prepared an aqueous system of 4% (w/w) polyethylene glycol (PEG) (mol. wt: 20,000; Fisher Scientific)/4% (w/w) dextran (mol. wt: 2,000,000; Pharmacia) in Hepes-buffered Hanks saline solution (pH = 7.3) and allowed it to equilibrate overnight. The
FIGURE 3 - 1

Schematic diagram of experimental apparatus.

(From Boyce et al., 1983; reproduced with permission).
solution separates at equilibrium into two phases - a PEG-rich top phase, which was decanted off and used as the tissue bathing medium, and a dextran-rich bottom phase which we used for test fluid droplets.

Seven male New Zealand White rabbits (2 - 3 kg) were killed with an overdose of sodium pentobarbital administered intravenously. A 4 to 6 cm long section of descending thoracic aorta was quickly dissected free of the animal and immersed in physiological saline solution. The aorta was cut longitudinally between the intercostal branches to expose the endothelium.

We bonded the outer surface of the tissue to a small brass mounting bar with cyanoacrylic adhesive (Loctite), and placed the entire preparation inside a small viewing chamber filled with the PEG-rich bathing medium. A micromanipulator fitted with a micropipette was used to place small droplets (0.1 - 0.2 mm diameter) of the dextran phase at random locations on the tissue surface. By illuminating the preparation from behind with polarized light, the droplet profiles could be observed through a dissecting microscope and photographed (Fig. 3-1). We measured the contact angles of droplets sitting on the arterial surface. All testing of the intact endothelium was done at room temperature (21°C) and was completed within 1 hour of removal from the animal. Two tissue specimens were inspected on the
TABLE 3 - 1

Summary of calibration experiments for saline jet removal of endothelium.
<table>
<thead>
<tr>
<th>Volume flow rate (1 \cdot s^{-1})</th>
<th>Fluid velocity (m \cdot s^{-1})</th>
<th>Number of aortae</th>
<th>Number of jetted regions</th>
<th>Damaged Area on SEM specimen* (mm$^2$)</th>
<th>Damaged Area estimated for fresh tissue (mm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0 \times 10^{-4}</td>
<td>4.0</td>
<td>5</td>
<td>5</td>
<td>Intact</td>
<td>Intact</td>
</tr>
<tr>
<td>6.7 \times 10^{-4}</td>
<td>5.3</td>
<td>5</td>
<td>5</td>
<td>Intact</td>
<td>Intact</td>
</tr>
<tr>
<td>8.3 \times 10^{-4}</td>
<td>6.7</td>
<td>5</td>
<td>5</td>
<td>1.0 \pm 0.2</td>
<td>1.3</td>
</tr>
<tr>
<td>1.0 \times 10^{-3}</td>
<td>8.0</td>
<td>3</td>
<td>4</td>
<td>1.8 \pm 0.3</td>
<td>2.3</td>
</tr>
<tr>
<td>1.2 \times 10^{-3}</td>
<td>9.3</td>
<td>3</td>
<td>4</td>
<td>3.5 \pm 0.5</td>
<td>4.6</td>
</tr>
</tbody>
</table>

* MEAN \pm SEM
scanning electron microscope (SEM) to ensure that the removal and preparation of the artery had not damaged the endothelium.

3.2.2.2 Removal of the Endothelium

To remove the endothelium to expose a smooth, homogeneous subendothelium, we adapted a fluid jet technique first suggested by Patel et al. (1979). Physiological saline was forced through a capillary tube (inside diameter 0.4 mm) at 140 kPa pressure onto the tissue while it was immersed in a saline bath. The jet orifice was positioned 1 mm above and perpendicular to the opened arterial surface with a micromanipulator. The flow rate and hence the amount of damage to the endothelium can be accurately controlled with a calibrated micrometer valve (Table 3-1).

We performed several control experiments to determine the minimum flow rate required to remove the endothelium and the approximate diameter of the jetted area.\(^2\) Samples of fresh artery were exposed to a stationary jet for 10 seconds at flow rates between \(5 \times 10^{-4}\) and \(1.2 \times 10^{-3}\) l \cdot s\(^{-1}\). The areas of denudation were calculated from photomicrographs using a computer.

\(^2\) This series of experiments was performed by Mr. Philip C. Wong who worked with me as a summer student in 1980.
FIGURE 3 - 2

A. Silver-stained scanning electron micrograph showing the intact endothelial surface.

B. Subendothelial surface adjacent to the area above, where the endothelium has been removed with a saline jet.

The markers in both micrographs are 10 μm long. (From Boyce et al., 1983; reproduced with permission).
a digitizer interface. To estimate the amount of shrinkage due to tissue dehydration, we lightly scratched the surfaces of two fresh artery pieces with a grid pattern of known dimensions. A graduated micromanipulator fitted with a 25-gauge syringe needle was used for this purpose. After the sample had been prepared for the SEM, the grid dimensions were determined from photomicrographs and compared with the original measurements for fresh tissue. The corrected diameter of the jet path was used to determine the number of successive passes that would be required to denude the entire endothelial layer. To ensure that the endothelium had been completely removed from the arterial surface, the tissue specimen was surveyed with the SEM at low magnification (Fig. 3-2).

Tissue samples were prepared for scanning, first by staining with a 0.25% AgNO₃ solution for 20 seconds, followed by fixation in a 1% phosphate-buffered glutaraldehyde:paraformaldehyde (1:1) solution for 8 hours. The tissue specimens were taken through a ethanol dehydration series, critical point dried and coated with gold-palladium before viewing on a Philips 501 SEM.

After we had tested the endothelium with the polymer system, we transferred the mounted artery from the viewing chamber to a bath of Hepes-buffered physiological saline. Then we removed the endothelium with several successive passes of the saline jet, using
a flow rate of $1.2 \times 10^{-3} \text{ l} \cdot \text{s}^{-1}$ (orifical flow velocity: $9.3 \text{ m} \cdot \text{s}^{-1}$). At this flow rate we had determined the width of the jet path to be at least 2.4 mm and each pass was incremented by slightly less than this dimension to ensure overlap. The tissue preparation was reimmersed in the PEG solution and tested in exactly the same manner as described previously. Two sections of artery were exposed to the jet immediately after removal from the animal, without pretesting the intact surface. We observed no difference in the results we obtained for these samples. After the subendothelium had been tested, two tissue specimens were viewed on the SEM to verify that the endothelium had been removed from the experimental sample.

3.2.2.3 Dextran Adsorption Studies

Since the contact angle of the dextran droplet was extremely low on the subendothelial surface, we designed the following experiment to determine whether this result was an artefact of dextran adsorption, or if the partially spread droplet was a true indicator of the surface properties of the tissue. Since dextran is a polymer of glucose, if adsorption were taking place, it should occur on the exposed saccharide groups of glycoproteins on the subendothelium. Concanavalin A (Con A) is a lectin known to interact with saccharide units on the cell surface (Wang et al., 1980). We
FIGURE 3 - 3

Typical results observed for the two experimental groups. The test droplet is dextran within a PEG-rich bulk fluid. The top photograph shows a droplet on the intact endothelium with a contact angle ($\theta_1$) of 84°. $\theta_2$ is 22° in the bottom photograph and was measured for a droplet on the subendothelium. Both droplets are approximately 0.2 mm in diameter. (From Boyce et al., 1983; reproduced with permission).
TABLE 3 - 2

Summary of contact angle measurements on arterial surfaces. The cosines of the mean contact angles are significantly different for intact endothelium and subendothelium \( p < 0.05 \).
<table>
<thead>
<tr>
<th></th>
<th>Endothelium intact</th>
<th>Endothelium removed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean angle ± SEM (in degrees)</td>
<td>86.0 ± 1.1</td>
<td>20.0 ± 0.8</td>
</tr>
<tr>
<td>(cosine)</td>
<td>(0.0698)</td>
<td>(0.9397)</td>
</tr>
<tr>
<td>No. of observations</td>
<td>64</td>
<td>61</td>
</tr>
<tr>
<td>No. of tissue samples</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>
presumed that Con A would block or mask any available saccharide adsorption sites at the interface on the arterial wall.

Two fresh segments of aorta were jetted in the usual way to remove the endothelium. The tissue was cut into 1 cm lengths, and the outer surface was bonded to small plastic stubs. This ensured that only the luminal surface was exposed to the dextran solution. The arterial segments were incubated at room temperature for periods of 5, 10, and 20 min, in 5 ml of the 4% dextran solution, which contained 1.25 μCi of 14C-labeled dextran, mol. wt: 70,000 (New England Nuclear). The tissue was washed in saline, solubilized overnight in NCS tissue solubilizer (Amersham-Searle), and counted in a liquid scintillation counter. Two additional aortae were used in a second test group and treated with the same protocol, except the tissue was pretreated with 1.25 μCi of tritiated Con A (New England Nuclear) in 10 ml of physiological saline for 15 minutes before exposure to the dextran solution.

3.2.3 Results
3.2.3.1 Contact Angles

Typical examples of droplets on fresh and jetted aortae are displayed in Figure 3-3, and the results are summarized in Table 3-2. A variance ratio test (Zar, 1974; p. 101-103) justified pooling data from
FIGURE 3 - 4

Dextran adsorption on the subendothelium with and without pretreatment with Con A. Each data point is the mean of four test samples. Vertical bars indicate S.E.M.
individual sections into experimental groups. We performed an unpaired Student's t-test (Zar, 1974; p. 105-107) on the cosines of the measured angles (see Equation 3 – 3) and found the difference between the means of the two test groups to be significant \((p < 0.05)\).

3.2.3.2 **Jet Removal of Endothelium**

The results of our calibration tests of the saline jet have been summarized in Table 3 – 1. The shrinkage experiments indicated that tissue dehydration decreased the arterial surface area by about 30\% therefore, the calculated areas of denudation were adjusted accordingly. This 30\% value is consistent with other results for shrinkage artefact reported by McGarvey *et al.* (1980).

3.2.3.3 **Dextran Adsorption**

The results for the adsorption study are illustrated in Figure 3 – 4. The two curves show no significant difference in activity per unit area for the dextran and Con A pretreatment groups. Although the concentration of the labeled Con A pretreatment solution, labeling of the subendothelium with Con A was eight times higher than dextran, for the same incubation period. \((5.6 \times 10^6 \text{ vs } 7.0 \times 10^5 \text{ disintegrations } \cdot \text{s}^{-1} \cdot \text{m}^{-2})\).
3.2.4 Discussion

3.2.4.1 Statement of Results

The results in Table 3-2 show that there is a significant change in the contact angle of the dextran droplet when the endothelium is removed from the arterial surface. A contact angle close to 90°, such as we measured on the intact endothelium, indicates that the substrate/droplet (dextran) and the substrate/bathing fluid (PEG) interfacial free energies (γ_{SL} and γ_{SB}) are almost the same. In other words, the endothelial surface has equal affinity for the dextran and PEG phases. However, after removal of the endothelium, the droplet almost spreads on the subendothelium (contact angle: 20°), indicating a greater similarity between the surface and the dextran-rich droplet. If this result were due to significant dextran adsorption on the subendothelium, we would have expected a substantial difference in the adsorption curve pretreatment with Con A. This was not observed (Fig. 3-4). The amount of dextran label remaining in the tissue may be due to incomplete washing. The hyperbolic nature of the dextran curve indicates that dextran may be trapped in tissue unstirred layers, as suggested by Podesta (1977) and Luckie et al. (1974). We therefore conclude that the low contact angle measured on the
subendothelium is an accurate indicator of the tissue's surface properties, not an artefact of dextran adsorption.

3.2.4.2 Physical Interpretation

As I discussed in the previous chapter, the contact angles obtained with the aqueous polymer system unfortunately cannot be converted to absolute substrate interfacial free energies. It is possible however to estimate relative changes of the substrate's interfacial free energy and predict how adhesion may be affected.

Arrangement of Young's equation [3 - 3]
yields:

$$\gamma_{SB} - \gamma_{SL} = \gamma_{LB} \cos \theta \quad [3 - 4]$$

The right side of this equation may be easily determined. Since the PEG bathing liquid/dextran droplet interfacial free energy ($\gamma_{LB}$) is constant, the difference between the substrate interfacial free energies ($\gamma_{SB}$ and $\gamma_{SL}$) increases as $\theta$ approaches zero. The 20° contact angle measured on the subendothelium indicates that the substrate/dextran interfacial free energy ($\gamma_{SL}$) has decreased relative to the substrate/PEG interfacial free energy ($\gamma_{SB}$). In terms of molecular interactions, a decrease in interfacial free energy indicates a greater chemical similarity between the substrate and the dextran droplet. After removal of the
endothelium, the substrate becomes more dextran-like or more hydrophilic, since dextran is more hydrophilic than PEG (Albertsson, 1971).

3.2.4.3 Biological Interpretation

While an almost continuous basement membrane material forms the subendothelium of capillaries, venules and arterioles, a similar electron dense material is neither continuous nor distinct in larger vessels such as the aorta (Baumgartner, 1974; Ts'ao and Glagov, 1970). The subendothelium of the aorta consists of a fibrillar collagen network resting on the elastic lamina and embedded in an amorphous polysaccharide component. Unfortunately, because tissue dehydration is required for scanning electron microscopy, these methods cannot be used to visualize the same hydrated subendothelial surface that the test droplet experiences.

In our test system, the hydrophilic subendothelium is adjacent to a less hydrophilic (hydrophobic in relative terms) PEG-rich bathing medium. This produces a surface of higher interfacial free energy because the two adjacent phases are chemically dissimilar. Sherman (1981) has reported the surface tension of blood in air to be $53 \pm 3$ (SEM) mJ m$^{-2}$. We have measured the surface tension of our PEG bathing medium to be somewhat higher, about $62 \pm 2$ mJ m$^{-2}$. These
measurements imply that blood is less hydrophilic (more hydrophobic) than the PEG bathing medium, which in turn is less hydrophilic than the subendothelium. Therefore, removal of the endothelium in vivo may expose a surface of higher interfacial free energy because a hydrophilic substrate (the subendothelium) forms an interface with a chemically dissimilar bathing medium (the blood).

3.2.4.4 Biophysical Model

Considering these findings, one can suggest a physical explanation for how endothelial injury and removal may initiate platelet adhesion to the arterial wall. The intact endothelium presents a low interfacial free energy surface (low $\gamma_{SB}$) to the blood constituents which may inhibit platelet adhesion by making it thermodynamically unfavourable. However, damage and removal of the endothelium exposes a new surface of significantly higher interfacial free energy (high $\gamma_{SB}$). This would shift the energy balance of the system favouring platelet adhesion by covering a high interfacial free energy surface with a lower one. The condition described by Relation [3 - 2] would be met in this situation, because the sum of the platelet/blood and subendothelium/blood interfacial free energies is greater than the platelet/subendothelium interfacial free energy. We assume that the platelet/blood interfacial free energy is low. This is not
unreasonable, considering the work of van Oss (1978) on the thermodynamics of phagocytic engulfment, which shows that cells with surfaces of high interfacial free energy are rapidly engulfed by phagocytes of the immune system. This would likely be the fate of platelets if they did not have surfaces of relatively low interfacial free energy. However, the thermodynamic theory as outlined here is applicable for only the first few seconds of endothelial injury, because it precludes platelet activation. Once the first platelets become adherent, they secrete substances which cause marked changes in platelet activity, leading to an active aggregation of platelets at the injury site (Schafer and Handin, 1979).
3.3 The Effects of Feeding Protocol, Time (on/off diet), and Location on the Surface Properties of Atherosclerotic Rabbit Arteries

3.3.1 Introduction

Our original study (Boyce et al., 1980) and the work described in the preceding section (3.2) indicate that changing interfacial free energy of the arterial wall may be an important factor in the initiation and development of atherosclerosis. The early results with the oil/saline test system showed that the lesion surface in rabbits has a significantly higher interfacial free energy than plaque-free regions of the same artery and of control vessels (0.36 ± 0.08 (SEM) vs 0.035 ± 0.01 mJ · m⁻²). The oil/saline system was useful in this application because the lesion and non-lesion situations probably represent extremes in the range of interfacial free energies present on the arterial surface. For reasons that I have discussed earlier, the oil/saline system lacks the sensitivity required to indicate what are probably more subtle surface energy changes that would occur during atherogenesis. In this

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study we have used the now familiar PEG/dextran system to investigate the surface properties of aortae from rabbits on atherogenic diets, prior to lesion development. Our purpose was to see if a gradual change in the arterial surface precedes the appearance of gross plaque.

3.3.2 Materials and Methods

3.3.2.1 Experimental Design

Rabbits fed a high cholesterol diet for four weeks and then returned to regular chow (regression protocol) develop lesions in locations quite different from rabbits fed a high cholesterol diet continuously (progression protocol) (Roach and Fletcher, 1979; Roach et al., 1976). Our original experimental design was to compare relative interfacial free energies at specific locations in atherosclerotic arteries from rabbits on "progression" and "regression" diets. Unfortunately it was necessary to modify this original plan because an interruption in the cholesterol feed supply caused the "regression" group to be withdrawn briefly from the atherogenic diet during the feeding program. We continued the investigation with a revised design which examines how arterial wall surface properties are affected by three different factors: 1) feeding protocol
(interrupted feeding vs a continuous cholesterol diet),
2) time on or off the atherogenic diet, and 3) location
on the vessel surface.

3.3.2.2 Feeding Protocol

Thirty-three male New Zealand White rabbits
weighing between 2 and 3 kg were used in the study.
Twenty of the animals, the "interrupted group" were fed
a 2% cholesterol diet (ICN Nutritional Biochemicals,
Cleveland, OH) for 40 days with a 5 day interruption
(returned to normal chow) in the middle of the program.
For the data analysis this group was further subdivided
into two sections of 10 animals each (A and B) so that
the diet of each animal within a section was interrupted
at the same time of the feeding period. This division
was necessary because one group (B) was started on the
cholesterol diet one week later than the other (A). At
the end of feeding, the animals were killed 0, 7, 14, 21
and 42 days after returning to a normal diet. Each time
class contained 4 animals. A second group of 10 rabbits
was fed 2% cholesterol with no interruption and animals
were killed after 30, 43 and 50 days on the diet. These
sub-groups contained 4, 3 and 3 rabbits respectively. By
killing rabbits at different times we hoped to get an
indication of disease progression. Three rabbits fed
normal rabbit chow formed a control group.
3.3.2.3 **Test System and Sample Preparation**

The method used here is essentially the same as the one described in the previous investigation (section 3.2.2.1): contact angle measurement with a test system of 4% dextran (mol. wt: 2,000,000)/4% PEG (mol. wt: 20,000). A section of descending thoracic aorta (4 to 6 cm long) was cut longitudinally down the ventral surface to expose the intercostal branches. To investigate the importance of location, we defined on the artery surface 4 regions: dorsal, ventral, proximal and distal. Although 23 of the 30 rabbits fed cholesterol exhibited some lesion development, only those areas where no visible plaques had yet formed were tested. We were only concerned with the surface changes leading up to plaque formation, therefore lesion areas were ignored. With the tissue immersed in the PEG-rich bulk fluid, we placed droplets of dextran at the specified locations which showed no plaques. We measured advancing contact angles from photographs of sessile drops. After the measurements the aortic sections were stained with Sudan IV to confirm that those areas where droplets were placed had not yet developed lesions.

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4 The experiments on atherosclerotic arteries were performed by Mr. Philip C. Wong who worked with me as a summer student in 1981.
3.3.2.4 Strain Experiments

The results that we obtained on the various arterial surfaces tended to have a large amount of scatter associated with them. Although when mounted, the artery was stretched to the length measured in situ, it is unlikely that the strain was uniform over the tissue surface. Perhaps the large scatter in our contact angle measurements could be attributed to different surface strains at different locations.

To test this hypothesis we designed a chamber which allowed us to apply a longitudinal strain to the arterial tissue as we placed drops on its surface. As with our other chambers, a pivoting support for the sample mount permitted tilting of the specimen so we could clearly see the contact points of the droplet on the artery. Thoracic aortae from 5 rabbits were used in this study and the in situ length of each vessel was recorded before it was removed from the animal. The tissue was secured with end clamps to a mounting bar which was designed to draw apart as tension was applied through a nylon cord attached at one end. By changing the weight hanging from this cord various strains on the artery could be produced. For each extension, the length of the tissue was recorded and 5 or 6 droplets were placed at random locations on the arterial surface. We
FIGURE 3 - 5

Variation of $\cos \theta$ vs Strain (%) for arterial samples.
measured advancing contact angles from photographs taken of the droplets. Longitudinal strains of between -15% and +30% were tested.

3.3.3 Results

3.3.3.1 Strain Experiments

From the in situ size of the artery and the elongated length for each test position, it was possible to calculate the strain on the vessel:

\[
\text{strain (\%)} = \frac{\text{elongated length} - \text{original length}}{\text{original length}} \times 100
\]

We constructed a plot of cosine of the contact angle vs strain to test if stretching an artery affected its surface properties. These results appear in Figure 3-5.

A linear regression was performed on both data sets and correlation coefficients were calculated. For the contact angle vs strain plot, the correlation coefficient was 0.002 while treatment of the cosines of the contact angles gave a value of -0.004. Neither of these values are significant at the 95% confidence level for a sample size of 100 measurements. Therefore, we must conclude that there is no significant linear correlation between the surface properties of the artery (as indicated by contact angles) and the longitudinal strain on the tissue.
3.3.3.2 Statistical Analysis

3.3.3.2.1 Analysis of Variance - Design

For analyzing the main experiment (section 3.3.2.1) for the effect of the three factors: 1) feeding protocol, 2) time on/off diet and 3) location, a three factor analysis of variance (ANOVA) is the appropriate statistical treatment (Sokal and Rohlf, 1981; p. 374-387). While the dietary protocol and droplet location factors had 3 and 4 levels respectively, the time factor had a more or less continuous distribution. We therefore blocked the time factor into 3 discrete levels based on the number of weeks off or on the atherogenic diet. For the two "interrupted groups", 3 levels were classified as follows: up to 1 week off the diet, 1 to 2 weeks off the diet and more than 2 weeks off the diet. To compare fairly the continuously fed rabbits with the ones on an interrupted diet, we classified them according to the weeks on the atherogenic diet beyond the initial 4 week feeding period. An initial 4 week feeding period was common to all groups. Therefore, the classification levels for those rabbits fed cholesterol chow continuously were: up to 1 week on the diet, 1 to 2 weeks on the diet and more than 2 weeks on the diet beyond the initial feeding period. The analysis of variance tests for the effects of: the duration of feeding, the feeding protocol and the test location as measured by the cosine of contact angle of the dextran
droplet. The individual effects of each factor as well as the effects due to multiple interactions between factors are considered in the analysis.

3.3.3.2.2 Analysis of Variance - Results

Bartlett's test for homogeneity of variances (Sokal and Rohlf, 1981; p. 402-412) justified using an analysis of variance to treat the data. The three factor ANOVA indicated that the time on or off the diet and the feeding protocol had no significant effect (at the 95% confidence level) on the contact angle of the dextran droplet. However, the analysis did indicate that the location of the droplet did have a significant effect on the measured contact angle (p < 0.05). There were no significant two or three way interactions between the various factors.

3.3.3.2.3 Analysis of Covariance - Design

To increase the number of replicates in each cell for the analysis of variance, the time factor was grouped according to the number of weeks on or off the atherogenic diet following the initial four week feeding period. It is possible that blocking the data in this manner could mask significant effects caused by the other two factors: the feeding protocol and the drop location. To explore this possibility a second analysis on the data was performed.
By considering the actual number of days on or off the diet beyond the initial feeding period, the time factor may be treated as an independent variable or covariate with the contact angle (the dependent variable). Since this gives rise to two variables in the analysis, a different statistical procedure known as analysis of covariance (abbreviated: ANCOVA) must be used (Sokal and Rohlf, 1981; p. 509-530). The analysis of covariance is useful because differences in a variable between groups can be adjusted before comparing the effects of the main factors. The analysis employed here tested for the effects of feeding protocol and droplet location on the contact angle, after adjusting for differences in time on or off the atherogenic diet.

3.3.3.2.4 Analysis of Covariance – Results

Once again Bartlett's test was used to confirm homogeneity of group variances. The analysis of covariance indicated that while the feeding protocol did not have a significant effect on the contact angle, the location of the dextran test droplet did ($p < 0.05$). There was no significant interaction between the two factors tested. This analysis confirms the earlier results obtained with the 3 factor analysis of variance. While analyses of variance and covariance will indicate that a factor has a significant effect on a variable, it will not identify which groups are significantly
different from others. A Student–Neumann–Keuls multiple range test (Zar, 1974; p. 151-155) was used to identify the distal location as having a significantly different contact angle from the other vessel locations (i.e. proximal, dorsal and ventral). This result was independent of feeding protocol.

3.3.3.3 Experiments on Normals

Because we were looking for surface changes due to disease progression, normal animals were not considered in the original experimental design. However, after finding that the time on or off the atherogenic diet had no significant effect on the contact angle, another question became important. Did the different surface properties measured at the distal location exist in normal vessels, or were they a result of the disease process? To answer this question, we designed a final experiment where tissue from normal animal controls was tested.

Since the previous analysis showed that the dextran contact angle was not significantly different at the proximal, dorsal and ventral locations, we collected data only from regions proximal and distal to branch orifices. The thoracic aortae from 3 rabbits fed a diet of normal rabbit chow were tested as before with the PEG/dextran system. We compared these results with the data obtained in the previous experiment for proximal
TABLE 3 - 3

Summary of contact angles on aortic surfaces.
Results from all proximal locations and the distal control are not significantly different. The results at distal locations for rabbits on atherogenic diets differ significantly from all other values \( (p < 0.05) \). There is no significant difference between the results at distal locations for rabbits on interrupted and continuous diets.
TABLE 3 - 3

<table>
<thead>
<tr>
<th>Location</th>
<th>Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Interrupted</td>
</tr>
<tr>
<td></td>
<td>Degrees ± SEM</td>
</tr>
<tr>
<td></td>
<td>90.3 ± 2.3</td>
</tr>
<tr>
<td>Proximal</td>
<td>(-0.0052)</td>
</tr>
<tr>
<td></td>
<td>n = 75</td>
</tr>
<tr>
<td>Distal</td>
<td>77.1 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>(0.2233)</td>
</tr>
<tr>
<td></td>
<td>n = 36</td>
</tr>
</tbody>
</table>
and distal locations in atherosclerotic arteries. The results from the two interrupted feeding protocols, A and B, were pooled into a single group, but the difference between the interrupted and continuous feeding programs was maintained throughout the analysis.

Bartlett's test justified using a two factor analysis of variance to analyze the three experimental groups (interrupted and continuous cholesterol feeding plus normals) at two different test locations (proximal and distal). A summary of these results appears in Table 3-3. The ANOVA indicated that there was a significant difference between group means ($p < 0.05$) and these were identified using a Student–Neumann–Keuls multiple range test. The mean cosines of contact angles measured at all the proximal locations, and at the distal location of the control group, were not significantly different ($p > 0.05$). However, the measurements made at distal locations for rabbits on atherogenic diets, both interrupted and continuous, did differ significantly from all other test groups. As in the previous analyses, no significant difference in results was found between rabbits on interrupted and continuous feeding programs.

3.3.4 Discussion

Unlike the results reported in our original study on atherosclerosed arteries, it is not possible to convert the contact angles measured here into absolute
values of interfacial free energy; I have mentioned earlier that Neumann's equation of state does not apply to the PEG/dextran system. This inability however, does not affect the significant difference in contact angle which we have measured between proximal and distal locations in aortae from cholesterol-fed rabbits.

3.3.4.1 Interpretations

A decrease in contact angle at locations distal to branch orifices in atherosclerotic vessels indicates an increase in the interfacial free energy (relative to the PEG-rich bulk medium) for those regions. This change in surface character occurs at those locations on the arterial surface where lesions are predicted to develop, given enough time on the atherogenic diet (Roach et al., 1976). The lower contact angle at distal locations is probably not due to endothelial denudation because our earlier experiments indicated that arterial subendothelium gives a mean contact angle of 20.0 ± 0.8° (SEM) when tested with the same system. This clearly was not what we observed here. Therefore it appears that some factor in the cholesterol-fed rabbits is causing a subtle change in the arterial surface at distal locations. This alteration occurs before any gross morphological or histological change is apparent. We have no evidence to
show whether the observed change in surface properties causes the visible lesion to form, or is a correlate of the factors leading to the formation of a lesion.

3.3.4.2 Biophysical Model

The lumenal side of endothelial cells is covered with a polysaccharide layer known as the glycocalyx (Schwartz et al., 1978). Roach and Smith (1983) have speculated that the glycocalyx of endothelial cells located distally to branch orifices may be damaged by the chronic high shear forces present in these areas. The decreased contact angle that we measured at distal locations implies an increase of hydration, and/or an increased interaction of polymers with the surface components. The high shear in this region may have the effect of reducing the structural organization of the glycocalyx, thus permitting a greater interaction between the constituent glycoproteins and the dextran polymers of the test drop. A glycocalyx damaged in this way could initiate atherosclerosis by increasing the permeability of the arterial wall to circulating lipids in the blood (Roach and Smith, 1983).
3.3.5 Appendix to Section 3.3

At this point a caution should be issued about comparing the results presented here, with the results from our original investigation which was mentioned in the introduction to this section (Boyce et al., 1980). Substantially different diets were used to induce atherosclerosis in the two experiments. In the original study, rabbits were fed a standard atherogenic diet consisting of 2% cholesterol in 6% corn oil mixed with ground rabbit chow. Since that time, a pelleted 2% cholesterol chow with no carrier fat has become commercially available and this diet was used in the present study. There is evidence that the type and quantity of the carrier fat used in an atherogenic diet has an effect on lesion development (Kritchevsky, 1967, 1968, 1970). Therefore it is possible that the hydrophobic character of lesion surfaces that we found in the original study, could have been partially the result of the very high levels of carrier fat in the diet.
CHAPTER 4

GENERAL DISCUSSION AND SUGGESTIONS
FOR FUTURE RESEARCH

"There was no doubt about the Magic this time. Down and down they rushed, first through darkness and then through a mass of vague and whirling shapes which might have been almost anything. It grew lighter. Then suddenly they felt that they were standing on something solid. A moment later everything came into focus ...."

C.S. Lewis - The Magician's Nephew
4.1 Overview – Surface Properties of the Arterial Wall and Atherogenesis

The results and interpretations presented in the third chapter may now be brought together to form a revised hypothesis of how atherosclerosis may develop. There is a large body of literature which treats endothelial injury (by whatever mechanism) as the initiating event of atherosclerosis Ross and Glomset, 1976; Kottke and Subbiah, 1978). Rather than being an acute event which causes sudden endothelial desquamation, the injury process appears to be more gradual, progressing over a long period of time. For example, Langille (1984), assuming a haemodynamic injury mechanism, has exposed normal endothelium to acute shear stresses of 80 N · m⁻² without producing denudation. This value is more than eight times the maximum shear stress estimated to occur at the arterial wall in vivo (Chien, 1976). Assuming that high shear forces do injure the endothelium, it must be chronic rather than acute exposure to high shear which is most damaging to the cells.

A gradual or chronic injury mechanism is consistent with the results reported in Section 3.3 of the preceding chapter. The 80° contact angle that we have measured on "pre-lesion" locations in atherosclerotic vessels shows that the arterial surface here is significantly different from other areas where
plaque is not expected to develop ($\theta = 90^\circ$). An altered surface of the endothelium may indicate, or be the result of, a decreased resistance of the endothelium to various damaging agents. Weakened resistance of the endothelium therefore, could permit sustained cell injury eventually resulting in endothelial desquamation.

It is now well accepted that endothelial denudation and exposure of the subendothelium is followed by rapid adhesion and accumulation of platelets, independent of the injury mechanism (balloon catheter [Groves et al., 1979]; treatment with ethylenediaminetetraacetate (EDTA) [Sheppard, 1972]; high fluid shear produced by stenosis [Ts'ao and Glagov, 1970]). My results from Section show that denuding the normal endothelium ($\theta = 90^\circ$) exposes a subendothelial surface of significantly higher interfacial free energy ($\theta = 20^\circ$). While platelets do not normally attach to intact endothelium, adhesion to a higher energy subendothelium would be favoured because the free energy change for adhesion could become negative - although we cannot actually calculate it.

After attachment to the subendothelium, the platelets interact with the collagen fibrils, become activated and disgorge the contents of their granules into the arterial wall. Smooth muscle cell
proliferation, lipid accumulation and fibrosis would follow (as detailed in Section 1.3) resulting in the development of a mature atherosclerotic plaque.

4.2 **Significance of the Surface Alteration in "Pre-Lesion" Areas**

The nature of the pre-lesion change of the endothelial surface is unknown and can only be speculated upon. The change is almost certainly confined to the glycocalyx of the cell surface because our own unpublished results show a dextran contact angle that is substantially higher (140-150°) on a bare phospholipid monolayer. If we assume that the relation between the cell/bulk fluid interfacial free energy and the dextran contact angle is similar to that depicted in Figure 2-2, a 10° change in contact angle around 90° reflects a relatively small change in the interfacial free energy of the cell surface. (A change of 10° at higher contact angles would represent a much larger change in substrate interfacial free energy).

An alteration of the surface properties of the glycocalyx must arise from a change in composition, and/or a change in structure, which permits greater interaction with the free polymers of the test droplet. The glycocalyx is made up of various carbohydrate-rich molecules (glycoproteins and glycolipids) and plasma proteins. Whereas the glycoconjugate material is firmly
anchored within the hydrophobic regions of the bilayer, the plasma proteins are adsorbed, or in some cases specifically bound to the bilayer surface (Peters, 1984). In a high shear region (such as those areas distal to branch points), the material most likely to be removed from the glycocalyx would be the weakly bound plasma proteins. It is possible that the altered blood chemistry produced by the high cholesterol diet facilitates the removal process by changing the affinity of the blood for specific proteins. Loss of plasma proteins from the glycocalyx could have two effects which might explain the decreased contact angle which we measure at distal locations: 1) Since the plasma proteins contain little carbohydrate, their removal from the glycocalyx would increase the proportion of carbohydrate in the cell surface coat. Such a change will increase the hydrophilic character of the glycocalyx. 2) The physical presence of plasma proteins in the glycocalyx must at least partially restrict the mobility of the long carbohydrate chains of the glycoproteins. Removal of the plasma would increase the mobility of the carbohydrate chains thus allowing for a greater degree of interaction with the free dextran polymers of the test droplet. Increased interaction between the constituents of the test droplet and substrate will reduce the interfacial free energy and cause the contact angle to decrease.
This hypothesis should be testable using fluorescently labelled antibodies for those proteins thought to be bound to the endothelial surface; albumin and immunoglobulin G are prime candidates. If proteins are absent at distal locations in cholesterol-fed rabbits, the fluorescence of those regions should be significantly less than those areas where protein removal is not expected, i.e. areas where lesions do not normally develop.

Few studies investigating how the endothelial glycocalyx is affected by atherogenesis have been published. Those studies that do exist report results which conflict with one another. For instance Bálint et al. (1974) found a decrease in the thickness of the endothelial surface coat in rats fed a high cholesterol diet, while Weber et al. (1973) reported just the opposite; the glycocalyx became thicker in the early stages of cholesterol induced atherogenesis in rabbits. Unfortunately, neither of these studies recorded from where on the aortic surface (relative to branch points) the histological sections were taken. The thickness of the glycocalyx was determined from transmission electron micrographs of samples stained with ruthenium red. However I question the accuracy of these measurements because the normal (hydrated) state of the glycocalyx will be radically altered by the tissue dehydration that is required for transmission electron microscopy.
4.3. Platelet Adhesion

4.3.1 Introduction

I first became interested in an alternative explanation for cellular adhesion because the widely accepted ligand-receptor theory (see Grinnell, 1978 for a review) just did not make sense to me. I felt that the main deficiency of the theory was that it did not explain at all well why cells, and platelets in particular, readily stick to so many different natural, and artificial materials. I also thought that extending many of the kinetic principles that worked well at the molecular level (e.g. enzyme-substrate interactions) to explain phenomena at the much larger cellular scale was unjustified. A thermodynamically-based theory had existed for decades to explain adhesion between materials in physical systems, and I could see no reason why it could not be applied to biological systems. The advantage of a thermodynamic theory is that it is completely general and can be used to explain cellular adhesion to synthetic materials just as easily as to natural substrates. The key to testing the theory was the development of sensitive methods to assess the surface properties of biological materials in their natural, aqueous environment.

In this thesis I have taken the thermodynamic viewpoint and measured a bulk property - the interfacial free energy, which thermodynamics relates directly to
adhesion. This approach is valid whether or not proteins are involved in the mechanism of platelet adhesion. As a first study, plasma proteins were excluded from my experiments for the sake of simplicity. However, since many investigators feel that plasma proteins are important as mediators of platelet adhesion, it is worthwhile considering the behavior of proteins in this context.

4.3.2 Proteins and Platelet Adhesion to the Subendothelium

While most researchers feel that an adsorbed layer of protein is essential for platelet adhesion to foreign surfaces (Vroman et al., 1977; Forbes, 1981; Salzman and Merrill, 1982), there is no conclusive evidence that protein adsorption to the subendothelium is a prerequisite for platelet attachment (Turitto and Baumgartner, 1982). If protein adsorption is necessary for adhesion in vivo, it has to be fast since platelets have been found to adhere within 30 seconds of endothelial denudation (Baumgartner and Muggli, 1976). Within this time, the adsorbed protein layer must be altered so that a different substrate surface appears to the platelet. If the protein did not change, the platelet would be exposed to the same protein on the
surface as it encounters in the bulk plasma, and there would be no reason for the platelet to preferentially attach to the arterial substrate.

4.3.2.1 Fibrinogen

Immunofluorescent techniques and other surface diagnostic methods have indicated that the first and predominant protein adsorbed, at least on artificial surfaces, is fibrinogen (Vroman et al., 1971; Baier, 1983). Brash and Uniyal (1979) have shown that the thrombogenicity of a foreign surface is correlated with the amount of fibrinogen the surface adsorbs relative to other proteins. By measuring the time required for an adsorbed layer of fibrinogen to become unrecognizable by an antifibrinogen antibody, Vromen and Adams (1969) were able to estimate that the transformation time for fibrinogen was 30 to 60 seconds. This information about the natural situation would seem to preclude fibrinogen as a necessary mediator of platelet adhesion, because platelets attach to exposed subendothelium in less than 30 seconds. This conclusion is further supported by Weiss et al. (1978), who reported normal platelet adhesion in an individual lacking fibrinogen in his blood (alibrogenemia).
4.3.2.2 **Fibronectin**

Other proteins have been suggested as important for platelet interaction with collagen of the subendothelium: Fibronectin, a high molecular weight glycoprotein has been implicated by Bensusan *et al.* (1978) as the collagen receptor on platelets. However, fibronectin has not been identified on the platelet surface (Hynes *et al.*, 1978), and Zucker *et al.* (1979) have shown that fibronectin is released only after the platelet has been activated by collagen or thrombin. It is possible that fibronectin could be adsorbed on the subendothelium from the plasma. Other work (Engvall *et al.*, 1978) indicates that fibronectin has a significantly stronger affinity for denatured, rather than native collagen. Experiments performed by Santoro and Cunningham (1979, 1981) have led these workers to conclude that fibronectin has no unique role as a mediator of platelet adhesion.

4.3.2.3 **Von Willebrand's Factor/Factor VIII**

Von Willebrand's factor (Factor VIII) appears to be essential for optimal platelet adhesion (Tshopp *et al.*, 1974; Berndt and Philips, 1981). The importance of the Factor VIII complex is indicated by patients with von Willebrand's disease, a bleeding disorder characterized by a decreased ability of platelets to adhere to the subendothelium. Little is known about the
Factor VIII mechanism other than digestion studies of the subendothelium showing it is primarily involved with the platelet-collagen interaction (Baumgartner et al., 1976).

The platelet-collagen interaction, which may be affected by fibronectin and von Willebrand's factor, is probably a secondary event to initial adhesive contact because naked collagen is not exposed on the subendothelial surface. Rather, an amorphous basement membrane-like material composed mostly of proteoglycans and glycoproteins is the main structure at the surface, usually covering the other components (Turitto and Baumgartner, 1982).

4.3.3 Physical vs. Biological Interpretations of "Adhesion"

In this thesis I have taken the physical view of adhesion treating it as the simple physical contact and attachment of a cell to a substrate. However, the previous paragraph illustrates a source of confusion in the biological literature, that is a vague interpretation of adhesion. Most biological researchers view cell adhesion as attachment and/or something additional where there is a specific biochemical interaction with components of the substrate, for example the platelet-collagen interaction. Unfortunately, most published investigations do not say
explicitly how adhesion has been defined in the study and this makes the results difficult to interpret. Notable exceptions are the work of Grinnell (1978), who discusses "specific" and "non-specific" adhesion, and Turitto and Baumgartner (1982) who define platelet adhesion as a two step process: 1) Contact-initial attachment of platelets, and 2) Spreading-interaction of platelets with the surface that requires an extensive change of shape and the release of granular contents.

The thermodynamic scheme that I have proposed for platelet adhesion is only concerned with the first step of the adhesive process outlined above. If proteins do play a role in "adhesion" (as the biologists interpret it), they are probably involved in the second stage, where specific interactions between the platelet and the substrate take place.

4.4 Antithrombic Surface Properties of the Endothelium

In the same way that many investigators have tried to explain platelet adhesion through a protein mediated mechanism, others have used similar arguments to account for the antithrombic properties of endothelium. Moncada and Vane (1979) have suggested that the formation of prostacyclin by the endothelium prevents platelet adhesion to the endothelial surface. However in vitro and in vivo experiments with cultured
endothelial cells and aortic segments have shown that platelets do not adhere to endothelial cells even when their prostacyclin synthesis is inhibited by aspirin (Dejana et al., 1980). Further evidence against an active role of prostacyclin in inhibiting adhesion is the fact that the subendothelial region contains smooth muscle cells which also produce prostacyclin (Moncada et al., 1977); platelet adhesion to this surface is certainly not inhibited. These results therefore seem to eliminate prostacyclin as an active inhibitor of platelet adhesion.

4.5 Surface Properties of Platelets – Suggestions for Future Investigation

As I discussed in the previous chapter, the underlying assumption of the thermodynamic theory of platelet adhesion is that the platelet/bulk fluid interfacial free energy is low. Only if this is true will the overall free energy of the system be minimized by covering a high energy surface (the subendothelium) with a lower interfacial free energy material (the platelet). The conventional means of determining cell/bulk fluid interfacial free energies involves contact angle measurements on cell layers formed by centrifugation (Neumann et al., 1979, Schürch et al., 1981). The highly reactive nature of platelets prohibits this type of treatment without using anticoagulents,
which must have some effect on platelet surface properties. While the actual measurement of contact angles on cell layers may not be feasible one may calculate a hypothetical contact angle using an equation derived by Albertsson (1971, p. 63) for the phase partition of cell particles:

$$\frac{N_i}{N_2} = C \exp \left( \frac{A}{\gamma_{1,2} \cdot KT} \right) \left( \gamma_{P,1} - \gamma_{P,2} - \gamma_{P,2} \right)^2 \quad [4-1]$$

where $N_i$ is the number of cells at the interface, $N_2$ is the number of cells in the bottom phase, $C$ is a partition constant, $A$ is the surface area of the cell, $K$ is the Boltzmann constant, $T$ is absolute temperature, $\gamma$ is the interfacial free energy, and the superscripts: $P,1$ and $2$ represent the platelet, top and bottom phases respectively.

While the absolute values of $\gamma_{P,1}$ and $\gamma_{P,2}$ cannot be calculated, the difference may be obtained from Young's equation:

$$\gamma_{P,1} - \gamma_{P,2} = \gamma_{1,2} \cos \theta \quad [4-2]$$

Substituting for $\gamma_{P,1} - \gamma_{P,2}$ in equation [4 - 1] and rearranging gives:

$$\cos \theta = \left[ \frac{\ln N_i \cdot KT}{N_2 \cdot C} \right]^{1/2} + 1 \quad [4-3]$$

where $\theta$ is the hypothetical contact angle formed by a drop of phase 2 within the bulk phase/on a layer of platelets. Using our reference polymer system of 4% PEG
(top)/4% dextran (bottom), a calculated contact angle greater than 20° implies a lower interfacial free-energy cell surface than the subendothelium that we tested earlier.

Because equation [4 - 1] was derived assuming that Brownian motion determines the particle distribution, there may be a question whether it is applicable to objects as large as cells. While evidence does exist which suggests that this equation is not valid for red blood cells (Sharp et al., 1983), it has not been directly tested for particles as small as platelets (≈1 μm diameter). The equation might be tested using glutaraldehyde fixed platelets which have similar partition characteristics to normal platelets (Brooks, 1983; personal communication), and which can probably be chemically bonded to polymeric surfaces for contact angle measurements. Should the distribution characteristics of the cells in both phases not be governed solely by thermal agitation as is assumed in the equation, an investigation into the mechanical and shear forces acting on cells adsorbed at the phase boundary would be necessary (Brooks et al., 1983).

4.6 Calibration of the PEG/Dextran System

Suggestions for Future Experimentation

As I discussed in Section 2:3.3, Neumann's equation of state cannot be used to calculate substrate
interfacial free energies with aqueous polymer systems. With the equation of state eliminated, and no other theoretical approaches suggested, we are left with attempting an experimental calibration. While we have not been successful to date the most promising method appears to be Schürch's double drop technique (Schürch and McIver, 1981) where the substrate interfacial free energies can be easily manipulated. An insoluble surfactant is spread on a dense fluorocarbon substrate drop and the tension of the monolayer is adjusted by adding or withdrawing fluorocarbon material. We believe our difficulties with this approach result from the low affinity of the dextran droplet for the surfactant, i.e. the test droplet will not form contact angles below approximately 150°. The key to success with this method may be the development of new surfactant materials, such as glycolipids with long carbohydrate chains which have an increased affinity for the dextran polymer.
CHAPTER 5

SUMMARY AND CONCLUSIONS

"Yes, of course you'll get back to Narnia again some day. But don't go trying to use the same route twice. Indeed, don't try to get there at all. It'll happen when you're not looking for it ... What's that? How will you know? Oh, you'll know all right. Keep your eyes open. Bless me, what do they teach them at these schools?"

C.S. Lewis - The Lion, the Witch and the Wardrobe
5.1 Summary and Conclusions

This thesis demonstrates how a technique, originally developed for analyzing physical systems, has been successfully and usefully applied to a biological problem. I have established an argument for the thermodynamic properties of the arterial surface playing an important role in atherogenesis. The involvement of changing interfacial free energies has been implicated here in two ways:

1) In atherosclerotic arteries, interfacial free energies at pre-lesion locations are significantly different from those areas where plaque does not usually develop. I've interpreted this as a change in the endothelial glycocalyx which may weaken the resistance of the endothelium to various injury factors (e.g. haemodynamic shear, hypercholesterolemia, etc.) leading ultimately to cell denudation.

2) Injury to the endothelium exposes a subendothelial layer of significantly higher interfacial free energy. A higher energy surface could cause the free energy change for initial adhesion of platelets to become negative, thereby favouring adhesion as a spontaneous process. On the other hand the intact endothelium may prevent platelet adhesion by providing a low energy surface which could yield a positive free energy change on adhesion. Such a process would not be favoured to occur spontaneously.
Although my attempts to calibrate the PEG/dextran system were unsuccessful, the experiments were valuable for extending our understanding of the dextran/substrate interaction. Had these experiments succeeded, we might have learned more.

To my knowledge this thesis is the first study to report subtle thermodynamic changes on the arterial surface and relate this to the pathology of atherosclerosis. To accomplish this, a new approach had to be developed and new methods pioneered. Now that the problems have been solved and the techniques mastered, the range of application for this type of biophysical analysis seems almost limitless.
REFERENCES


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