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MECHANICAL AND STRUCTURAL STUDIES
OF ELASTIN

A Thesis submitted for the Degree
of
DOCTOR OF PHILOSOPHY

by
WILLIAM N. GRUT

Department of Mechanical Engineering
University of Surrey
July 1977
ACKNOWLEDGEMENTS

First and foremost I would like to thank Dr. J. Edwards and Dr. E.J. Evans for supervision of the work. Without their continued help and encouragement the project would have met with little success.

I would also like to thank Dr. K.L. Dorrington, Dr. N.G. McCrum, Dr. S.M. Partridge and Dr. P. Richards for various theoretical and experimental assistances, and Professor J.M. Zarek for his invaluable support.

Finally, I wish to express my appreciation to the numerous members of the technical staff who assisted with the project and particularly to Miss Gillian Hunter for expert typing of the script.
Purified elastin was examined by means of dynamic and static mechanical testing, scanning and transmission electron microscopy, and a study performed of its swelling characteristics and density in various solvents.

Purification of the material was effected by a combination of autoclaving and solvent extraction and the method investigated for suitability by gravimetric analysis, chemical testing and scanning electron microscopy.

Investigation of previously reported mechanocalorimetric experiments on elastin (one of the major foundations of the globular theory of elastin structure) revealed a misinterpretation in the results and a re-interpretation could be made which fully supported the concept of elastin as an amorphous elastomer. This re-interpretation was backed by experimental results of swelling studies on the material.

Dynamic investigation of the temperature dependence of the shear modulus using a free oscillating inverted torsion pendulum between 0°C and 70°C revealed water and ethanol/water (up to 50 vol % ethanol) saturated elastin to display modulus/temperature characteristics corresponding with those of a rubber at the higher temperature end of its glass transition region. Formamide saturated elastin displayed the linear positive shear modulus relationship with temperature associated with equilibrium elastic rubbers. The effect of varying saturation was also examined by the method. Decreasing saturation was found to increase the glass transition temperature of the water/elastin composite. The glass transition temperatures and secondary relaxations of fully saturated elastin/water and elastin/formamide were investigated by measurement of logarithmic decrement at below 0°C.
Further studies of the mechanical states of the various elastin/liquid systems were carried out using apparatus based on the 'Instron' tensile testing machine. The stress responses to step and steady state strains were monitored over varying temperatures. 'Constant swelling' elastin solvents of 20:80 v/v ethanol/water, 30:70 v/v ethanediol/water, and 40:60 v/v glycerol/water were used as well as water and formamide alone. The results confirmed the viscous element of the transition region state of the elastin/water and elastin/solvent/water systems and the equilibrium rubber elasticity of the elastin/formamide system. Further steady-state strain tests on the elastin/water system at varying elongations yielded data which could be analysed to provide an assessment of the energy contribution to the retractive force of the material \( (f_e/f) \). This ratio appeared to lie in the region 0.0 to 0.1, a value typical of amorphous rubbers and one which re-affirms the random coil concept of elastin structure.

Structural examination was performed by scanning and transmission electron microscopy. Elastin from both elastin/water and elastin/formamide systems was used from both purified bovine ligamentum nuchae and porcine aorta. For high resolution transmission electron microscopy the technique of negative staining was employed to enhance contrast. Specimen preparation was by a method of fragmentation followed by air or freeze drying. Results indicated that previously observed fibrillar structures within the material were probably an artefact caused by the preparation process and not typical of elastin in its rubbery, in vivo condition.

The overall conclusion of the work was that the structure of solvated elastin conforms to the classical random coil model and not to the globular, fibrillar or 'oiled-coil' concepts suggested in the literature.
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Chapter 1

INTRODUCTION

1.1 The Connective Tissue

A large part of the mammalian body is composed of tissues which are designed in such a way as to enable them to perform mechanical functions. These tissues usually lie in essentially intercellular positions and are present in the form of fibres, membranes or surrounding matrices.

One group of these tissues is known as 'elastic tissues' owing to their ability to undergo large mechanical strains of several hundred percent without undergoing permanent deformation. Elastic tissue is found in widely varying amounts in many locations in the body. Generally, however, in man it is found in greatest abundance in the cardiovascular connective tissues, and in herbivorous animals in their ligamentum nuchae. These are thus the most common specimen sources for research purposes into elastic tissue and elastin, the soft yellow-white rubber-like protein which is responsible for supplying elastic tissue with its high strain tolerance.

It is with elastin that the work for this thesis is concerned. Although considerable effort has been put into attempting to understand the mechanisms which are operative in the formation, maintenance and disintegration of connective tissues, the mechanisms are still not very well understood. The tissues are subject to a wide variety of transformations under the influence of many normal and pathologic stimuli of a local or general nature.

Elastic tissue is a highly resilient 'composite' material, the prime function of which is the return of distended or distorted structures to their original shape. It chiefly consists of elastin which can be
differentiated from other connective tissue fibres on the basis of its unique physical and chemical properties. Care must therefore be taken to distinguish between 'elastic tissue' or 'elastic fibres' on the one hand and the protein 'elastin' on the other. It has long been known that the yellow-white fibres of elastic tissue are rarely homogeneous; they contain occluded collagen and are contaminated with all the usual ground substance components together with lipids and lipo-proteins. The term elastin refers to the component of elastic fibres which appears amorphous under the optical microscope and the electron microscope at lower magnifications, which takes up specific stains as defined by the early microscopists and finally, which endows the fibre with the characteristic property of rubber-like extension and elastic 'snap' on recoil.1-70

1.2 Early History of Elastic Tissue Research

Hass1-7 has made a detailed study of the historical aspects of elastic tissue research. In his opinion the classical Greeks must have been familiar with the elastic qualities of the various tissues even though Hippocrates1-2 makes no reference to this important property. Galen1-3 is quoted as making extensive, though often erroneous observations on the pulse and blood vessels, but failing to reveal the importance of the elastic nature of the arterial wall and it was in fact Harvey who started coming near the current conception of elasticity in blood vessels in his work of 16281-5.

The first careful studies and comments on the elastic properties of the connective tissues came in the last half of the 18th century1-5, although it was not until the rise of histology some 50 to 100 years later
that any classification of connective tissue into its respective elements came about\textsuperscript{1-6,1-7}. It was in fact the founder of histology, Jakob Henle, who was the first to make comprehensive studies on elastic tissue and identify the elastic lamellae in the walls of blood vessels\textsuperscript{1-8}.

The accumulated observations as recorded by Von Kolliker\textsuperscript{1-9} and by Donders\textsuperscript{1-10} disclose the eager progress made by the histologists and embryologists in the field, and soon pathologists began to add contributions dealing with the behaviour of elastic tissue in disease.

Unna\textsuperscript{1-11} perfected a stain by which the alterations in the character of elastic tissue became more apparent. Solubilization of elastin was described by Horbaczewski\textsuperscript{1-12} and Chittenden and Hart\textsuperscript{1-13} and later Richards and Geis\textsuperscript{1-14} demonstrated its reversible precipitation from solution with heat.

The first amino acid analysis was performed on elastin in 1938\textsuperscript{1-15} by Stein and Muller, and since then the rate of elastin and elastic tissue research has increased significantly and now spans many widely dispersed fields. Those relevant to this work will be discussed in later sections.

\section*{1.3 Distribution of Elastic Tissue}

\subsection*{1.3.1 General}
Although elastic tissue is always found in association with collagen fibres, the proportions of each vary within wide ranges. In general, however, elastic tissue is less abundant in those places where the connective tissue is of loose texture, but localized in areas subject to periodic stress such as the walls of the major arteries, lungs, skin, ligamentum flavum, ligamentum nuchae of ruminant animals, and capsules of various visceral organs such as pleura, pericardium and peritoneum\textsuperscript{1-1,1-16}. 
Rare fibres may be demonstrated in fibrocartilage, and in bone there are a few thick fibres which may be traced into the periosteum.

There is notable association of elastic tissue with smooth muscle to such an extent that some histologists have described the combination as one, myoelastic tissue. The abundance of myoelastic fibres varies but they are numerous in the ocular muscles and those muscles which are attached to soft parts such as those in the tongue and face. Cardiac muscle contains few fibres.

The review by Hass divides the body into various systems and considers the arrangement of elastic tissue in each separately. By his account it is 'those systems which are designed chiefly for the mechanical transportation of materials from one part of the body to another which are supplied with an abundance of elastin, the cardiovascular and lymphatic systems being of primary importance'.

The cardiovascular system can be classified into the heart, the arteries and the veins, while the lymphatic system is classified into the spleen, the lymph nodes and the lymphatic channels.

The arteries and more specifically the aorta are of particular importance to this thesis, as it was the porcine aorta which was the predominant source of the purified elastic specimens: these vessels, therefore, will be considered in more detail than the other systems.

1.3.2 Arteries

1.3.2.1 General

Arteries in general may be roughly subdivided into four groups: elastic types, distributing types, arterioles and precapillary arterioles.

It is customary to divide the walls of these (as well as the walls of the veins) into three regions:
i) the tunica intima - the innermost tissue layers;
ii) the tunica media - the middle region;
and iii) the tunica adventitia - the outermost region.

1.3.2.2 The Elastic Arteries

The elastic arteries include the aorta, the innominate, the subclavian, common carotid and pulmonary. One of their physical functions of prime importance is maintenance of the 'circulatory flywheel' effect, the smoothing out of the pressure fluctuations generated by the pumping action of the heart. Blood at systolic pressure delivered to the elastic arteries by the contracting ventricles both distends and lengthens the arteries, the strain being taken up by the elastic tissue of the arterial wall. As the pressure drops to its diastolic value this strain is released, so helping to maintain blood flow through the system.

The tunica intima of the elastic arteries generally consists of approximately one-sixth of the total wall thickness, and is lined on the inside with a layer of endothelial cells. Behind the endothelium lies the 'sub-endothelial layer' consisting of approximately one quarter of the total thickness of the tunica intima. It consists of an array of fairly delicate elastic fibres that are arranged in a generally longitudinal orientation, parallel to the axis of the vessel, and are surrounded, together with some collagen fibres, by 'amorphous' intercellular substances. A few fibroblasts and macrophages may also be present.

The remainder of the intima is known as the 'deep' or 'external' layer and consists of somewhat coarser elastic fibres, again embedded along with collagen fibres in 'amorphous' intercellular substances, but containing somewhat more cells than the sub-endothelial layer.
In the region where the tunica intima joins the tunica media, the elastic fibres of the former are condensed to form a thick 'fenestrated' plate of elastic tissue called the 'internal elastic lamina' which is similar to the elastic lamina of the media.

The tunica media constitutes the bulk of the wall and consists chiefly of concentrically arranged fenestrated laminae of elastic tissue similar to the internal elastic lamina of the intima. Collagen fibres, delicate elastic fibres and 'amorphous' intercellular substances, together with fibroblasts and smooth muscle fibres, fill the spaces between adjacent laminae. The outermost layer of the tunica media is called the 'external elastic lamina'.

The tunica adventitia in elastic arteries is thin and consists of irregularly arranged connective tissue which contains both collagenic and elastic fibres. Small blood vessels are usually present, known as the vasa vasorum.

1.3.2.3 Distributing Arteries

In distributing arteries, the group to which most arteries belong, the tunica intima is relatively thin, and its most prominent feature is a well developed internal elastic lamina. This consists of a single, thick layer of elastic fibres in youth (forming in the larger distributing arteries a fenestrated plate), but in later adulthood splits into two layers. Between the internal elastic lamina and the endothelium lies a very delicate layer of connective tissue.

The tunica media is a fairly thick layer and consists chiefly of circularly arranged smooth muscle fibres held together to form a cohesive mass by reticular, collagenic and delicate elastic fibres. The proportion of intercellular substances in relation to smooth muscle varies with the size of the vessel.
The tunica adventitia in distributing arteries varies in thickness, but usually is from one half to two thirds the thickness of the media. It consists chiefly of elastic fibres but also contains some collagen. The elastic fibres of the adventitia are condensed to form an external elastic lamina that is continuous with the outer border of the media. Vasa vasorum are present in the adventitia, particularly in the larger arteries.

1.3.2.4 Arterioles and Precapillary Arterioles

The arterioles have a thin internal elastic lamina composed of a network of delicate fibrils. There is no external elastic lamina, although there are a few fibres in the adventitia. As the arterioles diminish in calibre to about 62 micrometers the internal elastic lamina disappears and the adventitia soon loses its elastic network so that the precapillary arterioles as a rule are devoid of elastic tissue.

1.3.3 Other Locations

1.3.3.1 Lymphatic System

All lymphatics which are large enough to have valves and smooth muscle in their walls also have elastic fibres in their walls. Lymphatics with an approximate diameter greater than 2 mm have a certain amount of tangentially oriented elastic tissue in the adventitia, delicate intermuscular fibrils in the media and longitudinally interlacing fibres in the intima.

Several intimal layers of longitudinally directed delicate fibrils are found in the thoracic duct. An internal elastic membrane is found where the fibrillar network near the junction of the intima, and the media, has condensed, and from this structure branching fibres penetrate the media and connect with the thick longitudinal fibres of the adventitia.
Although the lymph nodes are poorly supplied with elastic tissue, fibrils may be found in the capsules and occasionally in the trabeculae of the nodes.

An abundance of elastic tissue is present in the spleen, especially plentiful in the inner layers of the splenic capsule. From here the fibres continue into the trabeculae where the amount of elastic tissue often exceeds the amount of collagenous tissue.

1.3.3.2 Respiratory System

The nasal mucosa contains sparsely distributed delicate elastic fibrils, and in the larynx the arytenoid cartilages have numerous elastic fibres in the matrix of the uppermost regions.

The true vocal cords are chiefly composed of bands of elastic fibres, and in the lamina propria of the trachea are to be found many delicate networks. The elastic fibres are so closely arranged around the tracheal cartilages that a compact membrane is formed and the smooth muscle fibres which pass between the free ends of the incomplete cartilaginous rings are inserted principally into dense bundles of elastic tissue which surround the trachea and its cartilages.

In the bronchi the lamina propria is richly supplied with elastic fibres, continuous with the fibrillar network enmeshing the smooth muscle cells of the bronchial walls and extending throughout the enveloping connective tissue and condensing around bronchial cartilages. The myoelastic membrane is very prominent in the respiratory bronchioles, and closely arranged straight, thin fibrils are found in the walls of the alveolar sacks. The interalveolar septums have a compact meshwork of reticular fibres, but the elastic fibres are few. The visceral pleura contains several layers of elastic tissue which lie at various angles to the plane of the surface.
1.3.3.3 Central Nervous System

With the exception of the elastic tissue that is found in the blood vessels, the only elastic tissue found in the central nervous system lies in a few networks in the dura and leptomeninges.

1.3.3.4 Digestive System

In general the digestive system has considerably less elastic tissue than the respiratory system. The mucous membrane of the mouth contains elastic tissue networks in similar manner though in more abundance than the skin.

There is an unusually abundant supply of elastic tissue in the mucous membrane of the cheek as well as in the derma. Dense networks in the soft palate lie between the lamina propria and the mucous glands of the submucosa and on the nasal side of this area, arrangements of elastic tissue separate the mixed glands of the lamina propria from the muscle. No elastic fibres are found in the peridontal membrane but beneath the epithelium of the tonsils lies a certain amount of elastic tissue, which is continued into the cores of the tonsillar folds.

The pharynx has a thick layer of fibres which lies in a longitudinal direction, and where it merges with the oesophagus the elastic tissue becomes thinner as the networks are succeeded by the muscularis mucosae which retains a few delicate fibres.

In the fornix the layer of elastic tissue blends into the periosteum of the skull, while throughout the lamina propria delicate fibrils are found in both the pharynx and the oesophagus. The oesophagus also has coarse networks which continue throughout the sub-mucosa.
Very little elastic tissue is to be found in the wall of the stomach while the intestine has a few delicate networks which surround the vessels in the lamina propria and accompany the smooth muscle of the muscularis mucosa. Although in the sub-mucosa the networks increase in prominence, the muscular layers contain only a few fibrils.

1.3,3.5 Urogenital System

With the exception of the elastic tissue associated with its vessels, the kidney has no elastic tissue.

The lamina propria of the renal pelvis, ureter and bladder has a few delicate networks which are continuous with the fairly abundant elastic tissue of the tunica muscularis.

The lamina propria of the urethra is rich in elastic networks.

A high proportion of elastic tissue is found in the genital tract of the male, while that of the female is less well supplied. Elastic networks are found in the tunica albuginea, mediastinum and septula of the testis and many very delicate fibrils are present in the singular basement membrane of the seminiferous tubules.

Elastic tissue is also present in the corpora cavernosa, in the tunica albuginea as well as between the cavernous blood channels. There is no elastic tissue in the cortex of the ovary, but there are numerous networks in the medulla and the fallopian tube has a few fibres in the muscularis.

In the uterus there is no elastic tissue in the endometrium or in the subjacent muscle though fibres are present in large numbers in the external portions of the uterine musculature, in the cervix and in the vaginal wall.

Normally there is no elastic tissue in the labule of the breast, but in the lactating gland elastic fibrils may be found, especially around excretory ducts.
1.3.3.6 Ear, Eye, Bone, Biliary and Pancreatic Ducts

The auricle of the ear has an irregular plate of elastic cartilage about which there are dense elastic networks. Delicate elastic fibrils are also present in the tympanum, most prominently in the central zone and the eustachian tube is enclosed partially by elastic cartilage.

The eye has an abundant supply of elastic tissue. The substantia propria of the cornea contains a series of very delicate networks and the rich meshwork of the lamina suprachoroidea and the lamina cribrosa is formed by the joining of the delicate networks of the sclera and the choroid.

Bones have a scant supply of elastic tissue. The periosteum contains a few networks, more prominent in the external layer from where they occasionally may be traced into cortical bone. There are also a few fibres present in the intervertebral discs and in the capsules of joints.

The biliary system has a small amount of elastic tissue around the intrahepatic ducts which increases in approximate proportion to the increase in calibre of the ducts.

The walls of the extrahepatic ducts and the gall bladder exhibit the largest number of fibres, but the pancreas has only a few delicate networks which are located around ducts.
1.4 Elastic Tissue, the Elastic Fibre and Elastin

Fig. 1.1 illustrates the typical architecture of a piece of purified bovine ligamentum nuchae. At the macro (low power light microscope) level of magnification (approx. x 100) the elastic fibres of the unpurified tissue are found embedded within a matrix of collagen fibrils which act to limit the extent to which the elastic fibres can be extended under tension\textsuperscript{1-19}. This is true of virtually all elastic fibres found in any location in the body. Mechanically, although highly elastic, elastic fibres are weak and so an ideal two phase composite is usually formed with the tough but high modulus collagen fibres taking up the bulk of the loading when necessary.

It has also been shown\textsuperscript{1-19} that on an individual basis, i.e.: down to approx. 150 nm, the elastic fibres are also closely associated with collagen; the collagen surrounds each elastic fibre as a knitted sheath.

The removal of collagen as well as other constituents such as mucopolysaccharides and lipids from the elastic tissue at this level of magnification (approx. 150 to 500 nm) leaves 'elastic fibres'. Such fibres prepared for electron microscopy by routine methods have been shown to consist of two morphologically and chemically different components\textsuperscript{1-10}: centrally located seemingly 'amorphous' material surrounded by tubular microfibrils (approx. 10 nm diameter) of different amino acid composition. Both these components have been observed in fibres from foetal and new-born calf ligamentum nuchae\textsuperscript{1-17,1-20,1-21}, developing rat tendon\textsuperscript{1-21} and various other tissues\textsuperscript{1-22,1-23}, and both have different staining properties. Research has led to the conclusion that each structure has a different surface charge and hence the interaction between the two may be partly ionic.
Fig. 1.1. Elastic fibre structure.

Purified ligamentum nuchae.

~2 μm

~1 μm

~10 nm Tubular microfibrils.  
(see ref 1-71)

~150 nm Micro-elastic fibres.  
(see chapter 2)

"Amorphous" Elastin.
Several investigations have shown that the earliest recognisable elastic fibres appear as a homogeneous collection of only one of the components - the microfibrils - arranged in aggregates and usually oriented in a parallel array. The 'amorphous' component increases in amount with increasing foetal age so that by birth the elastic fibre is several microns in diameter and consists largely of this component.

The terms 'elastic tissue', 'the elastic fibre' and 'elastin' appear in the literature to have no strict definition and in general are very loosely used. Although each has its place in the structural hierarchy of the connective tissue many researchers apply the terms quite casually, creating considerable areas of overlap in their meanings. Utilised, therefore, in this work are three definitions to clarify the situation based on current structural connective tissue knowledge.

The term 'elastic tissue' is used to define the bulk tissue within the body as it would be observed 'in vivo'. 'Elastic fibres' are considered as the product after the removal of collagen, mucopolysaccharides and other loosely associated components, and elastin the 'amorphous' component of the elastic fibres as observed by Ross and Bornstein.

1.5 Elastogenesis

It is now generally accepted that elastin is formed from a soluble precursor 'tropoelastin' which is composed mainly of the amino acids valine, alanine, glycine and proline. It is hydrophobic or uncharged, and is synthesized on the ribosomes of the elastin producing cells. Lysine forms a hydrophilic shell around the hydrophobic amino acids, rendering the complex soluble. The tropoelastin is then transported
outside the cell where, in the presence of the copper containing enzyme lysyl oxidase the tropoelastin is converted into elastin by the formation from lysine of the cross-linking amino acids desmosine, isodesmosine and lysinonorleucine. The hydrophilic lysine is thus essentially removed and the resultant elastin is hydrophobic and insoluble.

The role of the microfibrils appears to be that of shaping the elastin into elastic fibre form. They tend to be aligned longitudinally in depressions on the cell surface\textsuperscript{1-71}.

The cells which synthesize the components of the elastic fibre depend on the tissue in which the elastic fibre is found. In ligaments and tendons these cells are fibroblasts whereas in arteries they tend to be smooth muscle cells. This is shown by the fact that labelled proline fed to prepubertal rats is incorporated into smooth muscle cells and subsequently secreted within 4 hours into both collagen and elastic fibres\textsuperscript{1-37}. The $^3\text{H}$ proline is found in both the elastin component and the microfibrils of the elastic fibre.

Tissue culture studies reveal that smooth muscle can synthesize microfibrillar protein and 'amorphous' material identical in appearance to elastin\textsuperscript{1-38}. In culture, these microfibrils appear earlier than the amorphous material, just as with in vivo elastogenesis. It has therefore been suggested that the smooth muscle cell is able to synthesize both tropoelastin and microfibrils. Furthermore, cultures of human fibroblasts have been shown to produce lysyl oxidase\textsuperscript{1-39}.

The theory has been put forward that microfibrils represent a continuum within connective tissue and are the organised morphological precursors of collagen and elastic tissue\textsuperscript{1-23}. Other workers have proposed that the microfibrils are the precursors of collagen and elastin themselves\textsuperscript{1-17,1-33}. 

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1.6 Cross-linking of Elastin

It is only within the last two decades that the structure of the cross-links in elastin has become well understood. Prior to this the polymerization processes of both elastin and collagen were effectively unknown.

The disulphide bond, a known covalent mechanism for uniting polypeptide chains, is supplied by the amino acid, cystine, but this amino acid is virtually absent from both elastin and collagen. Instead, cross-linking predominantly occurs through two previously unrecognized amino acids, desmosine and its isomer isodesmosine, which are synthesized from lysine and incorporated into the elastin polymer. Early work on the subject has been well reviewed and so will only be outlined here.

The synthesis of the two polyfunctional amino acids desmosine and isodesmosine has been demonstrated using radioactive lysine. The radioactivity appeared first in the lysine and then in the desmosines of aortic elastin, in accordance with the expected precursor-product relationship. Later lysine labelled in specific positions was utilized to confirm the incorporation of four lysyl residues into each residue of desmosine and isodesmosine, and it was also demonstrated that the content of the desmosines in elastin from chick aorta increased during maturation, while the lysine content fell proportionately. Mature elastin contains about three residues of the desmosines per 1000 total amino acid residues, and the very low lysine content of mature elastin suggests that nearly every available residue is eventually used to make cross-links.

During the desmosine forming process three of the four lysines incorporated into each desmosine must lose its ε-amino groups and consequently it was suggested that the δ-semialdehyde of α-amino adipic acid
was an intermediate\textsuperscript{1-42}. After formation of this compound in peptide linkage, appropriate condensations, dehydrations and an oxidation could lead to the desmosines. The steps that are probably involved are outlined in Fig. 1.2 and proof that these kinds of reactions do occur has come from several sources. The first was the identification of a new amino acid, lysinonorleucine\textsuperscript{1-48} whose structure suggested that it was derived from two residues of lysine, and could be generated by reduction of a Schiff base, as shown in Fig. 1.2 - reaction (3). Later it was shown\textsuperscript{1-49} that the Schiff base itself was also present in trace amounts in elastin.

The demonstration that enzyme solubilized elastin contained groups which reacted with an aldehyde reagent\textsuperscript{1-50} opened up a second line of investigation and suggested mixed aldehydes of the type expected from reactions (1) and (2) Fig. 1.2. The δ-semialdehyde of α-amino adipic acid, the product of reaction (1) was identified as one of the aldehydes in elastin by oxidation to α-amino adipic acid\textsuperscript{1-51}.

The same aldehyde had been found earlier in collagen and there were strong suggestions that the aldol-condensation product of reaction (2) Fig. 1.2 was the intramolecular cross-link in rat-skin collagen\textsuperscript{1-52}.

Further addition to the evidence was the finding of small amounts of a new amino acid in reduced elastin, merodesmosine\textsuperscript{1-53}, which had a structure suggesting it was derived from three residues of lysine, and therefore was probably the reduced product of an intermediate in desmosine and isodesmosine synthesis.

A possible scheme for the biosynthesis of the unreduced form of merodesmosine is shown in reaction (4) Fig. 1.2, although an alternative mechanism of apparently equal probability would be the condensation of the Schiff base shown in reaction (3) with a residue of the δ-semialdehyde of α-amino adipic acid.
Fig. 12.

Cross-linking Reactions

(1) \( P-(CH_2)_4-NH_2 \rightarrow P-(CH_2)_3-CHO \)

Lysine \( \delta \)-semialdehyde of \( \alpha \)-amino adipic acid

(2) \( P_1-(CH_2)_2-CH_2 + OHC-(CH_2)_3-P_2 \)

\[ \begin{align*}
P_1-(CH_2)_2-C=CH-(CH_2)_3-P_2 & + H_2O \\
\end{align*} \]

(3) \( P_3-(CH_2)_3-CHO + H_2N-(CH_2)_4-P_4 \)

\[ \begin{align*}
P_3-(CH_2)_3-C=NH-(CH_2)_4-P_4 & + H_2O \\
\end{align*} \]

\[ \begin{align*}
2H & \\
\end{align*} \]

\( P_3-(CH_2)_3-CH_2-NH-(CH_2)_4-P_4 \)

Lysinonorleucine

(4)

(5)

Desmosine
Isodesmosine
The final formation of desmosine may occur by the addition of a fourth residue of the aldehyde as shown in reaction (5) Fig. 1.2 and isodesmosine could arise in a similar fashion as shown in reaction (6), although another possibility is the formation of desmosine and isodesmosine by the condensation of the Schiff base produced by reaction (3) with the aldol product produced by reaction (2).

The amounts of the various intermediates in elastin have been estimated for various tissues.

In bovine ligamentum nuchae approximately one residue of lysinonorleucine and a quarter residue of the Schiff base precursor per total 1000 amino acid residues was found\(^1\text{-}\text{49}\), and a total aldehyde content of ten equivalents per 1000 amino acid residues\(^1\text{-}\text{49}\). Chick aorta was found to contain about six equivalents per 1000 amino acid residues\(^1\text{-}\text{50}\) and four equivalents of the δ-semialdehyde of α-amino adipic acid\(^1\text{-}\text{51}\), although there may be less in mature elastin\(^1\text{-}\text{54}\). Since the content of the desmosines is about three residues per 1000 amino acid residues, equivalent to twelve lysine residues, they appear to constitute the major type of cross-link\(^1\text{-}\text{54}\).

1.7 Structural Theories of Elastin

1.7.1 General

The difficulty in establishing the exact ultrastructure of elastin has led to a number of models being put forward.

From the onset it has been recognized that one of the key issues in the modelling of the molecular structure of elastin must be the ability to account for the large elastic extensions that the material is capable of sustaining without significant permanent deformation. A similar
problem was experienced by the materials scientist when faced with the phenomen of rubber elasticity and the eventual solution then led to the revolutionary concepts of the random molecular coil and entropy induced elasticity. These ideas are described in further detail in Chapter 2.

The problem of knowing which ultrastructure to associate with elastin is currently a major area of controversy, and is largely the subject with which this thesis is concerned. The basic features of the major theories are thus introduced in the following sections and covered more fully in later chapters.

1.7.2 Early Theories and 'Classical' Model

From the earliest ultrastructural studies\textsuperscript{1-55,1-56} there has been disagreement concerning the structure of elastin.

As early as the 1930's the mechanical experimental work\textsuperscript{1-55} had led to the conclusion that the material was a classical rubber, while in the same decade an X-ray study labelled it a form of collagen\textsuperscript{1-56}.

Later, Astbury\textsuperscript{1-57}, carried out a considerably more detailed X-ray investigation from which he suggested that elastin was a member of the collagen group whose thermal transformation temperature was below room temperature. This offered a reconciliation between the two schools of thought. The proponents of the classical rubber structure, however, were further supplied with evidence for their cause in 1943 when a second mechanical study\textsuperscript{1-58} drew similar conclusions to that seven years earlier.

In 1955 the apparent transformation of collagen fibres into a structureless, elastin-like material was reported\textsuperscript{1-59} by treatment with various chemical reagents. Prior to this, however, the similarity of shrunk collagen and elastin had already been observed by a finding\textsuperscript{1-50} that collagen was attacked and 'solubilized' by elastase if it had been
shrunk thermally or by treatment with phosphotungstic acid, but remained unattacked in the normal state.

Following these findings two views were put forward:

i) that elastin was formed by the in vivo degradation of collagen\textsuperscript{1−54},

and ii) that elastin was the precursor of collagen\textsuperscript{1−61}.

Although it is now known that the conditions required for shrinkage of collagen fibres apparently could not be achieved in vivo\textsuperscript{1−54}, and that the chemistry of elastin precludes its derivation from collagen\textsuperscript{1−62} (as well as the derivation of collagen from elastin), the models cannot be completely ruled out. Strong similarities between collagen and elastin (such as high glycine, alanine and proline contents, the presence of hydroxyproline and common initial steps leading to cross-links\textsuperscript{1−54}) suggest that collagen may have been the evolutionary precursor to elastin.

It is probable that elastin appeared only relatively recently in the evolutionary tree, as it is apparently confined to vertebrates, whereas collagen is present in the earliest multicellular animals\textsuperscript{1−54}. In this perspective elastin may be viewed as being collagen-like, but as having lost the properties necessary to maintain a stable secondary and tertiary structure, and having gained thereby the properties necessary for elasticity possibly arising from a classical-rubber type structure\textsuperscript{1−54}.

Such a structure consists of a system of randomly oriented molecular chains, lightly cross-linked together at various random points and free to adopt a virtually unlimited number of possible molecular configurations or conformations. By means of a mathematical analysis of such a system it is possible to derive the source of elasticity of the system, being essentially related to its entropy. Such systems are described in more detail in Chapter 2.
It should be stressed, however, that with such systems it is the freedom of the molecular chains to pass from one conformation to another without hindrance which is the source of the elasticity of the material. Any model which postulates the holding of the molecules in fixed positions must also postulate an essentially different source of elasticity to that of the random coil mechanism.

1.7.3 Globular Theory

This model put forward by Partridge\(^1-63\) assumes the structure of elastin to be constituted of an array of molecular bundles, globular in shape, each some 5 nm in diameter, linked together by desmosine cross-links (see Fig. 1.3).

The model was postulated following an attempt to estimate an 'equivalent diameter' for the size of the pores in the water swollen elastin gel. A filtration tube was packed with elastin and used as a gel filtration column for the chromatography of sugars, glycols, and alcohols of differing molecular weights\(^1-63\). The results indicated that water swollen elastin could be regarded as a gel containing pore spaces equivalent to cylinders 3.2 nm diameter; or alternatively, within the accuracy of the measurements, regarded as a structure with about 35% free water and composed of randomly disposed hydrated rods about 1.6 nm diameter. The water between hydrated rods behaved as 'free water' and was available for the solution of solutes up to a molecular weight of about 1000\(^1-64\).

Electron microscopy, carried out in conjunction with Fitton-Jackson\(^1-65\) in which the collapse of the pore structure was prevented using fillers of a polyglycol compound revealed a structure of spheriodal particles of about 4 - 5 nm diameter.

This data, plus considerations deriving from the biogenesis of elastin, led to the suggestion of the globular or corpuscular model.
Fig. 1.3 shows the model viewed in two dimensions. It was assumed that when elastin is swollen in water, the centre part of the spheres is largely hydrophobic with a hydrated interface between the outer globular particles and the water spaces surrounding them.

The concept is similar in principal to that of Richards and Knowles\textsuperscript{1-66} who studied the reactions of gluteraldehyde with protein crystals. It was observed that gluteraldehyde cross-links occurred at the interface of the globular protein molecules in such a way as to render the whole material of the crystal insoluble. For several proteins the reaction did not disorder the crystal structure, and very similar single crystal X-ray patterns resulted from both the treated and untreated protein. In other cases some disordering did occur because the cross-links themselves resulted in the enforcement of new conformations.

If such a cross-linked structure is dried out, the water interface disappears and the globular shape of the particle is lost, although it may be recovered again on rehydration.

1.7.4 Oiled Coil Model

This model was put forward in 1973 by Grey, Sandberg and Foster\textsuperscript{1-67} and is outlined in Fig. 1.4.

Each monomer is fibrillar and is made up of alternating sections of cross-linked regions and 'oiled coils'. The mechanical properties of these are quite distinct, being rigid and flexible respectively.

Each monomer is linked to many others, forming a network resembling a three-dimensional mattress spring.

Chains may be cross-linked at various angles or bent so that the network can be isotropic despite the fibrillar nature of the monomer.

The diameter of the oiled coils is about 1.2 - 1.5 nm and the hydrophobic core can accommodate non-polar molecules, especially straight-chain aliphatic compounds.
Fig. 1.3. **Globular model.**

![Globular model diagram](image1)

Fig. 1.4. **Oiled coil model.**

![Oiled coil model diagram](image2)
The gel-filtration data of Partridge\textsuperscript{1-63} already mentioned showed the preferential partitioning of aliphatic alcohols within the gel particles, the effect being greater for n-alcohols than for branched. Grey et al suggested that such alcohols might be fitting within the core of the coils rather than absorbing on to the surfaces. From the composition they estimate that 20 - 25\% of the molecule should be in cross-linked regions with the rest as coil; the two forms then contribute equally to the length of the relaxed monomer. Extension of the coil proceeds smoothly until the whole molecule is 2 to 2.5 times the original length. Stretching increasingly exposes the hydrocarbon core to water.

1.8 Summary

The broad spectrum of information given in this chapter is designed to supply the foundation on which the more specific aspects of this thesis are built. The first section (1.1) shows that man's awareness of tissue elasticity in the body is by no means a new aspect of his scientific thinking and can be dated back over 200 years. As with the bulk of science, however, real progress in the field of elastin studies has awaited the development of suitable techniques and even then progress has not been as fast as with than on other connective tissues.

The reason for this is probably that elastin has few of the easily identifiable characteristics which label the more specialized tissues, such as the periodicity of collagen and muscle.

Another feature is that elastin is nowhere found in vivo in its ready purified state. It is always associated with collagen and various other intercellular substances. Hence direct studies on pure, freshly dissected elastin specimens are impossible and some form of extraction process is necessary prior to experimental work (chapter 3).
Section 1.2 provides a detailed summary of the many locations of elastic tissue in the human body. Special mention is given to the blood vessels, the area where contribution of elastin studies to the field of medical science promises to be the greatest, owing to the widespread occurrence of arterial disease. Indeed, it is just because of this medical interest that much research has been carried out on elastin which would otherwise not have been done. For example, an equivalent rubbery protein, resalin, found only in insects, has attracted considerably less interest and has been the subject of only limited research over the last two decades \(^{1-69}\).

The composition of elastin is essentially independent of animal source or location in the body \(^{1-63}\), a fact emphasized by the marked similarity of amino acid analyses from a variety of animal sources and body locations \(^{1-63,1-68,1-18}\). It is thus possible to accept elastin as being a unique material and not simply the general name for a class of materials all of slightly differing chemical composition. Extrapolation of results obtained from elastin from animal sources to the human being is consequently valid.

Elastogenesis and cross-linking have both been the subjects of a vast amount of research over the last two decades. The outlines of their progress in Sections 1.5 and 1.6 are, of necessity, only a very brief sketch of the enormous quantities of information that have now been amassed during their evolution. For more detailed studies reference should be made to the excellent reviews by Piez \(^{1-54}\), or Franzblau \(^{1-68}\) or to the various sections of Chapter 4 of ref. \(^{1-70}\).

The structural theories of elastin introduced in Section 1.7 provide only a very brief outline of their essential features. This is because they are covered in far greater detail in further sections of the work. For example, the mechanisms by which elasticity is generated according to
each of the theories is discussed in Chapter 2, while further considerations of their molecular biology are discussed in Chapter 8. It is these models which are the centre of argument currently raging in the scientific literature of elastin and are consequently central to the theme of the thesis.
Chapter 2

PREVIOUS MECHANICAL STUDIES OF ELASTIN AND ELASTIC TISSUE

2.1. Introduction

Before outlining and discussing any mechanical studies on elastin and elastic tissue, one must ask the question 'What is the point of such studies?'.

Basically, the objectives can be split into three groups:

(a) To obtain mechanical data which can be used to predict the performance of the material in vivo conditions;

(b) To examine the mechanical deterioration experienced by the tissue due to disease or physical damage,

(c) To provide data for the postulation of a structural model for the material.

It is with (c) that this thesis is mainly concerned, and as a consequence the review of previous work set out in this chapter will have its emphasis predominantly on this aspect.

The in vivo mechanical functions of elastic networks are five-fold\(^2\). By their elastic compliance they

(i) co-ordinate the rhythmic and arhythmic motions of the body parts, and

(ii) help to conserve energy by the maintenance of tension during the relaxation of muscle elements;

(iii) disseminate stresses originating at isolated points, and

(iv) provide a defence against excessive forces;

(v) assist organs in returning to their undeformed configuration once all forces have been removed.
The mechanical functions of elastin have been generally accepted as being its most important in vivo function, (though its chemical role in the development of atherosclerotic plaques in the aorta perhaps vies for this position). Due to this, mechanical studies on the tissue must be considered of primary importance in the general field of elastin research.

2.2 Early Work on Elastic Tissue

The earliest mechanical studies\(^2\) of elastic tissue were invariably made on the tissue in its unpurified form and generally on the aorta where the diastolic blood pressure is maintained chiefly by the elastic recoil of the wall\(^2\). In the main these studies were aimed at obtaining force-extension curves from the tissue. For a comprehensive review of this work, reference should be made to the paper by Burton\(^2\) (1954). Generally an ultimate tensile strength of approximately 1 MN/m\(^2\) was reported for human aortic tissue and a tensile modulus of 300 kN/m\(^2\). The curves appeared of similar though not identical form to those of natural rubber\(^2\) and showed two regions. The first was an early reversible straight line phase, obeying Hooke's Law, while the second was a curve convex to the stress axis attributed to the presence of collagen\(^2\). Also noted\(^2\) was the fact that in humans the ultimate tensile strength of aortic tissue decreased after the age of 25, whereas in rats\(^2\) tensile strength increased throughout life. Work on ligamentum nuchae, the richest source of elastin in any animal tissue (78-83% dry wt), produced similar force-extension curves to aortic tissue\(^2\),\(^10\),\(^11\),\(^12\). Force-extension curves were also obtained from fibres dissected from whole ligamentum nuchae\(^2\). With these the shape of the curves appeared to be of elastomeric type,
although their slopes indicated less long range elasticity than with both whole and purified ligament. It was suggested from these observations that slack in the interconnecting fibres of the ligament was reflected in certain regions of the stress-strain curve. Early work on elastic tissues from other areas of the body included a review on the lung and on the skin where it was shown that there is less elasticity in the male and in old age.

Since these early mechanical studies, emphasis appears to have shifted from simple tests on isolated and dissected pieces of tissue, to tests aimed more directly at establishing the properties of a specific area of tissue under normal or pathological conditions. Examples of this are recent studies on rabbit aortas under normal and hypertensive conditions as well as studies on the dynamic elastic properties of the canine left circumflex coronary artery. Such studies, however, are beyond the scope of the thesis, the philosophy of which is aimed more in another direction - namely that of using mechanical and physical tests to help establish the structure of the elastic component of elastic tissue, (ie: elastin).

2.3 Development of the 'Classical Theory' of Rubber and its Application to Elastin

2.3.1 General

Ever since its origin in the 1930's the development of the so-called 'Classical theory' of rubber elasticity has proceeded hand in hand with its application to elastin and other connective tissues. It was these studies which also pioneered the concept of a melting process occurring during thermoelastic shrinkage and crystallization occurring
during stretching, both concepts being of great importance in the theory today. Mathematical details of the theory are supplied in Appendix 1 of the thesis and a brief descriptive account of its fairly complex development given in the section below. For a fuller account of the theory the book of L.R.G. Treloar is to be recommended·2-26.

2.3.2 The Theory of Rubber Elasticity

Rubbers are unique because of their ability to recover from high extensions almost completely without permanent deformation. This 'long range elasticity' is related predominantly to entropy2-19 as opposed to energy contribution to the retractive force. The definition of an 'ideal rubber' can be made as one whose elastic free energy is totally devoid of energy contributions, being entirely dependent on those of entropy2-20. An analogy can be made with an ideal gas where energy is independent of volume. However, in the same way that most real gases deviate from ideal behaviour, so most elastomers experience energy changes on deformation2-21,2-22, but these usually represent only minor factors in the stretching process2-23,2-24.

Entropy as a mechanism for rubber elasticity can be explained in a qualitative fashion without undue difficulty2-19,2-25,2-26. Long polymer chains, containing covalent bonds along their backbones (predominantly single bonds) can assume numerous geometrical shapes because of internal rotations (fig. 2.1). As each backbone bond rotates, so the chain continuously alters its path through space. Each separate distinct path is referred to as a conformation. The number of conformations increases as the number of rotatable bonds increases, that is as the molecular weight of the chain increases. At any instant, in the process of spanning a specified end-to-end distance, a chain may adopt any of a considerable number of conformations. The number of these available conformations
increases as the end-to-end distance is decreased and decreases as the end-to-end distance is increased\(^2-^20\). Ultimately, when the end-to-end distance is at its greatest, there is only one conformation available to the chain. For a chain having its ends free, the probability of a small end-to-end distance is considerably greater than that of a large distance, and should the contour length of the chain be large, the probability of the former can be mathematically shown\(^2-^19,^2-^25\) to be overwhelming.

It is the ready availability of conformations which gives rise to the retractive force, not simply the fact that they exist. A polymeric glass can possess the same number of conformations as a strip of rubber but the glass is incapable of long range elasticity with spontaneous recoil because its molecular chains lack sufficient kinetic energy to utilize all the available conformations. In a rubber, the chains have sufficient kinetic energy to continually pass from one conformation to another. 'If, however, the rubber is cooled, forces are generated prohibiting vibrational chain movement and the rubber will become glassy.

Using the well-known Bolzman relationship which connects entropy and probability, it can be shown that highly coiled chains possess high entropy, while extended chains, low entropy\(^2-^19,^2-^25\). It can therefore be stated that uncoiling a chain decreases its entropy. Because it is a fundamental law of nature (2nd Law of Thermodynamics) that entropy 'strives' for a maximum, a stretched chain exerts a force in opposition to that deforming it, and the sudden removal of this force results in an immediate recoil.

An energetic contribution to the retractive force, however, will arise if internal bond rotations are restricted by hindering potentials\(^2-^27\). Such barriers to rotation result from interactions among substituent atoms or groups of atoms attached to adjacent and other backbone units in exactly
the same fashion that hydrogen atoms influence rotation about the carbon-carbon bond in ethane. Because such interactions depend upon the distances separating the atoms in question, restricting potentials are periodic functions of bond rotational angles (fig. 2.3). For ethane, the periodicity is every $120^\circ$, but polymer rotations are somewhat more complex\textsuperscript{2-28}. Factors other than attractive-repulsive interactions between non-bonded atoms also appear to play a role, but the quantum mechanical origin of such intrinsic factors is not yet understood. The rotational potentials as functions of the rotational angles consist of a series of maxima and minima, depending on the size and nature of the constituent atoms. The majority of bonds - with the exception of those few in transition from one minimum to another, take up residence in the various minima, their relative population being governed by the respective energy levels. This distribution is often referred to as 'the principle of rotational isomerism'\textsuperscript{12-29}: each isomeric form represents a conformation. The rate of transition from one conformation to another depends upon the height of the maximum between adjacent minima. A bond must possess at least this amount of energy to surmount the barrier. Normally these peaks are only a few kilocalories per mole, but special requirements can sometimes prohibit certain transitions altogether. Whether the chain is relatively extended or coiled depends on the positioning of the minima with least potential. This is because not all minima are of the same energy content and an energy contribution to the chain end-to-end displacement force can arise. Part of the displacement work alters the population distribution in the various minima, resulting in an absorption or emission of energy depending on which way the change occurs. This type of energetic contribution, however, will move towards zero as the temperature of the system increases. Another possible type of energy contribution arises from orientational dependent intermolecular forces and these are generally independent of temperature.
Fig. 2.1. Internal rotation in a polymer. Bonds along the polymer chain backbone can rotate as shown by the rotational angle $\Theta$. As rotation proceeds, the direction of the chain is altered. The angle $\Theta$, represents the fixed bond angle between covalently bonded atoms. Subsequent groups attached to the backbone are not shown.

Fig. 2.2. Chain conformations: Two paths a polymer chain might trace out in spanning the end-to-end distance $r$. Each path is a chain conformation. The total number of possible paths, or conformations available to the chain depends on the value of $r$ and the number of backbone atoms or links in the chain.

Fig. 2.3. Rotational potential for ethane. The potential $V(\theta)$ restricting the internal rotation of one methyl group relative to the other is shown as a function of the rotational angle $\Theta$. The maxima represent the eclipsed conformations – points of closest approach of the hydrogens of one carbon atom to those of the other carbon. The minima correspond to the staggered conformation when the hydrogens are separated by their greatest distance. The height of the maxima relative to the minima is about $1.26\text{kJ/mole}$.
Calculation of the probability that a chain of \( N \) backbone links (see fig. 2.2), each of length \( b \), has a given displacement vector \( r_e \) is a simple problem in principle, provided the orientation of each link is independent of all others. However, in practice difficulties arise and the probability cannot be written in a single closed form equation. Instead, two expressions are required, the simultaneous solution of which necessitates a power series expansion in terms of the argument \( r_e/Nb \).

The first term in the series - a valid solution when \( r_e \) is considerably less than \( Nb \) - is of Gaussian form, and as a result, the terms 'Gaussian chain' and 'Gaussian network' have come into being. Gaussian chains are suitable models for actual chains only when the deformations are small as they have the unrealistic capacity for infinite elongation. It is the higher order terms, neglected in the Gaussian approximation, which endow the chain with finite extensibility. Retention of one or more such terms results in a 'non-Gaussian distribution'.

The orientation of each chain link to its adjacent link, however, in real chains is not independent. Link orientations are limited by fixed valence angles and rotational barriers, and to circumvent this objection the argument is used that any real chain which is sufficiently long and flexible can be theoretically replaced by an equivalent, flexibly jointed chain having the same mean-square end-to-end distance and fully extended length. The reasoning is valid, but only for Gaussian chains. Failure in the non-Gaussian cases results from the fact that the quantities \( N \) and \( b \) do not appear in the same ratio from term to term. The problem then becomes one of incorporating fixed bond angles and restricted internal rotations into the model. A solution to this was found by the formulation of stochastic equations in such a fashion that the effects could be readily incorporated. This method was used to include fixed valence angles and finally to provide the complete solution for any real chain.
A useful result, however, still requires a series solution, the first term of which, again, is of Gaussian form.

Another way to approach problems in chain statistics involves the use of chain partition functions or conformational integrals\(^2\text{-}\text{35}\). There are two such functions: a partition function for a chain held at constant length, and a partition function for a chain held under constant force. The first is proportional to the Gaussian distribution function\(^2\text{-}\text{33}\) and leads to the concept of a fluctuating retractive force at constant displacement while the second is applicable to a situation in which the chain is under the influence of a constant force which biases certain conformations at the expense of others. In this case, because the chain ends are free, a fluctuating displacement is generated. Consequently, the important features of the theory are either an average force at constant displacement or an average displacement at constant force. The two results are not identical but tend to converge towards one another as the chain contour length increases.

Both approaches to the solution utilize the chain moment terms \(<r^x>_0\). (x = 2, 4, 6, 8 . . .) where the symbol \(< >_0\) represents the average length vector of the unperturbed chain. Each of these moments must be evaluated separately. The second moment \(<r^2>_0\), the mean square end-to-end distance, has been extensively investigated for a number of polymers\(^2\text{-}\text{37}\) but higher moments are often lacking. Fortunately only the second moment is necessary for the Gaussian approximation, although non-Gaussian solutions require one or more of the higher moments.

Knowledge of the chain probability function is equivalent to knowledge of the elastic free energy of the chain. However, the application of this knowledge to predict the characteristics of a network of chains is a far more difficult problem and remains to this day a centre of controversy.
Two approaches have basically been proposed; one by James and Guth\(^2\)\(^{-36}\)(Theory I), \(^2\)\(^{-38}, \(^2\)\(^{-39}\) and the other by Wall\(^2\)\(^{-40}, \(^2\)\(^{-41}, \(^2\)\(^{-42}\), Kuhn\(^2\)\(^{-31}, \(^2\)\(^{-32}, \(^2\)\(^{-43}\) and Hermans\(^2\)\(^{-44}\)(Theory II). All these authors viewed the network in a similar fashion; polymer molecules are cross-linked to one another at various points by covalent bonds; cross-links transmit forces and prevent flow; only those portions of the primary molecules that are attached at both ends to a cross-link are elastically effective; dangling ends (attached to but a single cross-link) are unable to transmit forces; chains are volumeless and freely pass through one another, i.e: entanglements are not taken into account; spacial packing requirements are not considered; the system is completely amorphous and homogeneous; and intermolecular interactions are ignored. Beyond these basic considerations, the two approaches diverge.

The James and Guth (Theory I) method is to compute the number of network conformations available by first establishing the number of available conformations for a system of chains with fixed and specified end-to-end distances. This is simply the product of the conformations of all chains. Since cross-links are capable of Brownian motion in the same fashion as chain-links, the chains joined to give a cross-link must move in conjunction with one another, a necessity that reduces the motion of a network to a level below that for a system of independent (uncross-linked) chains. To account for this, the chain conformations are integrated over all possible cross-link positions, maintaining chain independence. To do this a necessary assumption is that those cross-links residing on the sample surface are fixed in position while those lying in the interior are mobile. If this assumption is not made and the integration carried out for all cross-links, the result leads to naught.

The approach of Wall, Flory, Kuhn and Hermans (Theory II) follows a different reasoning, although they begin as Theory I by writing the
conformations of a specified (and fixed) set of chains as the product of the conformations of one chain, and the number of chains. Rather than integrate this, they multiply it by the total number of networks possible, arguing that since the actual network is not known and is only one of many possibilities, it is necessary to calculate the conformations for all possible networks. The multiplication factor is determined by counting the number of permutations among vectors of unequal length. This counts all possible connectivity patterns as well as all permutations within each single structure, provided the distribution of vectors is constant. Theory I maintains that this procedure overcounts because only one connectivity pattern need be considered. But the objection is parried by Flory by applying the preceding calculation not to a cross-linked network but to a system of restrained, independent un-cross-linked chains, and subsequently accounting for the cross-linking process in a separate step. Dispute, however, has broken out between Flory and Hermans on the cross-linking procedure with the result that three theories of network elasticity currently exist.

Provided the deformation occurs at constant volume, as it usually does, the deformational free energy calculated by Theory I is one half that given by Theory II, and this means that the elastic modulus will differ by the same amount. At first sight, this difference would appear easily to be measurable. The modulus, however, is also proportional to the number of elastically effective chains, some of which may result from the unknown factor of chain entanglements.

So far, the discussion has been restricted to Gaussian approximation. These principles have also been extended to non-Gaussian chains with varying degrees of success in both Theory I and Theory II. One assumption used regularly in developing the various theories is that of affinity. This is based on the mathematical assumption that the
interior of the material deforms in a proportional manner to the exterior when strain is applied. In actual fact, an affine deformation cannot occur\textsuperscript{2-50} as chains of low molecular weight (small contour length) cannot be deformed to the same extent as those of large contour lengths. Some internal re-adjustment of cross-linking positions is necessary. In the Gaussian approximation this issue is of no importance except in a few special cases such as, for example, semi-crystalline networks. In the non-Gaussian case, however, the affine-deformation assumption is critical only in Theory II approach. Theory I essentially eliminates it as an important factor by integrating the internal cross-link positions. The two cases can be compared\textsuperscript{2-48}:

(i) an affine deformation for chains having a most probable contour length distribution, and

(ii) an affine deformation for chains having a monodisperse contour length distribution.

The former yields a considerably higher stress for a given strain than the latter, and it is considered to be incorrect because short chains connected to long chains should distort the cross-link positions from an affine transformation. Application of the latter result to actual networks then rests upon the supposition that such distortions cause the network to behave (on average) more in accordance with a monodisperse system. Both assumptions have been applied to composite networks, i.e., cross-linked networks stretched and cross-linked again in the deformed state\textsuperscript{2-54}. The monodisperse system yielded an isotropic network, whereas the most probable distribution gave anisotropy. Rubber composites are in fact anisotropic\textsuperscript{2-55} but the anisotropy is the opposite to that predicted. This finding indicates serious shortcomings in Theory II as it is now presented.
Experimental studies on the mechanical behaviour of elastomeric networks on the whole reveal that the molecular theory is semi-quantitatively correct with a number of notable deficiencies\(^2-26\). One of these involves uncertainties in the region of high deformation where non-Gaussian behaviour becomes important and another, deficiencies in the moderate or low deformation zone where Gaussian theory ought to be applicable.

Rather than follow the simple Gaussian theory, most elastomers exhibit deviations that are more readily described by the Mooney-Rivlin phenomenological equation\(^2-56,2-57\) (see Appendix I). This equation contains a strain dependent term in addition to one of the same form as that given by the Gaussian theory. It is generally assumed, though not yet proven, that this extra term represents an addition to the Gaussian theory due to some of the factors omitted in the simple molecular model. Curiously, this term disappears if the network is swollen with increasing amounts of diluent\(^2-58,2-59\), leaving what appears to be true Gaussian behaviour at very high dilution. The origin of the term is unknown, but a number of proposals have been put forward; for example, the presence of heterogeneous regions\(^2-60\), non-equilibrium factors\(^2-61\), orientational entropy of the cross-links\(^2-62\), or intermolecular obstructions\(^2-63\) (the chains not in fact being volumeless). Probably the answer lies in a combination of several factors.

2.3.3 Application of 'Classical Theory to Elastin'

With the exception of two early studies\(^2-17,2-18\), the main progress in the application of classical rubber elasticity theory to elastin has awaited the 1950's, when renewed interest was applied to both elastin and other biological materials.
Generally the approach used has been that of studying the thermoelastic behaviour of the material by plotting force-temperature curves to establish the ratio of the energy component of the retractive force to the total force \( f_e/f \). The method relies on the thermodynamic equation of state derived by Wiegand and Snyder\(^2\)\(^{-7}\):

\[
f = \left(\frac{\partial H}{\partial l}\right)_{T,P} + T\left(\frac{\partial S}{\partial T}\right)_{P,1}
\]

as well as the expression\(^2\)\(^{-20}\):

\[
\left(\frac{\partial f}{\partial T}\right)_{P,1} = -\left(\frac{\partial S}{\partial l}\right)_{T,P}
\]

\( f \) = Retractive force  \( P \) = Pressure  
\( T \) = Temperature  \( l \) = Specimen length  
\( H \) = Enthalpy  \( S \) = Entropy  
\( \approx \) Internal energy

A plot of force vs temperature at constant length and pressure will thus yield a curve, the slope of which at any temperature \( T^1 \) will yield the quantity \( -\left(\frac{\partial S}{\partial l}\right)_{T,P} \). A positive slope will thus immediately indicate a decrease in entropy with length at constant temperature and pressure. Furthermore, if the tangent to the curve at point \( T^1 \) is extrapolated to the force axis, the intercept will give a measure of the increase in internal energy with length at constant temperature and pressure, i.e.: \( \left(\frac{\partial H}{\partial l}\right)_{T,P} \). Energy (\( f_e \)) and entropy (\( f_s \)) contributions to the retractive force (\( f \)) can thus be obtained. However, because the energy component \( f_e \) is defined under conditions of constant volume and length, experimental data obtained at constant (atmospheric) pressure must be converted to constant volume if \( f_e/f \) is to be obtained. Such a conversion requires an elastomeric equation of state which is commonly taken from the Gaussian theory of elasticity\(^2\)\(^{-21}\),\(^2\)\(^{-22}\). The results of these thermoelastic studies are only as good as the equation of state employed, and
because the molecular theory does not adequately describe elastomeric behaviour, even in the Gaussian region, variations in $f_e/f$ from its theoretical predictions can be expected. The fact that $f_e/f$ appears to deviate strongly at low deformations has been attributed\textsuperscript{2-64} to experimental error and at high deformations to the results of stress-induced crystallization\textsuperscript{2-20}. Elastin has the added complication that it must be tested in a saturated condition, since this is the only condition in which it exhibits rubber-like properties.

Early investigators\textsuperscript{2-17,2-18} working on the elastin-water system noted the occurrence of a large negative energy component of the stress ($f_e$) measured at constant length, pressure and equilibrium swelling and interpreted this effect in terms of stress-induced crystallization. This result was not questioned until comparatively recently when it was recognized\textsuperscript{2-65,2-66} that the conclusion was unwarranted without taking proper account of the large decrease in swelling with increasing temperature which occurs for elastin in water. As a consequence of this, a method was attempted\textsuperscript{2-65} whereby the swelling of the specimen was kept constant with temperature by employing a 30:70 v/v ethanediol-water mixture instead of water alone. It was claimed that in this mixture the retractive force was directly proportional to the absolute temperature, making the internal energy of the elastin chain independent of its conformation. This method was, however, later criticized\textsuperscript{2-67} on account of its neglect of the possible effects of temperature dependent water-ethanediol disproportionation during the measurements. As a result of this criticism the thermoelastic properties of elastin were re-examined\textsuperscript{2-68} using water alone as a single component diluent. It was shown that between the temperatures of 50°C and 70°C the volume-temperature (swelling) co-efficient was zero. Below 50°C it dropped sharply and for this case it proved necessary to apply a mathematical correction term to the data.
Experiments\textsuperscript{2-69} were also performed on specimens saturated in the swelling agent, dimethyl-sulphoxide, and in ethanediol. Results indicated low average \( f_e/f \) values of 0.15 in water, -0.02 in ethanediol and -0.04 in DMSO, results consistent with a predominantly entropic classical rubber model.

2.4 Elastin as a 'Liquid Drop' Elastomer

A new elastomeric structure named the 'liquid drop' elastomer was postulated following a series of microcalorimetric experiments by Weis-Fogh and Andersen\textsuperscript{2-70} in which strips of purified ligamentum nuchae were extended and relaxed in a microcalorimeter\textsuperscript{2-71} and the heat changes measured. The results showed that the amount of heat produced during stretching was always approximately equal to the heat absorbed on subsequent relaxation. Since the stress-strain curves were also found to be reversible, it was concluded that the sample plus the surrounding solvent in the calorimeter could be considered a reversible and closed thermodynamic system at constant temperature and pressure. Volume change was assumed to be negligible. It then followed that the reversible heat exchange \( Q(\text{Rev}) = TAS \) where \( Q \) was the heat absorbed by the system and \( \Delta S \) was the change of entropy. Also \( W(\text{Rev}) = -\Delta F \) where \( W(\text{Rev}) \) was the reversible work done by the system on the surroundings and \( \Delta F \) was the change in free energy. Since the fundamental thermodynamic equation for a closed system at constant temperature is:

\[
\Delta F = \Delta U - TAS
\]

the change in internal energy could be estimated by inserting the two measured quantities as:

\[
\Delta U = Q(\text{Rev}) - W(\text{Rev})
\]
Weis-Fogh and Andersen found that the amount of heat given off a water-saturated elastin specimen during approximately 35% extension over the undeformed length was approximately 5 times larger than the change in free energy (i.e., than the work put in by stretching). It was consequently concluded that elastin differs from 'ordinary' rubbers in that its deformation in water entails a reversible chemical change which is several times larger in terms of energy than that corresponding to the mechanical change.

Similar experiments using other solvents gave heat releases of various magnitudes as did ethanol-water solutions of varying concentrations.

Weis-Fogh and Andersen explained these phenomena in terms of chemical changes claiming (a) that when elastin is deformed under physiological conditions (in water) the strain introduces a reversible chemical change which involves changes in internal energy $\Delta U$ far greater than those corresponding to the change in Helmholtz free energy $\Delta F$; and (b) that these changes could contribute significantly to the increase in isometric force with temperature and that a thermodynamic analysis of elastin would have to take contributions from chemical reactions and interfacial forces into consideration.

For the application of these theories to elastin, Weis-Fogh and Andersen made use of the globular structural model proposed by Partridge (see Chapter 1) in which the material consists of a lattice of molecular globules or corpuscles bonded together by desmosine cross-links. The centres of the corpuscles are relatively hydrophobic and the inter-corporuscular spaces filled with water. It was considered that a structure of this type would result if the essential configuration of the soluble globular protein precursor of elastin could survive the process of cross-link formation via the lysine derived aldehyde residues. This process
has already been demonstrated to be possible in the cross-linking of protein crystals by double-ended aldehydes such as gluteraldehyde: some crystalline enzymes can be cross-linked into a three-dimensional network without disordering the crystal structure\textsuperscript{2-73}. The globules would be held together by hydrophobic secondary bonding. Tests for such interactions have been made by a variety of methods with positive results\textsuperscript{2-74,2-75}. Partridge argued that the centres of hydrophobic activity under physiological (i.e. aqueous) conditions would approach a compact globular shape to reduce the interfacial area to a minimum and ensure that the (Gibbs) free energy of the system as a whole is minimal.

From such a structure, Weis-Fogh and Andersen formed the general concept of the 'liquid drop' elastomer, stating\textsuperscript{2-79}:

"Consider long-chain hydrophobic molecules suspended in a hydrophilic liquid or hydrophilic molecules in a hydrophobic liquid. This is, of course, a dispersed two-phase system. Assume moreover that a chain is kinetically free when the links are surrounded by its own kind. Such a suspended molecule will tend to behave as a spherical droplet of liquid because the interfacial tension will tend to keep the interfacial area ('surface') at a minimum. Since the 'droplet' is in fact a thermally agitated chain, it is possible by means of suitable groups and reactions to cross-link neighbouring molecules at a few points and thereby to form an isotropic material consisting of spatially fixed spherical molecules surrounded by solvent, apart from where the covalent cross-links bridge the distance between the spheres. Such an assembly of interconnected spheres will behave as an elastomer, and we believe that this has not been recognized so far. On uni-directional deformation the spheres will tend to be drawn out into prolate spheriods and the interfacial areas will increase causing both a decrease in configurational
entropy of the chains (as in ordinary rubber but with steric restrictions) and an increase in free energy due to the work done against the interfacial forces. If it were possible to interconnect liquid droplets suspended in another liquid phase in which they were not soluble, such a system would behave as an ideal 'liquid drop' elastomer in which the changes in configurational entropy would be minimal and the elastic force would be caused mainly by the energy term, in this case due to work done against interfacial forces. Both the ideal (classical) random network and the liquid drop assembly could form stable elastic structures with no flow or faults, but, in other respects they would be very different and endowed with different potentialities with respect to modification and mode of formation."

Considering the concept more specifically in terms of elastin, Weis-Fogh and Andersen calculated a diameter for the globule of 5.4 nm and an area of about 90 nm² and concluded that as only 5% of the amino acids side chains in elastin carry polar side groups, these polar groups could not in any way cover the surface of the globules, and this meant that an increase of the surface area as a consequence of stretching would be highly likely to bring nonpolar hydrophobic groups to the surface where they would react with water. Furthermore, calculation of the theoretically expected heat output values using data derived for the transfer of alanine side chains from water to a nonpolar environment²⁻⁷⁶ yielded a result of the same order of magnitude and sign as their experimental microcalorimetric results.

All this data, coupled with that of Partridge et al (see Chapter 1) added up to powerful evidence in favour of a liquid drop-globular structure existing in elastin. In addition it provided the basic stimulus for the more recent 'oiled Coil' model (see Chapter 1) where a
'liquid drop' type mechanism was coupled with a helical structure of both α and β turns. The large β helices provide the same function as the 'globules' in the globular theory exposing on stretching hydrophobic groups hidden within the coils to the water interface. The swollen α helices provide the connection of the β turns to the desmosine cross-links.

2.5 Summary

As mentioned in Section 2.1, the general aim of this thesis is the study of the structure of pure protein elastin by mechanical and other means and to attempt some interpretation of the results in the light of current knowledge of polymer and general materials science. As a consequence, the most relevant background information is the type which pursues a similar general philosophy, for example the works of Meyer et al2-17, Wüllish et al2-18, Hoeve and Flory2-65,2-66, Mistrali et al2-68, and Weis-Fogh and Andersen2-70. Those might be considered as key papers on the background to the mechanical studies of this work.

The progress of research into elastin structure has been linked closely with that of the field of general polymer science, and more specifically with elastomers. It is only comparatively recently, with the development of the Globular Theory, that researchers have started turning away from 'classical' random coil principles to attempt to explain the long-range elasticity exhibited by the material. This has resulted in the consequential generation of much controversy. It is particularly towards the solution of this controversy that the present thesis is aimed.

Detailed discussion of the arguments for and against the classical and globular/oiled coil models are confined to subsequent sections, so will not be presented here. At the time of presenting this thesis, however, the argument remains one of great activity.
Chapter 3
SPECIMENS, PURIFICATION AND PURIFICATION STUDIES

3.1 Introduction

The worker wishing to make physical measurements on specimens of elastin is immediately faced with two problems:

(i) Where and from what creature to obtain specimens, and
(ii) How to treat the specimens to remove all contaminating materials except the elastin itself.

The problem must be considered carefully. Elastin is rarely found in mammals exceeding quantities of more than 40% dry weight of a particular tissue and usually in significantly smaller proportion. The exception to this rule is the nucheal ligament of herbivorous animals containing approximately 80% dry weight of elastin. Although this at first appearance would make it seem the obvious elastin source for the researcher, it has the disadvantage of considerable bulk anisotropy due to highly fibrous structure. On the other hand, for chemical and biochemical experiments and experiments whose results are not influenced by material anisotropy and bulk material properties, ligamentum nuchae may be ideal and have the added advantage of being available in large specimens, easily obtainable from the local abattoir. Another elastin source is from mammalian elastic arteries. These have the advantage of being more isotopic than ligamentum nuchae, with smaller fibres somewhat more randomly distributed (see Chapter 7). Elastin from the aorta is from a region of great interest to medical science, in particular to research into the causes and effects of atherosclerosis. The geometry of aortic elastin specimens presents also a greater range of mechanical testing possibilities without problems of specimen clamping - always a difficulty
with slippery biological tissues. For example, one testing method carried out in certain sections of this thesis has been to cut hoops of elastin from the vessel and loop these, in lieu of clamps, over two miniature rollers attached to the beams of an Instron tensile testing machine (see Chapter 6). Aortic elastin has the disadvantage, however, that in the adult human vessel the walls invariably contain the presence of small atheromatous plaques consisting of elastin of slightly changed composition, often partially or fully calcified. The field of atherosclerosis research is a vast one, beyond the scope of this thesis, and consequently the mechanism of formation of these plaques and their compositional details will not be enlarged upon here. For accurate mechanical tests to be carried out on human aortic elastin, however, these plaques must be removed, and although this is possible using such reagents as ethylene-diamine-tetra-acetate (E.D.T.A.), the vigorous chemical procedures required cause risk of degradation of the elastin itself. For a more detailed account of the mechanisms of atherosclerosis and plaque formation, consultation of reference 2 of this Chapter is recommended. Other sources of elastin have been mentioned in Chapter 1 and in general have been rarely used for specific elastin studies. Tissues such as lung, heart valve and skin, however, have been studied as composite structures in their own right, for example.

The problem of purification further presents difficulties to the elastin researcher. A method must be chosen which will efficiently remove all extraneous material and yet not be so aggressive as to start breaking molecular bonds within the elastin itself; the method must be able to produce reproducibly purified material and preferably be able to be monitored throughout its progress to check for purity level. It should also be equally efficient on specimens of all sizes. A number of methods have been used over the years with differing degrees of success. These are reviewed in the following section.
3.2 Background and Literature Review

3.2.1 Early Purification Methods

The first purification of elastin from elastic tissue was probably by Tilamus in 1840 (quoted in reference 3-6). He employed a method of cold water extraction to remove traces of blood and inorganic matter followed by dehydration with alcohol and ether. Tilamus claimed that if the residue was treated with boiling dilute acetic acid, it was free of sulphur and was pure elastin. Later, Tilamus's procedure was improved upon by Muller 3-7 who incorporated two additional steps involving treatment with boiling potassium hydroxide followed by cold hydrochloric acid. Although it is quite likely that these methods were partially successful, no accurate tests were available at the time to test the purity of the material produced, so it is impossible without repetition to judge them qualitatively.

In 1882 3-8 the purification of elastin from cervical ligament by repeated extraction in boiling water was described. It was recognized that this process would convert the collagen present to soluble gelatin and thereby remove it. Other impurities such as sulphur-containing mucopolysaccharides were not at that time mentioned. Shortly afterwards, however, comment was made on sulphur content 3-9, it being noted that the use of hot alkali during the preparation had the effect of lowering the sulphur content 3-9.

The first recognition of the rich source of elastin in ligamentum nuchae appears to be by Richards and Gies 3-6. They used a process involving cold lime-water instead of boiling potassium hydroxide, followed by successive treatment with boiling water, boiling 10% acetic acid, and 5% hydrochloric acid at room temperature. After removal of the hydrochloric acid by repeated cold-water washes, the residue which remained
was treated with alcohol and ether. Later\textsuperscript{3-10}, the use of the autoclave was introduced instead of boiling water.

Hass\textsuperscript{3-11} introduced a new chemical method in 1942 bypassing the need for boiling or autoclaving. It consisted of treatment with a 89\% formic acid solution at 45\textdegree C for 72 hours. For every 5 mg of tissue being purified, 1 ml of reagent was used, and under these conditions 90-95\% of the tissue by weight was claimed to be recovered.

3.2.2 Purification Methods of the last 30 Years

Since the early exploratory investigations of purification methods, three main types have emerged. Often a particular method will draw on techniques from the other two to supplement it. These methods are:

(1) Physical methods, ie: boiling, pressure, agitation.
(2) Chemical methods, ie: treatment with solvents, acids, alkali, etc.
(3) Biochemical or enzymatic methods, ie: treatment with enzymes, etc.

Although each type has points in its favour, none has emerged clearly superior to the others. In spite of the purely chemical, concentrated formic acid method introduced by Hass\textsuperscript{3-11} in 1942, physico-chemical methods still appear to be prevalent in the literature, with reports of the use of simple boiling water methods\textsuperscript{3-12,3-13,3-14,3-15}, methods using dilute boiling alkali\textsuperscript{3-16,3-17,3-18,3-19}, and methods using boiling dilute acetic acid\textsuperscript{3-20,3-21}. Research on the dilute alkali boiling method has shown it to yield the lowest values of retained mucopolysaccharides but also to cause considerable damage to the elastin itself if the extraction time is too long\textsuperscript{3-18,3-22,3-23,3-24}. The boiling dilute acetic acid method yields high values of retained mucopolysaccharides, and further purification is required, particularly of aortic tissue by dilute alkali or urea\textsuperscript{3-20,3-21}. In addition prolonged exposure to the acid causes blackening and brittleness of the tissue, related\textsuperscript{3-25} to the presence of sialic acid.
Hass's formic acid method$^3-11$ has been subsequently examined in
detail$^3-26,3-27$ and the results indicate that exposure of the tissue
to the reagent causes no change in the fibrillar meshed architecture
provided the exposure is not continued until dissolution of elastin
occurs. The amino acid composition of elastin purified by Hass's method
is similar to that of an alkali purification method$^3-28,3-29$. A more
recent study$^3-30$, however, disputes this evidence, reporting reduction
of desmosine and insufficient purification from collagen. In this study
it was found that a post-treatment with 5M guanidine hydrochloride was
necessary for complete collagen removal$^3-30$.

One of the most recently described physico-chemical methods also
employs a guanidine compound, guanidinium chloride$^3-31$ in what is claimed
as a greatly improved purification method. The elastic tissue is auto-
claved with the guanidinium chloride and the purified product is reported
to show a 5 to 10 fold decrease in free NH$_2$ terminal and groups over
elastin extracted by previous methods. This method, however, has yet to
gain wide acceptance.

Attempts to avoid physico-chemical methods resulted in the third
category of purification techniques - enzymatic methods. Probably
Hospelhorn and FitzPatrick$^3-32$ were the first to propose and employ such
methods. According to their procedure, elastin can be extracted with
1N NaCl, dried, defatted and then digested alternately with trypsin and
collagenase. The resulting product was found to have an amino acid
composition somewhat different from alkali extracted elastin, possibly
due to the fact that no measures were taken to remove glycoproteins
during the treatment.

A later chemico-enzymatic process$^3-33$, however, gave an amino acid
analysis of only slight difference from elastin prepared by an alkali
extraction method\(^3-^{17}\). In this process the tissue was extracted at neutral pH and 5°C for successive 48 hour periods with 3% Na\(_2\)PO\(_4\), 25% KCl and 5M guanidine-hydrochloride. These treatments were followed by several incubations of the residue with a commercially available purified collagenase, 1 mg of enzyme and 10 mg of insoluble substrate, interspersed with water washings.

A chemico-enzymatic method was used in a recent study\(^3-^{34}\) of the two different protein constituents of elastic fibres - the elastin and the microfibrils (see Chapter 1). The elastic fibres were extracted from the surrounding collagen using 5M guanidine followed by collagenase digestion and further treatment with sodium dodecyle sulphate. The microfibrillar component was then separated from the elastin using 6M guanidine dithiothreitol to dissolve the microfibrils away from the elastin residue leaving them as a supernatant which was then alkylated with ethylenimine for amino acid analysis.

Enzymatic and chemico-enzymatic purification techniques in general seem to hold out much promise for the efficient extraction of elastin and it is likely that they will be used with ever-increasing effectiveness in years to come. At the present moment, however, they have one major drawback. In spite of the high specificity of collagenase\(^3-^{35}\), the possibility of attack on the elastin itself with consequent peptide bond cleavage cannot be discounted since preparations of this enzyme (clostridiopeptidase A, EC 3, 4, 4, 19) are known to be contaminated by several proteolytic enzymes\(^3-^{36}\).
3.3 Experimental

3.3.1 Introduction

From the onset of the experimental work of this thesis it was never intended that a detailed study of purification techniques would be carried out. The literature review of this chapter has revealed that the field has already been studied in detail. The philosophy of this work has been rather to select the optimum well-documented and tested technique, examine it for suitability, and if acceptable, standardize the procedure for specimen preparation for all subsequent mechanical experiments.

Concerning the suitability of purification processes, Partridge has written\(^3\) - "The answer is that there is no general method for the isolation of the homogenous protein elastomer called elastin, and we have to choose our isolation procedure in relation to the kind of experiment we wish to perform and the kind of information we require".

This general statement is a valid one: the biochemist, for example, will require an ultra-pure elastin probably in a finely divided or homogenized form, whereas the materials scientist will probably require bulk sections for mounting in mechanical test-rigs where purity is important but not critically so. It is into this last category that the major part of the work of this thesis falls.
The following criteria were adopted as a guideline to the choosing of a suitable method; in order of importance:

(a) Aggressiveness: The method should not degrade the elastin itself. Breaking of amino acid chains can (and has been shown to\textsuperscript{3-30}) influence mechanical properties.

(b) Efficiency: The method should be able to remove the maximum amount of foreign material without infringing requirement (a).

(c) Applicability to bulk specimens: The method should be able to reach and purify the interior of bulk specimens as effectively as the external areas.

(d) Reproducability: The method should provide specimens of reproducible purity in order that a standard procedure might be adopted.

(e) Surveillance: An ability to monitor the state of purification as the process proceeds is an advantage as a guard against underpurification or excessive degradation.

(f) Cost: As it would be necessary to purify large quantities of specimen over several years, it was considered desirable to keep costs to a minimum.

Of the various combinations of purification techniques reported in the literature, four appeared to be most promising. These were:

(1) Hass's formic acid method\textsuperscript{3-11}

(2) Collagenase plus Guanidine method\textsuperscript{3-34}

(3) Boiling NaOH method\textsuperscript{3-28}

(4) An autoclaving plus cold dilute alkali plus solvent extraction method recommended by Partridge\textsuperscript{3-36}
The five criteria were applied to each of these methods in the light of knowledge gained from the literature survey, and the results of this are depicted in Table 3.1.

With the possible exception of the formic acid method, whose aggressiveness definitely appeared to affect mechanical properties\textsuperscript{3-30}, all the other methods seemed reasonably suitable. The collagenase method, however, did have a slight cost disadvantage and required methodology rather more in line with a biochemical laboratory than a materials testing laboratory. Its reproducibility was also slightly suspect, enzymes being very sensitive to factors such as storage conditions, batch number and humidity, etc. The purity of collagenase has also been questioned\textsuperscript{3-36}. Hence, although there have been many claims of considerable success with this method\textsuperscript{3-36,3-38,3-39}, it was decided that priority would not be allotted to it.

The autoclaving method finally adopted was recommended to the author by Dr. S.M. Partridge\textsuperscript{3-38} who kindly made his laboratory available for experience to be gained in its practice, and undertook to provide an amino acid analysis of the purified specimens as a check for purity. For these facilities and assistance the author is extremely grateful. Later purifications for various other parts of the work have all been carried out according to this 'standard' method. Experimental details of the method are presented in Section 3.3.3.

3.3.2 Specimens

3.3.2.1 Procurement

The specimens used for this work were mainly from porcine aorta, although occasionally bovine ligamentum nuchae were also used. Both types of specimen were obtained from freshly killed...
<table>
<thead>
<tr>
<th>METHOD</th>
<th>AGGRESSIVENESS</th>
<th>EFFICIENCY</th>
<th>BULK APPLICABILITY</th>
<th>REPRODUCIBILITY</th>
<th>SURVEILLANCE</th>
<th>COST</th>
<th>COMMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hass Formic Acid</td>
<td>Poor. Report of definite effects on mechanical properties(^3)-(^{30})</td>
<td>Excellent(^3)-(^{39}). Good, if subsequently treated with guanidine(^3)-(^{30}).</td>
<td>No data.</td>
<td>No data, but probably good if all parameters kept constant.</td>
<td>Good; aliquots of solution can be tested by Biuret test.</td>
<td>Good, formic acid comparatively cheap.</td>
<td>-</td>
</tr>
<tr>
<td>Guanidine + Collagenase</td>
<td>Claimed good(^3)-(^{30}) but not with complete certainty(^3)-(^{36}). Must depend on specificity of enzyme.</td>
<td>Good(^3)-(^{30}).</td>
<td>No data, but probably good if enzyme allowed sufficient time to work.</td>
<td>No data, but probably not as easy to ensure as with chemical methods, ie: enzyme deterioration.</td>
<td>Good; can monitor as above.</td>
<td>Poor, collagenase expensive.</td>
<td>Slow method (days).</td>
</tr>
<tr>
<td>Boiling NaOH</td>
<td>Good(^3)-(^{30}). Poor(^3)-(^{40}).</td>
<td>Good(^3)-(^{30}).</td>
<td>No data.</td>
<td>Probably good if parameters kept constant.</td>
<td>Good; as above.</td>
<td>Very good.</td>
<td>Quicker than enzymatic methods. (hours)</td>
</tr>
<tr>
<td>Autoclave Cold dil NaOH solvent extractions</td>
<td>Good(^3)-(^{30})</td>
<td>Good(^3)-(^{30})</td>
<td>No data, but probably good as pressure will force boiling water into interior of specimens.</td>
<td>No data, but probably good if times, temperatures etc maintained constant.</td>
<td>Good; can test residue solution in autoclave after each run.</td>
<td>Good.</td>
<td>Method allows flexible control by increasing or decreasing time in autoclave. Method recommended by Dr. S.N. Partridge.</td>
</tr>
</tbody>
</table>
2 or 3 year old adult animals at local abattoirs. The vessels and ligaments were extracted from the carcasses using a scalpel or sharp butcher's knife and forceps. In the case of the porcine aorta the specimens were removed from the 'pluck' or lung-vessel-trachea complex which was taken from the carcass by the slaughterers shortly after death. Co-operation was necessary with the abattoir health inspector, one of whose tasks is to slit, longitudinally, the aortas of the pluck to examine for parasites within the vessel. This destroyed the geometry of the vessel. The inspector agreed, however, to examine occasional vessels after scalpel excision by turning the sections inside out and hence eliminating the need for slitting. The bovine ligamentum nuchae were removed in segments from the suspended carcasses after they had been dressed (ie: skinned, hosed down, etc.), but before freezing. Unlike the porcine aorta they were part of the main, saleable part of the animal and therefore had to be excised with great care and cleanliness. Usually only part of the ligament could be taken from any one carcass to prevent the neck section from becoming semi-detached with the consequent spoiling of the aesthetic qualities of the carcass.

Following removal the specimens were placed in individual plastic bags and transported by road to the laboratory, a maximum journey time of 30 minutes. On arrival they were immediately washed clean of blood in a 0.9% (physiological) saline solution and placed in plastic bags in a freezer at -60°C temperature for storage before purification. This was always carried out within three weeks of arrival, or else the specimens were discarded. In certain cases, however, when time permitted, the purification process was embarked upon immediately.
3.3.2.2 Appearance and Classification

Typical specimens are depicted in Figs. 3.1 and 3.2. Before a superficial examination was possible it was necessary to remove a considerable amount of adherent fatty tissue and this was accomplished using a scalpel and forceps.

The appearance of nuchal ligament was significantly different from the aorta, although the saline saturated yellow-white colour and soft rubbery feel were similar. In addition, on drying overnight in air, both specimen types would undergo a similar transition through a more yellow leather-like phase to one resembling a brittle brown glass. The difference, however, lay in the fibrous appearance of the two specimen types. Whereas both the inner and outer surfaces of the aorta seemed smooth and homogeneous under eye and low power (x10) examination, the nuchal ligament retained what appeared to be a highly oriented fibrous structure with the fibre bundles running down the length of the specimen, forking at the intersection region (see Fig. 3.2) and continuing down the long and short arms respectively. Examination of the cut cross section confirmed this fibrous structure, showing the appearance of a sectioned rope with the ends of numerous fibre bundles being clearly visible.

To examine for any marked property variations from region to region in the specimens it was decided to adopt a categorization of the different areas. The scheme adopted is depicted in Figs. 3.2 and 3.3.

In the case of the aorta, each section was approximately 40 mm in length, the total area encompassing the aortic arch plus about 80 x 100 mm beyond the bifurcation of the left subclavian artery. Material beyond this lower part was rejected on the grounds that it
Fig. 3-1. Porcine aorta classification.

Region.
[1.] Heart to innominate artery. [approx. 40mm]
[2.] Region including innominate and left subclavian branches. [approx 40mm]
[3.] 40mm on from left subclavian.
[4.] 40mm on from end of region 3.
Fig. 3.2. Bovine ligamentum nuchae classification.

Region.
[1.] = 'Trunk'.
[2.] = 'Intersection region'.
[3.] = 'Short arm'.
[4.] = 'Long arm'.

~130 mm

~80 mm

~50 mm

~260 mm

~35 mm
has been shown\textsuperscript{3-4, 3-42} (for dogs and humans) that there tends
to be a marked decrease in the elastin:collagen ratio as the vessel
approaches the abdominal region; it was considered that this could
also apply to the porcine case. Furthermore, in this region the
vessel tapers to about 5 mm diameter making it a less geometrically
desirable area from which to cut specimens for mechanical testing.

Although the regions of the ligamentum nuchae specimens were
also categorized numerically (see Fig. 3.2) to avoid confusion with
the aorta region, they were also given names - 'long arm, short arm,
intersection region and trunk'. These names are more commonly used
in the text. The long and short arms and trunk of the specimens
had a generally anisotropic appearance with fibres lying parallel
and aligned with the long axis of the section. In the intersection
region, however, where the fibres forked into the two arms of the
specimen the appearance was of a far more homogeneous nature.

3.3.3 Purification

3.3.3.1 The 'Standard' Method

The saline washed and scalpel cleaned specimens were sliced
into their respective sections and placed in beakers half filled
with glass-distilled water. These beakers were in turn placed in
either a standard gas or electrically heated sterilizing autoclave
and the head of steam allowed to rise to 103.42 kN/m\textsuperscript{2} (15 psi) (121°C).
After 45 minutes the specimens were removed and placed in clean
beakers of continuously boiling glass-distilled water where they
were allowed to stand for a further 30 minutes to wash out the
decomposed collagen of the autoclaving process. The 45 minutes
autoclaving/30 minutes boiling water cycle was then repeated two
more times, and after the last time a sample of beaker water was
taken and Biuret tested (section 3.6.1) to check for purity from decomposed collagen. Usually by this time the residue water was found to be Biuret negative, ie: free from decomposed collagen, but if it was not, further 45 minute autoclaving/washing cycles were carried out until Biuret negative results were obtained. The following process of solvent washings were then carried out to remove any lipids and glycoproteins present. Each of these washings was undertaken in 500 ml round-bottomed flasks mounted on a twist-arm or oscillating tray flask shaker set at 100-120 oscillations per minute.

(a) To remove lipids

<table>
<thead>
<tr>
<th>Duration</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 minutes</td>
<td>50% Ethanol/50% distilled water</td>
</tr>
<tr>
<td>30 minutes</td>
<td>Ethanol</td>
</tr>
<tr>
<td>30 minutes</td>
<td>50% Ethanol/50% Diethyl ether</td>
</tr>
<tr>
<td>30 minutes</td>
<td>Diethyl ether</td>
</tr>
<tr>
<td>30 minutes</td>
<td>50% Diethyl ether/50% Ethanol</td>
</tr>
<tr>
<td>30 minutes</td>
<td>Ethanol</td>
</tr>
<tr>
<td>30 minutes</td>
<td>50% Ethanol/50% distilled water</td>
</tr>
<tr>
<td>30 minutes</td>
<td>Distilled water</td>
</tr>
<tr>
<td>30 minutes</td>
<td>Fresh distilled water</td>
</tr>
</tbody>
</table>

(b) To remove glycoproteins:

<table>
<thead>
<tr>
<th>Duration</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 minutes</td>
<td>0.1 M NaOH (room temperature)</td>
</tr>
<tr>
<td>60 minutes</td>
<td>Fresh 0.1 M NaOH</td>
</tr>
<tr>
<td>60 minutes</td>
<td>Fresh 0.1 M NaOH</td>
</tr>
</tbody>
</table>

(c) Finally to remove NaOH and any other remaining contaminants:

<table>
<thead>
<tr>
<th>Duration</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 minutes</td>
<td>Methanol</td>
</tr>
<tr>
<td>30 minutes</td>
<td>50% Methanol/50% Chloroform</td>
</tr>
<tr>
<td>30 minutes</td>
<td>Pure Chloroform</td>
</tr>
<tr>
<td>30 minutes</td>
<td>50% Chloroform/50% Methanol</td>
</tr>
<tr>
<td>30 minutes</td>
<td>Methanol</td>
</tr>
<tr>
<td>30 minutes</td>
<td>Distilled water</td>
</tr>
<tr>
<td>60 minutes</td>
<td>Distilled water</td>
</tr>
<tr>
<td>60 minutes</td>
<td>Distilled water</td>
</tr>
<tr>
<td>60 minutes</td>
<td>Distilled water</td>
</tr>
</tbody>
</table>

Total time of purification = approximately 18 hours
Following purification, the specimens were either
(i) used immediately,
(ii) stored in cold 1% acetic acid for up to 4 weeks,
or (iii) frozen at -60°C for up to 12 weeks.
If they had not been used, however, by 12 weeks following purification, they were discarded for fear of deterioration.

An amino acid analysis of a sample of the first batch of porcine aortas to be purified by the process was kindly undertaken by Dr. Partridge to check for purity. The results are given in Table 3.2 and compare favourably with results given in the literature on the subject.

3.3.4 Determination of Specimen Elastin Content

3.3.4.1 Introduction

As the proportion of elastin in the various tissue tends to vary with type and location it was considered that the elastin : collagen and other vessel wall material ratio should be monitored in order to:

(a) check the efficiency of the purification process, and
(b) correlate the elastin proportions with position from which the specimen had been taken.

The method employed to do this was of a physical nature, and involved weighings with a microbalance as described in the next section. The method was chosen for its comparative simplicity and accuracy.

3.3.4.2 Method

Several samples of scalpel cleaned unpurified aorta or ligamentum nuchae approximately 10 mm square or cube were cut from various regions
### Table 3.2

#### AMINO ACID ANALYSIS

<table>
<thead>
<tr>
<th></th>
<th>Residues/1000</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MA</td>
<td>Porcine aorta</td>
<td>Bovine ligamentum nuchae</td>
</tr>
<tr>
<td></td>
<td>Ref. 3-43</td>
<td>Present Study</td>
<td>Ref. 3-44</td>
</tr>
<tr>
<td>HYDROXYPROLINE</td>
<td>14.5</td>
<td>10.5</td>
<td>12</td>
</tr>
<tr>
<td>ASPARTIC ACID</td>
<td>8.8</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>THREONINE</td>
<td>7.4</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>SERINE</td>
<td>8.1</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>GLUTAMIC ACID</td>
<td>20.9</td>
<td>14.5</td>
<td>11</td>
</tr>
<tr>
<td>PROLINE</td>
<td>93.8</td>
<td>90.5</td>
<td>96</td>
</tr>
<tr>
<td>GLYCINE</td>
<td>328.9</td>
<td>323</td>
<td>320</td>
</tr>
<tr>
<td>ALANINE</td>
<td>233.3</td>
<td>230</td>
<td>234</td>
</tr>
<tr>
<td>VALINE</td>
<td>124.9</td>
<td>129</td>
<td>152</td>
</tr>
<tr>
<td>ISOLEUCINE</td>
<td>19.6</td>
<td>18.5</td>
<td>24.5</td>
</tr>
<tr>
<td>LEUCINE</td>
<td>57.4</td>
<td>58.5</td>
<td>67</td>
</tr>
<tr>
<td>TYROSINE</td>
<td>16.9</td>
<td>17.5</td>
<td>15</td>
</tr>
<tr>
<td>PHENYLALANINE</td>
<td>32.3</td>
<td>31</td>
<td>31.4</td>
</tr>
<tr>
<td>LYSINE</td>
<td>7.6</td>
<td>7.5</td>
<td>3.2</td>
</tr>
<tr>
<td>ARGinine</td>
<td>6.6</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>ISOIDESMOSINE/4</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>DESMOSINE/4</td>
<td>2.1</td>
<td>2</td>
<td>5.2</td>
</tr>
</tbody>
</table>
of the specimen and equilibrated for 3 hours in glass-distilled water at 20°C. They were then gently individually removed, surface water dabbed off with absorbent tissue paper and weighed before being returned to the distilled water. This process was called 'dab-weighing'. For each specimen ten dab-weighings were taken with at least 10 minute intervals between each. A similar method has been reported elsewhere\textsuperscript{3-45}. The mean result of the ten dab-weighings was calculated and the scatter was found to be within ±5% of this value and showed no noticeable progressively increasing or decreasing trends. This weighing was designated \( m_{\text{uwet}} \).

The weighed specimens were then placed in a vacuum oven at 40°C and 0.1 torr for 3 hours to dry, the temperature chosen being sufficiently low as to avoid any decomposition of collagen. The specimens were then re-weighed and returned to the vacuum oven for a further hour at 40°C and 0.1 torr to check for any further weight drop. In no case, however, was a further weight drop observed, and 3 hours was consequently deemed sufficient for complete water removal. This weighing was designated \( m_{\text{udry}} \).

Hence the water content of the unpurified specimens could be calculated:

\[
m_{\text{water}} = m_{\text{uwet}} - m_{\text{udry}}
\]

Specimens cut from the various sample regions were also put through the 'standard purification process', some being removed at each stage, ie:

(i) after first autoclave and boil
(ii) after second autoclave and boil
(iii) after third autoclave and boil
(iv) after NaOH treatment
(v) after full treatment
Each of these specimens was equilibrated with distilled water at 20°C and a similar dab-weighing and drying process carried out as on the unpurified specimens. From these measurements the following parameters (see overleaf) were calculated for each specimen.

3.3.4.3 Results and Discussion

The results are depicted in Tables 3.3 and 3.4 and are typical of results obtained in subsequent purification processes.

The $\alpha$ percentage gives a measure of the swelling of a particular sample from its dry state, and is a parameter of more relevance to subsequent chapters of this thesis than here (i.e. Chapter 4). Its value includes both a contribution from liquid dissolved within the actual material of the matrix and liquid contained within the inter-fibre spaces of the specimen. Consequently, accepting the assumption that elastin from the bovine ligament and the porcine aorta are essentially the same material, it can be concluded that there is a larger ratio of inter-fibre voids to bulk purified material in the case of the aorta giving rise to its overall greater $\alpha$ value.

For the purposes of this chapter $\beta$ and $\delta$ are the more relevant parameters. $\beta$ gives a measure of the dry weight loss of solid material due to each stage of the purification process. As can be seen, the main mass of material appears to be removed by the first two autoclavings and virtually no more by the third autoclaving. This is undoubtedly due to the breakdown and removal of the collagen component of the material, a process which appears to be complete by the end of the third autoclaving and which is confirmed by the negative results of the Biuret tests. The diethyl ether and NaOH rinses then appear to remove a little extra material, though somewhat more so in the case of the aorta with its higher glycoprotein
Parameters

\[ \alpha \% = \frac{m_{\text{treated, wet}} - m_{\text{treated, dry}}}{m_{\text{treated, dry}}} \times 100 \]

= Wt percentage water in treated saturated specimen

\[ \beta \% = \frac{m_{\text{untreated, dry}} - m_{\text{treated, dry}}}{m_{\text{untreated, dry}}} \times 100 \]

= Wt percentage dry material removed

The \( \beta \) percentage at the end of the purification process could be used to give \( \delta \% \), a measurement of the remaining elastin in the specimen, ie:

\[ \delta \% (= \% \text{ dry wt elastin in original unpurified specimen}) = 100 - \beta \]
### 'Standard' Purification Method

<table>
<thead>
<tr>
<th>Process after which measurements taken</th>
<th>α%</th>
<th>β%</th>
<th>δ%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aorta</td>
<td>LN</td>
<td>Aorta</td>
</tr>
<tr>
<td>Untreated</td>
<td>250.9</td>
<td>145.3</td>
<td>0</td>
</tr>
<tr>
<td>1st Autoclave and boil</td>
<td>207.1</td>
<td>163.3</td>
<td>39.82</td>
</tr>
<tr>
<td>2nd Autoclave and boil</td>
<td>209.1</td>
<td>164.0</td>
<td>44.43</td>
</tr>
<tr>
<td>3rd Autoclave and boil</td>
<td>207.7</td>
<td>161.2</td>
<td>44.45</td>
</tr>
<tr>
<td>Ether treatment</td>
<td>215.4</td>
<td>165.5</td>
<td>49.80</td>
</tr>
<tr>
<td>NaOH treatment</td>
<td>386.3</td>
<td>295.2</td>
<td>59.57</td>
</tr>
<tr>
<td>Fully pure</td>
<td>286.1</td>
<td>171.1</td>
<td>61.03</td>
</tr>
</tbody>
</table>
TABLE 3.4

Regional Comparisons

1. Aorta  Mean of 10 samples from 5 different aortas

<table>
<thead>
<tr>
<th>Region</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40.48</td>
</tr>
<tr>
<td>2</td>
<td>40.17</td>
</tr>
<tr>
<td>3</td>
<td>38.93</td>
</tr>
<tr>
<td>4</td>
<td>35.62</td>
</tr>
</tbody>
</table>

2. L.N.  &%

<table>
<thead>
<tr>
<th>Region</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trunk</td>
<td>79.33</td>
</tr>
<tr>
<td>Intersection</td>
<td>80.06</td>
</tr>
<tr>
<td>Short arm</td>
<td>78.99</td>
</tr>
<tr>
<td>Long arm</td>
<td>79.72</td>
</tr>
</tbody>
</table>
content. Finally, the last rinses remove any remaining NaOH and loosened extraneous material, to cause a further slight weight drop.

The purification process thus seemed reasonably efficient giving elastin values in agreement with those in the literature.

The second part of the experiment, to examine the distribution of elastin in various regions of the bulk specimens, yielded results which can be seen in Table 3.4. In the case of the ligamentum nuchae no variation above experimental error could be ascertained from region to region of the specimen. The aorta, however, did appear to exhibit a slight dropping off of elastin content in regions 3 and 4. It was therefore decided to concentrate on regions 1 and 2 when taking samples for future mechanical tests.

3.3.5 Purity Examination by Scanning Electron Microscopy

3.3.5.1 Introduction and Literature Review

Although the physical method of drying and weighing outlined in the previous section provided a useful quantitative measure of the amount of material removed during the purification process, it did not answer all the questions relating to quality of the product produced. The questions had to be asked whether, for example, there was an element of collagen fibres so highly adhered to the elastin that it remained undecomposed throughout the purification process, releasing no free peptides into solution to affect the Biuret test, and, also, what was the effect of the purification process at each of its stages. It was decided that the best method of answering these questions was to undertake a scanning electron microscopic study of the specimens at various stages of the purification process, and in fully purified form.
The scanning electron microscope has the advantage of a wide range of useful magnifications (from about x20 to x50,000), requires only a comparatively simple specimen preparation technique and can produce images of very great depth of field compared with the 'light' microscope. Its disadvantage is that, in similarity with other types of electron beam examination techniques, the specimen must be held in vacuo, and for this reason must be in a dry state. Although 'wet' cells for the instrument have been developed experimentally\textsuperscript{3-46}, their efficiency remains unproven.

Previous applications of scanning electron microscopic techniques to elastin and elastic tissues have been fairly limited. In 1969, however, Finlay used the instrument to examine the human dermis under uniaxial strain\textsuperscript{3-47} taking specimens from the operating theatre, stressing, fixing and sectioning them and carrying out a post-fixation mucopolysaccharide removal process. He observed closely-packed fibrous networks of collagen and elastic fibres which tended to align themselves with the applied load. Later\textsuperscript{3-43}, the surfaces of the epidermis were examined and scanning electron microscope images were compared with those obtained under the light microscope. Ehlers-Danlos syndrome skin has also been examined\textsuperscript{3-49} using a preparation technique similar to Finlay's\textsuperscript{3-47}. Disease and control specimens were compared and it was observed that the Ehlers-Danlos samples had loosened collagen fibre bundles of varying thickness and irregular orientation and the fine elastic fibre network was increased. From this it was concluded that changes in fibrillar components may have an important role in the hyperelasticity of Ehlers-Danlos type skin. Work on other diseased skins has also been undertaken\textsuperscript{3-50}.

The first scanning electron microscope observations specifically on elastin were preformed by Cotte, Mamm and Pezzin\textsuperscript{3-51} in 1972.
Samples of ligamentum nuchae were taken from the carcasses of two-year old oxen and purified by autoclaving and NaOH treatment. They were then swollen in several solvents and allowed to dry by evaporation in air. The purified elastin was observed at low magnification to consist of a uni-directional array of fibres which appeared to be arranged in bundles, connected by extended fibres. At higher magnification the surface seemed to be resolvable into smaller fibrillar units, also parallel to the fibre axis, and a single fibre was often found to generate two or more separated fibres.

Ligamentum nuchae purified enzymatically has also been examined in the scanning electron microscope \(^{3-52}\). A guanidine hydrochloride incubated sample digested with collagenase was compared with a control specimen treated only with guanidine hydrochloride. It was observed that the enzymatically treated sample was rather frayed, whereas the control specimen was microscopically unaltered. Histologically the collagen had been removed from the surface layer of the digested specimen, but the control sample was normal.

Although all these papers outline the main structural details of their elastic tissue or elastin specimens, one criticism can be applied to all of them; their method of specimen preparation. Each of the authors resorts to a drying method of simple evaporation - either of water or of solvent; in air, a method shown to be accompanied by considerable shrinkage and specimen distortion whichever solvent is employed\(^{3-53}\). It is quite possible that distortion during the air-drying process could cause a highly unrepresentative picture in the scanning electron microscope which would result in fallacious conclusions concerning the architectural microstructure of the specimen. For example, the differential shrinkage of two or more protein phases present could cause a complete misinterpretation.
of the amount of each substance to be found in the natural state, as could the drying induced opening up or disappearance of voids, vessel centres of interfibrillar pores. It was thus decided that it was necessary to develop a non air-drying method for scanning electron microscopic elastin examination, and this is described in the following section.

3.3.5.2 Freeze-drying Method of Specimen Preparation

The freeze-drying of biological tissues for electron microscopic examination has been recognized for several years to have a number of advantages over standard air-drying methods, although its application to elastin does not appear to have been attempted. The technique of freeze-drying in general has been reviewed in detail by Rowe and has been applied to ultrastructural studies of cell morphology with varying degrees of success, as well as the preservation of tissue grafts, micro-organisms and bovine semen.

The main difficulty with freeze drying specimens for microscopic examination is the necessity to avoid the formation, with consequent tissue damage, of ice crystals - often known as 'the ice crystal artefact'. Recrystallization of ice formed by rapid freezing at liquid nitrogen temperatures is believed to occur in the region -50°C to -60°C, so theoretically it is desirable to work below these temperatures, a requirement which many current freeze-drying units are unable to attain, and to make the specimens as small as possible to reduce the drying time to a minimum. Very rapid freezing is required for small pieces of animal or vegetable tissue that are to be fixed by freeze-drying for examination in the scanning electron microscope. The objectives basically are:
(i) to prevent gross structural distortion of the type produced by air-drying methods,
(ii) to reduce the ice crystal artefact to a minimum, and
(iii) to present to the researcher as far as possible the micro-architecture as it was in the instant when the sample was cut from living tissue.

Direct immersion in liquid nitrogen of specimens even smaller than 1 mm³ results in film or spherical boiling of the liquid gas so as to lower its latent heat, and this generates an insulating envelope around the specimen which retards freezing. To overcome this problem a refrigerant may be used, pre-cooled to -150°C in liquid nitrogen and the specimens immersed in this instead.

The specimens chosen for the initial testing of the method were from the purified porcine aorta samples, these promising a more detailed microstructure than the ligamentum nuchae. Four specimens were cut in approximately 5 mm squares from the wall of a 'standard' purified aorta in region 2 and placed in glass-distilled water for an hour at 20°C to equilibrate. They were then removed, their surface dabbed dry with absorbent tissue paper and their dimensions taken precisely with a micrometer screw gauge. Five readings were taken for each dimension, and the mean calculated. Following this, two of the specimens were placed in a desiccator at 20°C over P₂O₅ to dry, and the remaining two freeze-dried as follows:

A bath of 'Arcton 12' (ICI Chemicals, Runcorn, Cheshire) was prepared by condensing in a small aluminium container held by wires in a Dewar flask of liquid nitrogen. Into this refrigerant bath were then placed the two specimens where they were held for 30 seconds before being quickly transferred to the freezing platform of an Edwards EPD 2 freeze-drying unit pre-cooled to -55°C. Pressure was
reduced to 0.025 torr and the specimens allowed to 'dry' for 6 hours. P₂O₅ was used as the moisture trap in the system. After this 6 hour period the specimens were quickly transferred to a desiccator over P₂O₅ for storage.

Following further dimensional measurements on all the specimens, they were mounted on the aluminium specimen stubs which had previously been ultrasonically cleared in a bath of acetone. The adhesive used for the mounting was 'Pritt' non-stringing adhesive (Henkel Chemicals Ltd., Winsford, Cheshire) which was allowed to dry for one hour. At no time during these procedures were the specimens allowed to remain outside the desiccator for a longer period than 5 minutes. Of the two specimens in each preparation method, one was mounted on its edge to expose the cut cross-section of the wall, and the other split down its centre by gentle pulling with forceps, and the two sections mounted side by side, one with the adventitial surface lying uppermost and the other exposing the split medial region (see Figs. 3.3, 3.4).

For scanning electron microscope examination the specimens were vacuum coated with a 20 nm thick layer of 60/40 wt% gold/palladium with the stubs mounted on a rotating stub holder to ensure even coating. A thin conductive bridge of silver dag was painted from one edge of each specimen to its stub to ensure adequate earthing in the electron beam. Viewing was carried out at 45° tilt in a Cambridge Stereoscan Mk.IIA scanning electron microscope using an accelerating voltage of 20 kV, and micrographs recorded on Ilford FP4 film using an Exacta 35 mm camera.

Typical results are shown in plates 3.1 to 3.8. Plates 3.1 and 3.3, and 3.2 and 3.4 respectively, show views across the wall of the purified vessel. In all instances the elastic laminae are clearly visible although in the freeze-dried specimen the intervening voids
Fig. 3.3. Edge mounting.
Fig. 3.4. Split mounting.
are far larger, and occupy approximately 50-60% of the visible cross-sectioned area.

Micrometer measured dimensions resulting from the freeze-drying processes are shown in Table 3.6.

In the case of the air-dried specimens, the combination of fibre shrinkage and the virtual absence of voids would appear to be the major factor accounting for its large measured shrinkage of approximately 60% in the x direction, the void spaces collapsing and the fibres shrinking as the material dries out. In the y and z directions, however, the main shrinkage is probably due almost entirely to fibre dimension changes, accounting for the somewhat smaller shrinkage values. The micro-architecture of the porcine aorta specimen will be discussed in more detail in a later section of the work (see Chapter 7), so will not be enlarged upon here.

In the medial and adventitial views of plates 3.5, 3.6, 3.7 and 3.8 respectively, there appear a marked difference between the condition of the fibres although their diameters appear approximately similar. In the air-dried case there is considerable distortion and kinking of the fibres which themselves have a somewhat dented appearance quite dissimilar to the smooth-looking freeze-dried fibres. This was apparent in both the medial and adventitial sections. The appearance of the air and freeze-dried specimens was also markedly different on a 'macro' level. The air-dried specimens dried down to a translucent brown glass while the free-dried specimens retained their white colour, spongey texture and the overall appearance of the elastin in the water-saturated structure.

The ability of the freeze drying method to preserve fine detail is illustrated in plates 3.9 and 3.10 which show two views of the freeze-dried edge mounted specimens angled so as to be able to observe
<table>
<thead>
<tr>
<th>Measured % dimension change after drying process</th>
<th>Freeze dried ± 5%</th>
<th>Air dried ± 5%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>x    y    z</td>
<td>x    y    z</td>
</tr>
<tr>
<td>+ = shrinkage</td>
<td>-1.5 +0.7 +0.9</td>
<td>+62.5 +11.8 +13.5</td>
</tr>
<tr>
<td>- = expansion</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Plate 3.1
Magn. x 24

Plate 3.2
Magn. x 24

Plate 3.3
As 3.1 
Magn. x 300

Plate 3.4
As 3.2 
Magn. x 300

Plate 3.5
View of a medial layer of purified porcine aortic wall. (Blood flow top to bottom of plate) Prepared by freeze-drying. 
Magn. x 1000

Plate 3.6
View of medial layer of purified porcine aortic wall. (Blood flow top to bottom of plate) Prepared by air-drying. 
Magn. x 1000

Plate 3.7
View of adventitial layer of purified porcine aortic wall. (Blood flow top to bottom of plate) Prepared by freeze-drying. 
Magn. x 1000

Plate 3.8
View of adventitial layer of purified porcine aortic wall. (Blood flow top to bottom of plate) Prepared by air-drying. 
Magn. x 1000
Plate 3.9
View of cross-section and intima of purified porcine aorta. Prepared by freeze drying. Magn. x 80

Plate 3.10
View of purified intima of purified porcine aorta. (Blood flow top to bottom of plate) Prepared by freeze drying. Magn. x 770
their intimal regions - the innermost wall closest to the bloodstream. Here it was possible to observe the fine structure of longitudinally oriented elastin fibrils preserved by the freeze-drying process.

From these results it was concluded that freeze-drying presented a method superior to air-drying for the preservation of the structure of purified elastin by scanning electron microscopy, giving reduced shrinkage and distortion and generally presenting the structure in a dried condition more similar to its in-vivo state. As a consequence it was decided to adopt this method for the work of the next section as well as scanning electron microscope examinations carried out in subsequent sections of the thesis.

3.3.5.3 S.E.M. Examination of the 'Standard Purification Method'

Having established a suitable specimen preparation method for the scanning electron microscope, the second stage was to apply it to the 'standard' purification method to examine the efficiency of the method.

A sample of region 2 aorta was taken unpurified, and two specimens approximately 5 mm square cut from its wall. The sample was then put through the standard purification process, though after each major stage two more specimens were taken for scanning electron microscope examination.

The stages after which the specimens were taken are listed below:

<table>
<thead>
<tr>
<th>Specimens</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a and 1b</td>
<td>Unpurified but scalpel cleaned and distilled water equilibrated</td>
</tr>
<tr>
<td>2a and 2b</td>
<td>After 1st autoclaving plus boil (Biuret +ve)</td>
</tr>
<tr>
<td>3a and 3b</td>
<td>After 2nd autoclaving plus boil (Biuret -ve or faintly +ve)</td>
</tr>
<tr>
<td>4a and 4b</td>
<td>After 3rd autoclaving plus boil (Biuret -ve)</td>
</tr>
<tr>
<td>5a and 5b</td>
<td>After ether treatment</td>
</tr>
<tr>
<td>6a and 6b</td>
<td>Fully purified</td>
</tr>
</tbody>
</table>
The area of the specimen used for the purposes of comparison was a split medial surface (as described in the previous section); preparation was by the freeze-drying method. The results can be seen in plates 3.11 to 3.23, where typical views are given of areas of the specimens at low and medium scanning electron microscope magnifications respectively, at the various stages of the purification process.

Plates 3.11 and 3.12 show the specimens in their unpurified forms. A large amount of extraneous material is present, almost totally obscuring the collagen/elastin networks, and probably consisting largely of lipid and glycoprotein deposits and cellular material. After the first autoclaving and boil, however, all this extraneous material appears to have been removed, leaving a branched network structure of elastin fibres thickly sheathed with collagen. The second autoclaving and boil seems to remove a large proportion of this collagen component (plates 3.13, 3.14) but it is not until the third and final autoclaving and boil that clean elastin fibres are finally revealed (plates 3.17, 3.18) almost completely free of collagen sheathing, although still retaining small pieces of adherent material. The diethyl-ether treatment, however, appears to remove much of this adherent material, probably signifying that the bulk of it is fatty residue (plates 3.19, 3.20). The final stages of NaOH treatment and ultimate solvent washes leave clean, smooth and apparently completely residue-free fibres (plates 3.21, 3.22).

The process appears to be equally efficient in the case of ligamentum nuchae where round, smooth and clean fibres are yielded, free from visible impurities across the working range of scanning electron microscope magnifications (plates 3.23, 3.25, 3.26).
Plate 3.11
Medial view, unpurified but scalpel cleaned porcine aorta specimen. Freeze dried. Magn. x 70

Plate 3.12
As 3.11 Magn. x 1440

Plate 3.13
Medial view, porcine aorta after first autoclave and boil of purification process. Freeze dried. Magn. x 70

Plate 3.14
As 3.13 Magn. x 1440

Plate 3.15
Medial view, porcine aorta after second autoclave and boil of purification process. Freeze dried. Magn. x 70

Plate 3.16
As 3.15 Magn. x 1440

Plate 3.17
Medial view, porcine aorta after third autoclave and boil of purification process. Freeze dried. Magn. x 70

Plate 3.18
As 3.17 Magn. x 1440
Plate 3.19
Medial view, porcine aorta after diethyl-ether treatment stage of purification process. Freeze dried.  
Magn. x 72

Plate 3.20
As 3.19  
Magn. x 1440

Plate 3.21
Medial view, porcine aorta after full purification process. Freeze dried.  
Magn. x 72

Plate 3.22
As 3.21  
Magn. x 1440

Plate 3.23
Purified bovine ligamentum nuchae. Freeze dried.  
Magn. x 12

Plate 3.24
Purified bovine ligamentum nuchae. Freeze dried.  
Magn. x 120

Plate 3.25
Purified bovine ligamentum nuchae. Freeze dried.  
Magn. x 270

Plate 3.26
Purified bovine ligamentum nuchae. Freeze dried.  
Magn. x 2970
3.4 Discussion

The main object of this Chapter has been to describe a suitable preparation method with which to purify elastin specimens for the further experiments of the thesis. It was not the aim to formulate a new, previously untried method nor to try to significantly improve upon any existing methods. Consequently there was no attempt to break any new ground, but rather to critically examine the literature, select a suitable method from it, apply and test this method for suitability for adoption as a 'standard method' for the thesis. The question must thus be asked, whether the aim has been successfully achieved or not?

In so far as the selection of the method was concerned, details of this have already been discussed in the first part of the chapter, so will not be repeated here. A method was chosen which had been well tried and proven in the literature and which was personally recommended by an eminent worker in the field. The four tests used to examine the method - Biuret, amino and analysis, weight loss and scanning electron microscopy - each testified satisfactorily to the purity of the material. Although an N-terminal analysis, a test for degradation in the elastin, was not carried out, the work of Steven et al has shown that treatment of elastin with 0.1M NaOH up to one hour causes little change in its N-terminal pattern. Such treatment was considered to be of equal, if not of greater rigor than the treatment of the 'standard method'. Furthermore, the scanning electron microscope revealed clean smooth fibres apparently in good condition, with no signs of denting, excessive kinking or pitting, which might be expected if any significant chemical or physical attack had occurred on or within the fibres.
Although both ligamentum nuchae and porcine aorta were prepared with the 'standard method', more concern was generally applied to the latter, for two reasons. The first was that the bulk of the mechanical and physical experiments of this work use porcine elastin for their experiments, and the second was because the complexity of structure of the aorta is greater and contains only about half the dry weight percentage elastin of the ligament. Thus it is more difficult to purify and it was considered that if the method was suitable for the aorta it would certainly be suitable for the ligament.

In the field of elastin purification there remains a vast amount of basic experimental work still to be carried out to find the ideal method. It is part of elastin studies very often overlooked in the researcher's haste to commence 'more meaningful' experiments on their purified elastin. This, however, is a mistake, for improperly or over-vigorously purified elastin could yield totally erroneous results to workers both in biological and physical fields. It is for this reason that the studies in this Chapter were undertaken.
3.5 Conclusions

1. Elastin from specimens of bovine ligamentum nuchae and porcine aorta were successfully extracted by a method of repeated autoclaving followed by solvent extraction.

2. The elastin purified by this method showed:
   (i) a high degree of purity from collagen glycoproteins and lipids;
   (ii) no visible signs of degradation.

3. A freeze-drying elastin specimen preparation method for scanning electron microscope was successfully tested and compared with a standard air-drying method for fixative free specimen observations. The freeze-drying method was seen to produce better results than the air-drying method.
3.6 Appendices

3.6.1 The Biuret Test

3.6.1.1 General Theory

The Biuret tests test for the presence of free peptide linkages:

when urea is heated to about 180°C, it decomposes to form 'Biuret'.

\[
\text{urea} \xrightarrow{\text{Heat}} \text{NH}_2 - \text{C} - \text{NH}_2 \quad \text{at} \quad 180^\circ\text{C} \quad \text{NH}_2 - \text{C} - \text{NH} - \text{C} - \text{NH}_2 + \text{NH}_3
\]

and if a strongly alkaline solution of biuret is treated with very dilute copper sulphate a violet colour is obtained.

It is for this reason that the test is named the Biuret test.

A similar reaction, however, also occurs with peptide structures and their derivatives which contain

\[
\begin{align*}
\text{H} & \quad \text{H} \\
\text{CO} & \quad \text{NH} \\
\text{C} & \quad \text{CO} \\
\text{NH} & \quad \text{R}
\end{align*}
\]

linkages. The peptide bonds form complexes with the copper sulphate in alkaline solution which range from blue to pink in colour. Gelatin, however, (decomposed collagen) gives a blue colour.

The Biuret test apparently is due to co-ordination of Cu ions with the unshared electron pairs of peptide nitrogen and the oxygen of water to form a coloured co-ordination complex which may be represented as follows:

[Diagram of Biuret complex]
The biuret reaction is extensively used as a delicate test for the presence of free proteins in biological materials; it is also used as a method for the quantitative determination of proteins in blood serum and other fluids.

In this work it has been used in a qualitative manner for detecting the presence of free peptides resulting from the breakdown of collagen in the autoclaving/boiling process. For this test it was highly suitable, due to its comparative simplicity and easily visible colour changes.

3.6.1.2 The Reagent

Approximately 500 ml of glass-distilled water were taken and into this dissolved 1.5 g CuSO4.5H2O and 6.0 g sodium potassium tartrate. 200 ml of 10% high purity NaOH solution were then slowly added with continuous stirring and this solution made up to 1 litre with further distilled water. Storage was in a standard polythene bottle with a well fitting screw stopper. Generally, fresh reagent was made up before each purification run and on no occasion was reagent used which was more than 3 weeks old, or had shown any signs of precipitation.

3.6.1.3 Test Method

After each autoclaving, samples of the residual beaker water in which the specimens had been placed in the autoclave were taken with a new pasteur pipette and allowed to cool to room temperature. The 1.5 ml of these was then taken and to these added 6.0 ml of biuret reagent. They were then left to stand for an hour to allow any colour to develop. As the test was only being used qualitatively a colorimeter was not used and it was sufficient to hold the test tubes against a background of white card in good light to check for the change of colour visually.
3.6.2 Abattoirs

The abattoirs from which the specimens were obtained are listed below:

(1) Abingdon F.M.C. Abingdon, Oxfordshire
(2) Thame F.M.C. Moorend Lane, Thame, Oxfordshire
(3) Guildford F.M.C. Guildford, Surrey
4.1 Introduction

The sensitivity of the physical properties of elastin to water content is easily observed. On drying in air the material changes form from a soft, yellow-white rubbery phase to one brown in colour of considerably increased hardness and brittleness.

Elastin, immersed in water or saline, will imbibe or exude water with variations in temperature. This effect was often neglected by early experimenters, which consequently led to erroneous conclusions about elastin structure (see section 2.3.3). For the interpretation of the results of mechanical experiments on saturated elastin it is necessary to have knowledge of its swelling characteristics. In addition, considerable structural information may also be obtained from swelling information, a fact which has been demonstrated by Mukherjee. This chapter, therefore, investigates some of the basic physical properties of elastin/solvent composites as a first step in the understanding of the structure of the material.

Water in the elastin/water composite exists in one of two regions:

(i) Microvoid water - the water inbetween but unassociated with the elastin molecules.

(ii) 'Dissolved' water - the water chemically associated with the elastin molecules.

Elastin consists of a higher proportion of non-polar amino acids than polar amino acids and as a consequence non-polar interactions are
though to play an equally important part as polar interactions in its structure and swelling properties\textsuperscript{4-2}. Such interactions are known as hydrophobic interactions and taken individually are generally weaker than other protein side-chain interactions. They become important, however, if the protein has a large number of non-polar side chains, as is the case with elastin with approximately 93% hydrophobically side-chained amino acids. Hydrophobic interactions exhibit less specificity than other interactions, both with respect to steric requirements of side chain orientations and the number and the kind of side chains which can participate in their formation.

Another property unique to hydrophobic interactions is their dependence on the surrounding solvent medium for their existence. Generally they can only achieve their maximum predominance in aqueous systems. In non-aqueous solvents whose molecules consist partly of non-polar hydrocarbon type groups, interaction occurs equally favourably with the solvent as with the non-polar molecules of the chain themselves. Any stabilizing effect of these interactions within the tertiary structure of the protein is thus nullified and the chain may unfold. Even in solvent mixtures containing water as well as an organic solvent, the latter may form hydrophobic bonds with the non-polar side chains facilitating unfolding of the protein. Thus it can be seen that replacement of water by other solvents often leads to denaturation.
With the exception of the thesis of Mukherjee\textsuperscript{4-1} there are very few detailed studies of the swelling behaviour of elastin. The first mention of elastin appreciably swelling in any reagent appears to be in the papers of Lloyd\textsuperscript{4-3} and Partridge\textsuperscript{4-4} who reported elastin swelling in formic acid, acetic acid, thioglycollic acid, lactic acid, sodium salicylate, thiourea, zinc chloride and potassium iodide.

The swelling of bovine ligamentum nuchae as a function of pH has been examined\textsuperscript{4-5}. Maximum swelling was found to occur at pH 2.7 and pH 8.5 for both foetal and adult ligament. Above pH 13 there was complete dissolution.

Another study\textsuperscript{4-6} examined the effect of iodine on the water content of bovine ligamentum nuchae after anionic shielding of its positive charges. Iodide atoms have been found\textsuperscript{4-7} to be most effective in increasing the swelling properties of synthetic polyelectrolyte complexes having quaternary nitrogen-containing groups, and this phenomenon has been explained as being due to the site binding of iodide ions with the quaternary nitrogen group\textsuperscript{4-7}. Elastin contains compounds having such nitrogen groups (desmosines) and it was found\textsuperscript{4-6} that isomolar iodide leads to either swelling or shrinking of the elastic tissue depending on the salt concentration and pH of the aqueous medium.

In the general field of polymer science, the studies of swelling and polymer-solvent interactions have attracted much wider study. There are two major concepts currently in use to describe the degree of interaction between a polymer and a solvent.
The first is by use of the factor $\chi$, a parameter defined as a dimensionless quantity which characterizes the interaction energy per solvent molecule divided by $kT$. The $\chi$ factor has been interpreted as consisting of an energy component and an entropy component, and its numerical value gives an indication of the compatibility of a particular solvent with a polymer. Hence $\chi < 0.5$ represents a good solvent for a particular polymer and $\chi > 0.5$ a bad solvent. With regard to the practical application of the $\chi$ factor with elastin diluents, Mukherjee has pointed out the difficulty that it cannot be predicted or calculated but must be determined experimentally in dilute solutions.

The other concept, however, namely that of the 'solubility parameter', $(\delta_s)$ is relatively free from this objection. It is a parameter related to the energy required to separate one molecule from others in its vicinity, and hence can be correlated with the physical constants of the solvent. Any solute-solvent interaction can be described from the free energy concept that

$$\Delta F_m = \Delta H_m - T\Delta S_m$$

($\Delta F_m$ = Free energy of mixing
$\Delta H_m$ = Overall heat of mixing
$\Delta S_m$ = Entropy of mixing)

For reasonably non-polar molecules and in the absence of hydrogen-bonding $\Delta H_m$ is positive and is assumed to be the same as that derived rigorously for the mixing of small molecules. In this case

$$\Delta H_m = \nu_1\nu_2(\delta_{s1} - \delta_{s2})$$

where $\Delta H$ is enthalpy of mixing

$\nu_1$ and $\nu_2$ = volume fractions of the two phases

$\delta_{s1}$ and $\delta_{s2}$ = solubility parameters of the two phases

$\delta^2_s$ = cohesive energy density

= (for small molecules) the energy of vaporization per unit volume
The advantage of using solubility parameters is that $\delta_s$ can be calculated for both polymer and solvent. At a first approximation and in the absence of strong interactions such as hydrogen bonding, solubility can be expected if $\delta_{s1} - \delta_{s2}$ is less than 1.7 - 2.0, but not if it is appreciably larger.

Polymer-solvent interactions are unique in one aspect; generally there is a high gain in conformational entropy as the disentangling action of the solvent on the long polymer molecule bundles increases the number of possible conformations (see Chapter 2) the molecules can adopt. Hence their segmental motion is greatly increased. The entropy change is invariably positive and so the positive or negative sign of the free energy change depends on the relative value of $\Delta H_m$ with respect to the term $T \Delta S_m$. Hence the magnitude of $\Delta H_m$, the heat of mixing, will be a deciding factor in determining polymer-solvent compatibility.

The importance of the interactions of natural biopolymers with different solvents has been reviewed by Singer$^4$-10 who pointed out the special significance in the study of proteins in non aqueous solvents for understanding the nature of the forces which are responsible for the stability of the tertiary structure. Mukherjee$^4$-1 used swelling data of elastin in various solvents to attempt to predict the various bonding contributions (hydrogen bonds, polar bonds, etc.) within the material. For liquid mixtures there are a number of ways that the solubility parameter can be calculated, but for polymers there is no direct method. Mukherjee's method was to measure the swelling of his elastin in a number of different solvents; the amount of swelling is greatest in the liquid which has the same solubility parameter $\delta_s$ as the polymer.

The solubility parameter of a number of other polymers has also been determined$^4$-10,$^4$-11 using the above method. In many of these cases,
however, no consistent relationship was found between the solubility parameter of the solvent and the degree of compatibility of solvent and polymer, implying that the theory needed some modification. Such modification was introduced\textsuperscript{4-13} by an analysis in greater detail of the solubility parameter itself. It was shown that the solubility parameter is a vector composed of hydrogen bonding, polar (orientation and induction) forces and dispersion (London) forces. The modified theory was successfully applied to the solubility of several polymers and resins in solvents and plasticisers.

Mukherjee\textsuperscript{4-1} chose solvent systems specifically to study the role of hydrogen bonding, hydrophobic bonding and ionic interactions in the stability of the structure of elastin. The systems chosen were formamide-water, formamide-alcohol, alcohol-water and sodium iodide-water systems. Mukherjee concluded\textsuperscript{4-1} that normal physiologically saturated elastin is stabilized by a large amount of secondary forces and interactions. Removal of such secondary forces by a suitable solvent such as formamide, leaves the structure in a simple covalently bonded form.

The energy and entropy components of the stress required to characterize the molecular deformation of a polymer network in equilibrium swelling with a diluent are those measured at constant volume and at fixed composition. In practice, however, it is far more convenient to measure stress-temperature co-efficients under equilibrium swelling at constant (atmospheric) pressure and length. A method for converting these constant pressure results to constant volume results must thus be used if a proper interpretation of the fundamental elastic mechanism is to be made\textsuperscript{4-14}.

Early work\textsuperscript{4-15,4-16} on the measurement of stress-temperature co-efficients ignored the changes in swelling of water saturated elastin
with temperature\textsuperscript{4-15}. An attempt was made to circumvent this effect by performing a thermoelastic analysis\textsuperscript{4-17} in a 70:30 v/v water-ethanediol mixture where the total volume of the (unstressed) swollen polymer was found to be independent of temperature. This approach, however, was criticized\textsuperscript{4-18} for reasons concerning solvent-polymer interactions. It was pointed out that the use of multi-component diluents must be considered with caution on account of a possible effect due to solvent disproportion within the specimen at various temperatures. This disproportionation was later demonstrated experimentally\textsuperscript{4-19} by gas chromatography. A 70:30 v/v water-ethanediol mixture at 20°C was found to disproportionate within the specimen to 76:24 at 5°C and 65:35 at 48°C. It was pointed out\textsuperscript{4-19} that occurrence of this compositional change of the diluent must be of particular concern in the case of a protein network such as elastin, where strong selective interactions may drastically alter chain conformation and crystallization\textsuperscript{4-20}. As a result, Volpin and Ciferri\textsuperscript{4-19} performed a series of stress-temperature measurements in water alone. They observed that in water the dimensions of unstressed samples decreased with increasing temperature up to about 50°C and then remained constant to approximately 70°C. The specimens could thus be examined without volume change in the region from 50°C to 70°C. At temperatures below this region they used a thermodynamic correction factor\textsuperscript{4-18}.

Although far less work has been carried out on the swelling properties of elastin than on many of its other parameters, it is true to say that virtually all swelling results so far obtained have been exceedingly useful in adding to the overall knowledge of the subject, considerably more so than many more heavily researched topics. For mechanical studies it is essential to obtain prior data on the swelling properties of the specimens. History has shown\textsuperscript{4-15,4-16,4-17} the erroneous assumptions which can be
drawn without careful examination of such data. It was thus considered that the first step in examination of purified elastin of Chapter 3 must be a basic examination of its swelling properties.

4.3 Swelling Studies

4.3.1 Introduction

The most important studies reported in the literature of elastin relating to the work of this chapter were those of Weis-Fogh and Andersen (described in Chapter 2). Their conclusions constitute one of the main supports of the globular theory of elastin structure - a theory which has made great progress over the last few years towards replacing the classical, random coil theory. At the present moment, however, both theories have their supporters and it remains for time and experimentation to reveal which, if either, of the two is correct.

Weis-Fogh and Andersen used a microcalorimeter to monitor the heat output of specimens of purified ligamentum nuchae in water, in a series of ethanol/water mixtures and in a variety of other solvents, one of which was formamide. In water Weis-Fogh and Andersen observed a heat output approximately four times greater than that expected from the mechanical work put into the specimen. This appeared to contradict the theory of rubber elasticity, but they accounted for this by the postulation of a new structural model 'the liquid drop elastomer' (see Chapter 2).

Weis-Fogh and Andersen's experiments in ethanol/water solutions gave also a steadily decreasing heat output as the proportion of ethanol in the mixture was increased, until at approximately 20:80 v/v ethanol/water the heat output was that predicted by rubber elasticity theory. Further increases in ethanol concentration reduced heat output below the theoretically
predicted value and at approximately 30:70 v/v ethanol/water it became negative (ie: heat input).

Weis-Fogh and Andersen's findings are reproduced in Figs. 4.1 and 4.2. The results of their experiment using formamide and water solvents are shown in Fig. 4.2. It can be seen that the heat output in formamide was considerably less than that in water.

These findings appear very significant and seem clearly to contravene the theory of rubber elasticity. The question must be asked, however, whether it is necessary to postulate a new structural model to explain them. The random coil structure of the classical theory of rubber elasticity has by now accumulated a vast amount of experimental and theoretical backing (eg: see Chapter 2) and elastin would be almost unique in nature if its elasticity did not arise from such a structure. Such argument, however, is not sufficiently powerful to attempt to prove or disprove any theory, and so it was considered necessary to perform experiments on elastin under similar conditions to those of Weis-Fogh and Andersen to see if their results could in any way be re-interpreted in terms of the classical rubber elasticity theory.

It was considered that the first step in this task would be to examine the swelling properties of the material in solvents of the same type as used by Weis-Fogh and Andersen, and see if these could in any way have affected their results. In order, however, to perform any swelling measurements, it was first necessary to construct a suitable rig, a task described in the next section. The simplest and most accurate method of measuring swelling is to measure liquid uptake by weighing. If necessary, this can then be converted to volume using density measurements. Such density measurements are described in a later section of this chapter.
Fig. 4.1

Plot of ethanol content in ethanol/water mix against total heat output over a standard strain cycle on purified elastin specimen.

After Weis-Fogh and Andersen⁴⁻²¹

Fig. 4.2

Plots of differential heat outputs against time, during strain cycle for water and formamide solvents.

After Weis-Fogh and Andersen⁴⁻²¹
Fig. 4.1.

Fig. 4.2.
4.3.2 Design and Evaluation of Temperature-Swelling Rig

Several considerations were necessary in the design of such a rig. It had to be able to maintain the specimen at as constant a temperature as possible with minimum fluctuation. The temperature had to be fully and easily controllable from water freezing point to approximately 80°C. It had to be able to hold the specimen immersed in corrosive liquids, and to prevent excessive evaporation at any temperatures and over long periods of time. Finally it had to be able to be easily dismantled for cleaning.

The design adopted is depicted in Fig. 4.3. A 'Pyrex' reaction vessel (A) was mounted inside a stainless steel thermostatically controlled water bath (B). Four sealable inlets at the top of the vessel were used to admit

(i) a standard mercury/glass thermometer (C)
(ii) a manually operated stainless steel stirring paddle (D)
(iii) a stainless steel specimen wire and hook cemented to a tight fitting glass stopper (E)
(iv) a water-cooled 'jacket-and-coil' type condenser (F)

The heating bath (B) contained an electric stirrer (G) and the heating element (H) leading directly from the control box (I), which in turn was controlled by an adjustable mercury/glass triggering thermometer (J). On the side of the control box was positioned the on/off switch plus a normal/boost switch. The 'boost' position supplied an increased current to the heating element giving a higher heating rate, though with a subsequently increased thermal fluctuation in the bath when cycling. It was, however, found to be useful for rapidly increasing bath temperature, which it could raise at approximately 3°C/minute.
The swelling rig.
For cooling, a mains water-cooled copper coil (K) was also incorporated and was found to be highly effective, being able to cool the bath at approximately 3-5°C/minute. The surface of the bath was insulated with a layer of 1 inch diameter floating, expanded polystyrene balls (L).

To evaluate the rig's capability for maintaining constant composition and temperature a 50:50 v/v ethanol/water solution was carefully mixed and its composition accurately determined by density measurements at 20°C (3 measurements) using a specific gravity bottle and density/composition data from tables (Handbook of Chemical Physics, CRC, USA). The solution was then placed in the inner cavity of the rig, the lid sealed with wire clips (M), condenser turned on and the bath heated to 80°C on 'boost'. On achieving this temperature the element was switched to normal and the bath left to cycle around this temperature for one hour. At the end of this period the solution in the inner cavity was well stirred and two samples withdrawn with the aid of a Pasteur pipette, and put aside in sealed containers to cool to 20°C for further density measurements. At the same time continual stirring and monitoring of the solution temperature was commenced and continued for approximately 30 minutes. Over this period the limits of the cycle were observed to be less than ± 1°C.

Two density measurements were performed on each of the two samples withdrawn from the inner cavity. The densities measured were within ± 1% of the value of the initial solution, although as a check the bath was left for a further hour and the test repeated - with a similar result.

The specimens of elastin used were from the purified porcine aorta, cut perpendicularly to the axis of the vessel into rings of approximately 3 mm width. Their natural thickness was approximately 2 - 4 mm and their diameter 10 - 14 mm. These rings could be hung in the solution from the stainless steel wire specimen hook.
The method of weighing the specimens was to quickly withdraw the stopper and wire (replacing immediately with another stopper), quickly dab off surface liquid with absorbent tissue paper, and drop the specimen on to the weighing pan of a set of microbalances. Speed was essential between removing the specimen from the solution and placing on the pan in order to minimize cooling with its consequent loss or gain (from droplets on the specimen surface) of liquid. In practice, however, this stage could be completed in around 3 seconds. The technique found to be most efficient was to bring the specimen straight up and into a fold of the tissue paper, at the same time as slipping it from the wire hook. The specimen in the tissue was then transferred to the adjacent microbalances and the specimen shaken out on to the pan. From this stage onwards speed was less important, as any liquid lost from the specimen simply remained on, and was weighed with, the pan. The method of transferring the specimen within the tissue paper had the advantage that the removal of surface liquid occurred almost automatically during the transfer and also that it provided a certain degree of thermal insulation to the specimen. As an added precaution, when the bath was being used above 30°C the tissue paper was preheated by holding the appropriate area in contact with the warm surface of the top of the reaction vessel for about a minute.

To test the method, a fine copper-constantan thermocouple was constructed and the hot junction gently pushed into the centre of an elastin specimen. This was then placed in a bath of water in the swelling rig at 80°C for approximately 5 minutes before being removed and held in a piece of the pre-heated absorbent tissue paper for several more minutes. During this cycle the temperature at the hot junction was continuously monitored and this is shown in Fig. 4.4. Although this experiment was of a basic nature and only measured specimen core temperature, it was
Fig. 4.4

Diagram of specimen with embedded thermocouple and plot of data produced. Core temperature measured by thermocouple after: (1) immersion of specimen in bath at 80°C, and (2) removal from bath. Cooling curves shown for both pre-heated tissue drying and non-pre-heated tissue drying methods.
Figs. 4.4.

Evaluation of 'dab-weighing' method [see text].
nevertheless useful in demonstrating the order of magnitude of the cooling effect present in the most severe case to be encountered. The normal 3 seconds taken to transfer specimen to balances appeared to be within the time interval before any major temperature drop occurred. A similar experiment carried out with non-preheated tissue paper gave a faster cooling rate (see Fig. 4.3) although the main drop seemed still to occur outside the 3 second mark. Elastin appeared to be a good thermal insulator.

From the above test it was concluded that the method and apparatus were suitable for further experimentation.

4.3.3 x-Plots

4.3.3.1 Theory

This first experiment was designed to determine the sign and relative magnitudes of the partial molar heats of solution \( \overline{\Delta H} \) of elastin in the various solvents used by Weis-Fogh and Andersen, under the restraint of constant length.

This term \( \overline{\Delta H} \) consists of a mixing term \( \overline{\Delta H_m} \) and an elastic term \( \overline{\Delta H_{el}} \). In many cases the latter term will be minor, and in such cases \( \overline{\Delta H_m} \) can be equated to the heat of mixing only\(^\text{4-22}\).

Abe and Prins\(^\text{5-23}\) have derived the following equation to describe the partial molar heat of solution of a swollen filament in swelling equilibrium with a diluent:

\[
\overline{\Delta H} = -T \left( \frac{\partial f}{\partial N_i} \right)_{P,1,T} \left( \frac{\partial N_i}{\partial T} \right)_{P,1,eq} - \left( \frac{\partial N_i}{\partial l} \right)_{P,T,eq}
\]

where \( F \) is force, \( l \) length, \( T \) temperature and \( \text{eq} \) signifies that the specimen is in equilibrium with a reservoir of diluent from which it has absorbed \( N \) moles of diluent \( i \).
Of the three partial derivatives in equation 4.1, one only \( \frac{\partial N_i}{\partial T} P, l, eq \) is of particular interest, and this can be examined by plotting liquid uptake vs temperature curves as described subsequently. The other two \( \frac{\partial f}{\partial N_i} P, l, T \) and \( \frac{\partial N_i}{\partial l} P, T, eq \) can be assumed to be negative and positive respectively. They will be discussed in greater detail in Section 4.6. It should be stated here, however, that such an assumption is in accord with observations on other swollen polymer systems \(^{4-23}\).

4.3.3.2 Method

The method was aimed at the computation of a parameter, \( x \), where

\[
x = \frac{(m_T - m_{T_0})}{m_{T_0}}
\]

\( m_T = \) mass of saturated specimen at temperature \( T \)
\( m_{T_0} = \) mass of saturated specimen at reference temperature \( T_0 \)

The reference temperature \( T_0 \) was taken as \( T_0 = 20^\circ C \) and the parameter \( x \) thus represented a normalized measure of the mass increase or decrease per unit mass (at \( 20^\circ C \)) of the specimen with temperature. Curves plotted of \( x \) vs \( T \) in the different mixtures could thus be directly compared.

Specimens of porcine aorta rings were cut from region 2 of the aorta perpendicularly to the axis of the vessel. Their approximate dimensions were 3 mm wide x 3 mm thick x 12 mm diameter. Four specimens were taken (A, B, C and D) and two of these (C and D) stretched over two pre-weighed, stapel-shaped stainless steel wire frames. These were sized to give the specimens approximately 30-40\% strain and hence impose a constant length 1 on the rings throughout the experiment. The weighing method employed was as described in section 4.3.2 and relied on rapid absorbent tissue paper drying and
transfer to microbalance pan. At each temperature, after an hour's equilibration, five weighings were performed at 10 minute intervals in order to check for constancy of measurement. In no case were any upward or downward trends observed during the readings and consequently the mean of the five readings were taken in each case to give \( m_T \).

In order to obtain low temperature readings in the region 0–5°C crushed ice was used in the outer bath. For a reading between 5°C and room temperature the copper cooling coil could be used on its own. The mains water temperature was found to be within the range 5–15°C.

The diluents used in the experiment consisted of distilled water 10:90, 20:80, 30:70 and 50:50 v/v percentages ethanol/water solutions and pure formamide. These represented the solvents of most interest used by Weis-Fogh and Andersen\(^4-21\).

The standard experimental procedure for each specimen in each solvent was as follows:

(i) Solvent of known composition at room temperature placed in inner cavity and specimen hung on specimen hook; specimen gently lowered into solution and left for 2 hours to equilibrate;

(ii) Series of 5 dab-weighings taken at 10 minute intervals;

(iii) Inner bath surrounded with crushed ice/water mix and left one hour for specimen and temperatures to equilibrate; then as (ii);

(iv) Cooling coil turned on and bath left one hour to equilibrate;

(v) Heating element used to obtain heating stages up to around 50°C at approximately 10°C intervals;

(vi) Cooling coil used to return back through 10°C stages, thus completing the cycle;

(vii) Solvent changed for fresh of the same batch and the cycle repeated with a new specimen.
4.3.3.3 Results

Results were obtained by first plotting $m_T$ vs $T$. From this curve $m_{TO}$ (20°C) could be obtained and used on the original data to obtain the $x$ vs $T$ plot.

From these plots it became immediately apparent that the result from the specimens under strain was (within the random scatter of data) identical to that from the specimens under zero strain. Thus to within the precision of the measurements (within ± 5%) it could be concluded that $(\frac{dx}{dT})_{P, eq}$ was independent of strain within the range 0% to 30-40% and hence that

$$\left(\frac{\partial N_i}{\partial T}\right)_{P, 1, eq} \propto \left(\frac{dx}{dT}\right)_{P, eq}$$

The results are depicted graphically in the $x$ vs $T$ plots of Fig. 4.5.

4.3.3.4 Result Analysis and Discussion

The swelling of elastin with temperature can be clearly seen in the $x$ vs $T$ plots of Fig. 4.5. $(\frac{dx}{dT})_{P, eq}$ is seen to decrease progressively from its value in 50:50 ethanol/water, through 30:70 to achieve zero at 20:80. It then becomes negative, increasing through formamide to 10:90 ethanol/water and finally to water itself which exhibits the greatest de-swelling tendency with temperature.

It is hence interesting to compare the data with the composition vs heat output plot of Weis-Fogh and Andersen reproduced in Figs. 4.1, 4.2. If the slopes of the $x$ vs $T$ curves are measured at 20°C, a parameter, the volume expansion co-efficient $\beta_V$ can be derived, defined as

$$\beta_V = \frac{1}{V_s} \left(\frac{dV_s}{dT}\right)_{P, 1, eq} = \left(\frac{dlnV_s}{dT}\right)_{P, 1, eq}$$

where $V_s$ = the volume of the saturated specimen.
Plot of mean $x$ values obtained from temperature-swelling experiment in water. $x$ is defined as the difference in saturated specimen mass at temperature $T^\circ C$ (per unit mass at $20^\circ C$) and saturated specimen mass at $20^\circ C$ (per unit mass at $20^\circ C$) or:

$$x = \left(\frac{m_{T^\circ C}}{m_{20^\circ C}}\right) - 1$$

The curves thus give a normalized representation of the variation of swelling with temperature of purified porcine aortic elastin.
Fig. 4.5. $x$ vs. $T$ plots.

- $\triangle$ = water
- $\nabla$ = 10 eth/90 water
- $\square$ = 20 eth/80 water
- $\triangle$ = 30 eth/70 water
- $\bigcirc$ = 50 eth/50 water
- $\blacksquare$ = formamide
can be related to $\beta_v$ as follows:

\[ \rho_s = \text{apparent density of saturated specimen} = \frac{m_s}{V_s} \]

\[ \Rightarrow \ln \rho_s = \ln m_s - \ln V_s \]

Owing to the high water content of the composite, $\rho_s$ will only vary slightly with temperature, so can approximate:

\[ \frac{d\ln \rho_s}{dT} = 0 \]

hence

\[ \frac{d\ln m_s}{dT} = \frac{d\ln V_s}{dT} = \beta_v \]

Now in the x plot, x is defined as:

\[ x = \frac{m_T - m_{T_0}}{m_{T_0}} \]

\[ \Rightarrow \frac{dx}{dT} = \frac{1}{m_{T_0}} \frac{dm_s}{dT} \]

At 20°C, however, $m_{T_0} = m_T$

so

\[ \frac{dx}{dT} = \frac{d\ln m_s}{dT} = \beta_v \]

If the experimentally observed $\beta_v$ values are plotted against the heat output values observed by Weis-Fogh and Andersen, a linear plot is obtained, depicted in Fig. 4.6. This curve has a correlation of 0.95. Although Weis-Fogh and Andersen make no mention of the temperature at which their calorimetry was performed, there is no indication that it was at any but room temperature, in which case 20°C would be a reasonable approximation to their conditions. Furthermore, Mistrali et al.\(^{27}\) have measured $\beta_v$ in water for nuchael elastin and obtained the result at 20°C as $-14 \times 10^{-3}$ °C\(^{-1}\). Excellent agreement was thus observed between their result and the value of $13.4 \times 10^{-3}$ °C\(^{-1}\) obtained from the 'x-plot' (Fig. 4.6). These data presented a powerful validation of the method.
Plot of calorimetrically obtained heat output values of Weis-Fogh and Andersen\(^4\)-\(^21\) in various solvents and solvent mixes against volume expansion coefficients in corresponding solvents obtained from experiments described in section 4.3.3.3. The excellent correlation of the two parameters suggests a strong connection between volume change and heat output in saturated elastin.
Fig. 4.6. [see text.]
Examination of the data with reference to the Abe and Prins relationship where:

\[
\Delta H \propto \left( \frac{\partial N_i}{\partial T} \right)_{P,1,eq}
\]

shows that \( \Delta H \) the partial molar heat of mixing changes from a large negative value for water, to smaller negative values for 10:90 ethanol/water and formamide to increasingly positive values for 30:70 and 50:50 ethanol/water. By thermodynamic convention, a negative \( \Delta H \) implies a heat release while a positive, a heat uptake, precisely as observed by Weis-Fogh and Andersen.

This data thus allows a complete re-interpretation of the results of Weis-Fogh and Andersen in terms of standard rubber elasticity theory. The hypothesis can be put forward that these two workers neglected the effect of a stress-induced volume change in their specimens during the straining process. This volumetric change caused a small uptake of the surrounding solvent, with the consequent generation of heat of mixing \( \Delta H_m \) of positive or negative nature, depending upon the solvent. If the hypothesis is correct, then the postulation of a new type of molecular structure is unnecessary to explain their experimental observations.

Detailed discussion on the subject is confined to a later section in the chapter.

4.3.4 Additional Experiments

4.3.4.1 Absolute Liquid Uptake Tests

This experiment was designed to examine the absolute liquid uptake of aortic elastin samples in both the solvents used in the x-plots and various others at 20°C.
The solvents used were:

(i) Distilled water
(ii) Ethanol/water solution as per x-plots
(iii) Pure formamide
(iv) 70:30 v/v Water/ethanediol
(v) 0.1M NaOH solution
(vi) Methanol
(vii) Physiological (0.9%) Saline

The object was to obtain a comparison between various swelling agents and the parameter used to do this was 'α%' as defined in Chapter 3.

Three specimens were used for each solvent, being taken from regions 1, 2 and 3 of the porcine aorta respectively. Each of these was equilibrated in the solvent for 3 hours in the swelling apparatus at 20°C. Following this period, 5 dab-weighings were carried out on each specimen at half-hour intervals. They were then placed in the vacuum oven at 100°C and 0.1 torr for 3 hours to dry, and then re-weighed. Following this, as a final precaution, they were returned to the vacuum oven for a further hour and re-weighed at the end of this period to check for constant weight.

The results of this experiment are shown graphically in Fig. 4.7. For each solvent the mean was taken from the individual α% values from each specimen.

4.3.4.2 Drying Curves

This experiment was designed to examine the process of drying of the elastin specimens.

The method was to equilibrate the elastin in a particular solvent at 20°C, then remove, it dab the surface dry and weigh continuously in
a drying atmosphere until constant weight was achieved. The specimen was then vacuum oven dried at 0.1 torr and 100°C for 3 hours before re-weighing plus a further hour for constant weight check. α values were calculated taking the vacuum oven reading as \( m_{\text{dry}} \) and these plotted against time to produce a series of 'drying curves'.

The object of the experiment was to observe any discontinuities in the drying process and examine how near the material would spontaneously approach to complete (vacuum oven) dryness when dried at room temperature and pressure. The two solvents used were formamide and water, and in both cases drying was effected by suspending the specimens from a pre-weighed wire hook attached to the pan hanging-hook of the microbalances. A 250 ml beaker of \( P_2O_5 \) was placed in the balance case one hour prior to the first weighing to ensure a dry atmosphere and room temperature was checked to be 20° ± 1°C.

The curves obtained are depicted in Fig. 4.8.

4.3.4.3 Closed System Experiment

If any material is taken in equilibrium with its surroundings and a force applied such that there is a change in length, there may be an exchange of material with the surroundings - in which case the system is said to be 'open'; or there may be no exchange - in which case the system is said to be 'closed'. The application of heat to a piece of elastin in swelling equilibrium with a diluent will create an 'open' system, liquid being imbibed or exuded from the specimen. It was considered that if a method could be found to 'close' the system, i.e. prevent this transfer of solvent, it could well prove useful to further mechanical experiments to be performed in the thesis.
One possible method of 'closing' a system of elastin equilibrated with water is by surrounding the elastin/water composite with a hydrophobic liquid, thus essentially trapping the water within the material. In order to test out this method, the following experiment was performed:

Three hydrophobic liquids were taken:

(i) Silicone oil (BDH Chemicals, Poole, Dorset),
(ii) n-Decane (BDH Chemicals),
(iii) Perfluorodecalin ('Flutec PP9') (ICI Chemicals, Runcorn, Cheshire)

Specimens of porcine aortic elastin were equilibrated in water at 20°C before being dab-weighed and allowed to dry out for 10 minutes in air. They were then transferred to beakers containing the three hydrophobic liquids at 20°C where they were left for one hour before being re-weighed. In the case of perfluorodecalin it was necessary to hold the specimens on the bottom of the beaker with a small piece of wire gauze to prevent them floating to the surface. After weighing, the specimens were returned to their hydrophobic liquids, but were heated gently on an electric hotplate to 50°C and allowed to remain at this temperature for a further hour before being cooled back to 20°C for a further dab-weighing. The cycle was then repeated, using the higher temperature of 80°C (although this was not done with the n-decane due to excess evaporation - instead a second cycle to 50°C was carried out), and then again to approximately 4°C using a crushed ice/water mix to cool the beakers. Between and after these cycles further dab-weighings were carried out as demonstrated in Fig. 4.9.
'Additional Experiments' Results

Fig. 4.7

Graph showing absolute swelling in various solvents. The swelling parameter used is $\alpha\%$, defined as the ratio of the mass of the solvent within the specimen to the vacuum oven dried mass of the specimen - expressed as a percentage.

Fig. 4.8

Plots of liquid content ($\alpha\%$) of specimens against time for water and formamide saturated elastin specimens drying in air.

Fig. 4.9

Plots of liquid content ($\alpha\%$) against time for water saturated elastin specimens surrounded by three different hydrophobic liquids - perfluorodecalin, silicone oil and $n$-decane.
Fig. 4.7 Absolute swelling [20°C]
Fig. 4.8. Drying curves.
Fig. 4.9. 'Closed system' expt.

- Circle = perfluorodecalin.
- Square = silicone oil.
- Triangle = n-decane.

Temperature cycle:
- 20°C
- 50°C
- 80°C

Air drying [10 min]

Into reagent.
4.3.4.4 'Additional Experiments' Discussion

Although the major issues arising from these experiments are discussed in detail in Section 4.6.1 (General Discussion) of this chapter, some of the more specific points will be summarized here.

The absolute swelling data of experiment 4.3.4.2 can be compared with the results of Mukherjee\(^{-1}\) who determined \(M_C\) (the theoretical molecular weight of the polymer chains between cross-links) from his swelling data using the theory of rubber elasticity. Mukherjee's data was obtained using the equation:

\[
\frac{f}{A} = \frac{\rho RT_v}{M_C} \left( K - \frac{1}{K^2} \right)
\]

where 
- \(A\) = unswollen, unstretched cross-sectional area of specimen
- \(v\) = volume fraction of rubber in the swollen specimen
- \(\rho\) = dry density of the rubber
- \(R\) = gas constant
- \(K\) = extension ratio = \(\frac{\text{swollen length under force } f}{\text{swollen length unstrained}}\)

Now \(v = \frac{\text{vol dry}}{\text{vol swollen}} = \frac{V_{\text{dry}}}{V_s} = \frac{m_{\text{dry}}}{\rho} \frac{m_s}{\rho_s}\)

\(m_{\text{dry}} = \text{mass dry rubber}\)
\(m_s = \text{mass swollen rubber}\)
\(\rho_s = \text{density swollen rubber}\)

Let \(\frac{m_s}{\rho_s} = D\)

\[
\therefore v = \frac{m_{\text{dry}}}{m_s}
\]

Now \(\frac{\alpha}{100} = \frac{m_s - m_{\text{dry}}}{m_{\text{dry}}} = \frac{m_s}{m_{\text{dry}}} - 1\)

\(\Rightarrow \frac{\alpha}{100} + 1 = \frac{D}{v}\)

\(\Rightarrow v = \frac{100D}{\alpha + 100}\)
Now from 4.2

\[ M_c \propto \alpha^{-1/3} \quad \text{(assuming all other parameters constant)} \]

\[ \Rightarrow M_c \propto \frac{1}{3\sqrt[3]{\frac{100D}{\alpha + 100}}} \]

Let \[ 3\sqrt[3]{100D} = D^1 = \text{const} \]

Then \[ M_c \propto \frac{1}{\sqrt[3]{\alpha + 100}} \]

Hence \( M_c \) should be proportional to \((\alpha + 100)^{1/3}\) and in Fig. 4.10 this appears to be the case where \( M_c \) values from Mukherjee's work are plotted against \((\alpha + 100)^{1/3}\). The resulting curve has a correlation of 0.958.

Experiment 4.3.4.3 demonstrated that the drying processes for water and formamide were both continuous. There did, however, appear to be a great difference in affinity between the two solvents. The water saturated specimens dried out in air over the time period of the experiment to within a few per cent of their vacuum dried values, whereas the formamide saturated specimens retained a high percentage of solvent. Furthermore, at the end of the experiment - before the vacuum oven treatment, the formamide saturated specimens were highly rubbery, whereas the water specimens had dried out to their brittle dry state. The initial drop in the formamide curve was attributed to the evaporation of the macrovoid liquid, the dissolved formamide remaining highly chemically bound to the elastin molecules only being eventually removed by the heat and reduced pressure of the vacuum oven.

Experiment 4.3.4.3 demonstrated the feasibility of using a hydrophobic liquid to maintain a 'constant' state within the specimen when working with water as a solvent. The most efficient of the
Fig. 4.10

Plot of molecular weight between cross-links of an elastin chain (as determined by Mukherjee$^{-1}$) against the coefficient $(\alpha + 100)^{\frac{1}{3}}$, for different solvents.
Fig. 4.10. [see text.]

1 = water.
2 = 10% ethanol.
3 = 20% ethanol.
4 = 30% ethanol.
5 = 50% ethanol.
6 = methanol.
7 = formamide.
three liquids used appeared to be the Flutec PP9 (Perfluorodecalin) giving the minimum variation of weight readings, all of which were within the ± 5% experimental error of the dab-weighing method. Flutec PP9 also had the added advantages of very little change in viscosity over the temperature range (unlike the silicon oil which became very glutinous at the lower temperatures, making dab-weighing difficult) and high stability with low evaporation at the higher temperatures (unlike the n-decane).

Probably the ideal material with which to create a closed system would be mercury. The hazards of its use, however, outweigh its value by creating a serious safety problem. Flutec PP9 was reported by the manufacturers as being totally non-toxic.

4.4 Density Measurements

4.4.1 Introduction

Elastin in its swollen state can be considered in one of two ways:

(i) It can be regarded as a dry material swollen by a diluent to a new 'unnatural' thermodynamic state, i.e. in a similar fashion to the way in which the swelling of natural rubber in benzene might be regarded; or

(ii) it can be considered as a special liquid/protein composite-rubber in its natural condition, each phase being necessary for the mechanical functioning of the material as a whole.

In this second case, it is the bulk properties of the composite which are important, not one or other of the constituent phases individually. Hence it is the density of the bulk saturated composite which is important for relating swollen mass measurements to swollen volume.
Although density measurements on the specimens were not specifically required for the work of this chapter, they were nevertheless included at this point as they were considered to be more appropriate to the subject matter of this chapter than the next. It was, in fact, in the processing of the data of Chapter 5 where they were specifically required, as will be seen in due course.

The density of dry elastin has been measured to be $1.23 \text{ kg/l}$ at $20^\circ\text{C}$, which is somewhat lower than other fibrous proteins. Wool, for example, is $1.38 \text{ kg/l}$ and collagen $1.32 \text{ kg/l}$. There appear, however, to be no values quoted in the literature for the density of elastin/water, elastin/water-ethanol and elastin/formamide composites at $20^\circ\text{C}$.

Two methods of measuring density were used for greater accuracy and to serve as a cross-check on each other. These are described below:

4.4.2 Methods

4.4.2.1 Density Bottle Method

Using a standard 50 ml density bottle:

(a) Theory

Let $m_1 = \text{mass of liquid in bottle filled with liquid}$

$\quad m_2 = \text{mass of liquid + specimen, in bottle filled with liquid and specimen}$

$\quad m_s = \text{mass of saturated specimen}$

$\quad V_s = \text{volume of saturated specimen}$

$\quad V = \text{volume of bottle}$

$\quad \rho_v = \text{density of liquid}$

$\quad \rho_s = \text{density of liquid/elastin composite}$
Now \( m_1 = \rho_v V \)

\[
m_2 = \rho_v (V - V_s) + \rho_s V_s
\]

So \( m_2 = m_1 + V_s (\rho_s - \rho_v) \)

But \( \rho_s = m_s / V_s \)

\( \Rightarrow V_s = m_s / \rho_s \)

So \( m_2 - m_1 = m_s \left( \frac{\rho_s - \rho_v}{\rho_s} \right) \)

Now let \( m_2 - m_1 = \Delta m \)

\[
\therefore \frac{\Delta m}{m_s} = 1 - \frac{\rho_v}{\rho_s}
\]

or \( \frac{\rho_v}{\rho_s} = 1 - \frac{\Delta m}{m_s} = \frac{m_s - \Delta m}{m_s} \)

So \( \rho_s = \frac{m_s \rho_v}{m_s - \Delta m} \) \hspace{1cm} (4.3)

All the quantities on the right hand side of the equation are easily measurable: \( m_s \) by dab-weighing, \( \rho_v \) by a separate density bottle measurement or by tables, and \( \Delta m (= m_2 - m_1) \) by simple weighing of the dry density bottle, and bottle + liquid, and bottle + liquid + specimen.

(b) Method A number of specimens were cut from region 2 or 1 of three purified aortas of dimension approximately 5 mm x 5 mm x 3 mm. Solutions of glass-distilled water 10:90, 20:80, 30:70 and 50:50 v/v ethanol/water, and pure formamide were made up and allowed to attain 20° ± 1°C (room temperature). A 50 ml density bottle was taken and an accurate determination of its volume obtained using distilled water at 20°C as a standard 1,000 g/ml. Five such determinations were made and the mean taken. The following process was then carried out on three specimens for each solvent.
(i) The specimens were allowed to equilibrate for 2 hours in the swelling apparatus at 20 ± 1°C. Five weighings were then carried out at half-hour intervals, and the mean of these taken to establish \( m_s \).

(ii) The density bottle was then filled with solution from the swelling apparatus and weighed. This was repeated twice and the mean of the three readings taken to establish \( m_v \) and hence \( \rho_v \).

The specimen was placed in the solvent within the density bottle by gently pushing through the top with a pair of forceps, and the combination left for 30 minutes to ensure complete equilibration (topping up the density bottle if necessary). Weighing of this yielded \( m_2 \) although again the solvent was changed twice and the mean of the three weighings taken to increase the accuracy of the method.

4.4.2.2 Archimedes Method

(a) Theory

Using a method of specimen suspension on a wire in the solvent:

Let \( m_3 = \) mass of specimen + wire (suspended in air)

\[ m_a = \text{(apparent) mass of specimen + wire suspended in solvent} \]

\[ m_a = \text{mass of wire} \]

\[ m_s = \text{mass of saturated specimen} \]

\[ V_s = \text{volume of saturated specimen} = \frac{m_s}{\rho_s} \]

\[ \rho_v = \text{density of liquid} \]

\[ \rho_s = \text{density of specimen} \]
So \( m_s = m_B + M \)

\[ m_h = (m_B - \text{upthrust on specimen}) + (M - \text{upthrust on length } L^a \text{ of wire}) \]

Assume surface tension effects to be negligible

\[ m_h = (m_B - \rho_s V_s) + (M - \pi r^2 L^a \rho_v) \quad r = \text{radius of wire} \]

For wire used \( M = 1.7 \text{ mg} \)

\[ r = 125 \text{ micrometers} \]

\[ L^a = 20 \text{ mm} \]

So \( \pi r^2 L \rho_v \approx 80 \text{ microgram} \), which, compared with the typical 200 mg reading, is negligible.

Thus \( m_h = (m_B - \rho_s V_s) + M \)

But \( V_s = m_s / \rho_s \)

So \( m_h = m_s \left( 1 - \frac{\rho_v}{\rho_s} \right) + M \)

Remembering \( m_s = m_3 - M \)

\[ m_h = (m_3 - M) \left( 1 - \frac{\rho_v}{\rho_s} \right) + M \]

\[ \Rightarrow \frac{\rho_v}{\rho_s} = 1 - \frac{m_h - M}{m_3 - M} \]

\[ = \frac{m_3 - m_h}{m_3 - M} \]

\[ \Rightarrow \rho_s = \frac{(m_3 - M)\rho_v}{m_3 - m_h} \]

(b) Method

An approximately 50 mm length of .25 mm diameter stainless steel wire was taken, its ends hooked over and attached to the pan-hanging hook of a set of standard microbalances. It was then weighed to obtain \( M \). Ring specimens were cut from pieces of region 2 aorta approximately 3 mm x 2 mm thick x 12 mm diameter, and allowed to equilibrate in the first solvent (water) for 2 hours in the swelling rig at \( 20^\circ \pm 1^\circ \text{C} \). One of these was then taken and gently
placed on the lower hooked end of the stainless steel wire, and, with the balances in the 'caged' position, surface liquid was dabbed off using absorbent tissue. A reading was then taken to obtain a value for $m_3$. A 50 ml beaker was filled with the solvent in which the specimen had equilibrated and this was gently raised around the specimen and on to a small wooden stand straddling (though not touching) the balance pan. The length of submerged wire was measured to be 20 mm. This arrangement was allowed to stand for 5 minutes before the balances were uncaged and a reading of $m_n$ taken. The beaker was then removed, the balances recaged and the specimen dabbed dry for a second $m_3$ and $m_n$ weighing cycle. In all the process was repeated five times, and the results averaged to give the final $m_3$ and $m_n$ values. Throughout these readings no significant upward or downward trends were observed.

In the cases of the solvents other than water, their 20°C densities were determined using the density bottle, and these values compared with published data (Handbook of Chemical Physics). In all cases agreement was within 0.5%.

4.4.2.3 Results

The two experimental methods were found to give results in excellent agreement with each other (see Table 4.1). The data was consequently combined by averaging and these values used as standard densities in subsequent chapters of the thesis.

The results can be seen in Fig. 4.11 in the ethanol/water case, as a function of ethanol content and it was also possible to use the data in conjunction with the absolute swelling liquid uptake data of section 4.3.4.1 to obtain the volume fraction $\nu$ of elastin in the swollen composite (Fig. 4.12) as well as the variable $\nu^{-\frac{1}{3}}$ (see equation 4.2).
### Table 4.1

**COMPARISON OF DENSITIES OF SATURATED ELASTIN SPECIMENS**

MEASURED BY 'DENSITY BOTTLE' AND 'ARCHIMEDES' METHODS

<table>
<thead>
<tr>
<th>Vol % Ethanol in mixture</th>
<th>Density of solvent measured by density bottle method kg/l</th>
<th>Mean densities of saturated specimens measured by:</th>
<th>Mean of (1) &amp; (2) kg/l</th>
<th>Standard deviation from mean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(1) Density bottle method, kg/l</td>
<td>(2) Archimedes method, kg/l</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1.0096</td>
<td>1.0790</td>
<td>1.0780</td>
<td>1.0785</td>
</tr>
<tr>
<td>10</td>
<td>0.9871</td>
<td>1.0774</td>
<td>1.0768</td>
<td>1.0771</td>
</tr>
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<td>0.9752</td>
<td>1.0717</td>
<td>1.0701</td>
<td>1.0709</td>
</tr>
<tr>
<td>30</td>
<td>0.9635</td>
<td>1.0610</td>
<td>1.0590</td>
<td>1.0600</td>
</tr>
<tr>
<td>50</td>
<td>0.9375</td>
<td>1.0456</td>
<td>1.0446</td>
<td>1.0451</td>
</tr>
</tbody>
</table>
Let \( v = \frac{V_{\text{dry}}}{V_{\text{swollen}}} \) = volume fraction of elastin in swollen composite

But \( \frac{\alpha}{100} = \frac{m_{\text{swollen}} - m_{\text{dry}}}{m_{\text{dry}}} \)

\[ \Rightarrow m_{\text{swollen}} = \left( \frac{\alpha}{100} + 1 \right) m_{\text{dry}} \]

However, \( m_{\text{swollen}} = \rho_s V_s \) and \( m_{\text{dry}} = \rho_{\text{dry}} V_{\text{dry}} \)

From ref. 4-24, \( \rho_{\text{dry}} = 1.23 \text{ kg/l} \) \( \rho_s V_s = \left( \frac{\alpha}{100} + 1 \right) 1.23 V_{\text{dry}} \)

\[ \Rightarrow \frac{V_{\text{dry}}}{V_s} = v = \frac{100 \rho_s}{1.23 \alpha + 123} \]

4.5 Void Volume Determination

As mentioned previously, water in saturated elastin can be divided into two categories:

(i) Microvoid water, and
(ii) Dissolved water

Mukherjee\(^4\) determined the void volume of his purified ligamentum nuchae specimens by measuring the rate of diffusion of NaCl from a NaCl solution-treated sample, using conductivity measurements. He also used an optical method. He obtained:

Microvoid (defined by Mukherjee as Macrovoid) water: 28.2 vol % conductivity measurement
36.5 vol % optical measurement
Fig. 4.11

Plots of density of solvent saturated elastin specimens and solvent against solvent composition for ethanol/water solvents.

Fig. 4.12

Plot of volume fraction dry elastin \((v)\) in solvent swollen elastin composites against composition of solvent mixture. Also plotted is the parameter \(v^{-1/3}\) as used in equation 4.2.
Fig. 4.11. Density vs solvent composition.
Fig. 4.12 Volume fraction in ethanol/water mixtures.
The aorta, however, has a very different elastin content, typically:

<table>
<thead>
<tr>
<th>Source</th>
<th>Elastin % of dry fat-free tissue (wt%)</th>
<th>Collagen (wt%)</th>
<th>Other Constituents (wt%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ox</td>
<td>39.8</td>
<td>23.1</td>
<td>37.1</td>
</tr>
<tr>
<td>Rat</td>
<td>47.7</td>
<td>25.0</td>
<td>27.3</td>
</tr>
<tr>
<td>Pig (own work)</td>
<td>39.0</td>
<td>53.0</td>
<td>8.0</td>
</tr>
</tbody>
</table>

as compared with the 60-70% wt% of dry, fat-free ligamentum nuchae\(^4\-29\).

Hence there will be a great difference between the microvoid liquid content of the saturated purified ligament and the aorta.

Estimations of the 'dissolved water' content of elastin have also been made by Mukherjee\(^4\-1\) and Partridge\(^4\-29\), the former using his conductivity method and the latter a method of column diffusion. The values they obtained were:

<table>
<thead>
<tr>
<th></th>
<th>Dissolved water vol %</th>
<th>Dry Elastin vol %</th>
<th>Dry elastin and Microvoid water vol %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mukherjee(^4-1)</td>
<td>19.5</td>
<td>52.2</td>
<td>80.4</td>
</tr>
<tr>
<td>Partridge(^4-29)</td>
<td>17-20</td>
<td>-</td>
<td>80-83</td>
</tr>
</tbody>
</table>

Mean 19.08\%

Although the volume of microvoids will vary with location in the body and animal source depending upon the amount of collagen, smooth muscle and other materials present in the unpurified tissue, the same will not be true of the elastin/dissolved water composite structure (at constant temperature, pressure, volume, etc.). Elastin from various locations and animal sources constitutes the same material\(^4\-30\).

Hence it is reasonable to accept the estimates by Mukherjee and Partridge to give a dissolved water to saturated elastin (without microvoids) ratio of

\[
\frac{19.0}{52.2 + 19.0} = 0.2668 = \text{Fraction of dissolved water in saturated elastin phase}
\]
Using this the microvoid water content of the purified aorta specimens can be estimated. From Table 3.3 (Chapter 3) the α% of the purified porcine aorta in water

\[ \text{α} = \frac{(m_s - m_{dry})}{m_{dry}} \times 100 \]

Let \( m_{dry} = 100 \) mass units, then

\[ m_{wet} = \alpha + 100 = 386.1 \text{ mass units} \]

But from section 4.4, \( \rho_s \) (water at 20°C) = 1.0785

So \[ V_s = \frac{m_s}{\rho_s} = \frac{386.1}{1.0785} = 357.1 \text{ vol units} \]

Also \( m_{dry} = 100 \) mass units

\[ \rho = \text{Ref 4-24} \quad 1.23 \text{ kg.l}^{-1} \]

So \[ V_{dry} = \frac{100}{1.23} = 81.30 \text{ vol units} \]

Therefore the volume percentage dry elastin in the complete water saturated purified aortic elastin composite (including microvoids)

\[ \frac{V_{dry}}{V_s} \times 100 = \left( \frac{81.3}{357.1} \right) \times 100 = 22.7\% \text{ vol} \]

But the volume of dissolved water \( V_{dw} \), using the data of Partridge and Mukherjee (obtained from purified ligamentum nuchae) gives:

\[ \frac{V_{dw}}{V_{dry} + V_{dw}} = 0.2668 \]

\[ \Rightarrow V_{dw} = 0.2668 (81.30 + V_{dw}) \]

\[ = 21.69 + 0.2668 V_{dw} \]

\[ \Rightarrow V_{dw} = \frac{21.69}{1 - 0.2668} = 29.58 \text{ vol units} \]

Hence the volume percentage of microvoids in the complete water saturated purified porcine aortic composite (including microvoids)

\[ \frac{V_s - (V_{dry} + V_{dw})}{V_s} \times 100 \]

\[ = \frac{357.1 - (81.30 + 29.58)}{357.1} \times 100 = 68.95\% \text{ vol} \]
The result can be compared with a typical $\beta$% result (see Table 3.3). $\beta$ represents the dry weight percentage material removed from the elastic tissue during the purification process.

Hence $100 - \beta$ gives the dry percentage (wt) of remaining elastin ($\delta$%).

$\beta = 61.03\%$, so

$\delta = 100 - 61.03 = \text{remaining dry wt } \% \text{ elastin in tissue after purification} = 38.97\%.$

$p = 1.23\text{kg/l}^{1.24}$

Remaining dry volume percentage elastin = $38.97/1.23 = 31.68\% \text{ vol}$ which leaves $100 - 31.68 = 68.32\% \text{ dry vol } \% \text{ of material other than elastin removed from the tissue.}$

It is reasonable to assume that it will be this volume percentage which will proportionately fill with water to provide the microvoid water. The two derived microvoid values are thus in excellent agreement and an average of the two gives 68.64 vol %.

4.6 Discussion

4.6.1 Re-interpretation of the Results of Weis-Fogh and Andersen$^{4-21}$

Weis-Fogh and Andersen's interpretation of their microcalorimetric experimental results$^{4-23}$ has been one of the main supports of the globular theory of the structure of elastin. It was considered possible, however, in the light of the experimental findings of this chapter to offer a re-interpretation of their published data in terms of the theory of rubber elasticity.

Weis-Fogh and Andersen observed that when, for example, 1.33 m cal of work was done on a specimen immersed in water at room temperature, the
heat evolved was 5.53 m cal: according to the theory of rubber elasticity, the heat evolved should be close to 1.33 m cal. This experiment and others of a similar nature with water-ethanol solutions and formamide as a solvent led Weis-Fogh and Andersen to the conclusion that their calorimetric experiments could not be explained by the classical theory of rubber elasticity which they replaced by an interesting two-phase model, the liquid drop elastomer.

In approaching this anomaly the hypothesis was formulated that the apparent conflict between the theory of rubber elasticity and the calorimetric experiments was due to an erroneous assumption by Weis-Fogh and Andersen that the heat change due to the stress absorption of solvent was negligible.

The variation of the degree of swelling $v^{-1}$ (= volume swollen gel to volume dry polymer) with the tensile force for an amorphous network at equilibrium with an excess of a one-component diluent has been given by Treloar^{4-31}

$$t_1 = \frac{RT}{V_1} \left[ \ln(1 - v) + v + \chi v^2 + \frac{\rho V_1}{M_c} v v_0^{-2} K^2 \right]$$  \hspace{1cm} 4.4

Where $t_1$ = the true stress (force per unit area measured in the swollen unstrained state); $v_0$ is the volume fraction of polymer in the swollen, unstrained state; $\rho$ and $V_1$ are the polymer density and the diluent molar volume respectively; $\chi$ is an interaction parameter (see section 4.2); $M_c$ is the molecular weight of a network chain; $K$ is the extension ratio defined as the length of the sample in the direction of the applied force referred to the unstrained swollen length; $R$ is the gas constant, and $T$ temperature.

Equation 4.4 combines the free energy of dilution (as obtained from the Flory-Huggins theory of polymer solutions\textsuperscript{4-8}) and the elastic free
energy (as obtained from the rubber elasticity theory\textsuperscript{4-31,4-32}). It predicts an increase in the degree of swelling $v^{-1}$ on increasing the strain, and this has been verified experimentally for a number of polymer-diluent systems\textsuperscript{4-31}. Although equation 4.4 is only strictly valid for an amorphous network, Treloar\textsuperscript{4-31} has nevertheless shown an increase in swelling on increasing stress (which is a more significant factor than strain\textsuperscript{4-31}) in fibres such as hair and cellulose\textsuperscript{4-31,4-32}. It consequently appears that any material which exhibits an increase in length upon swelling will generally exhibit an uptake of diluent on the application of stress\textsuperscript{4-31}.

A possible cause of confusion on this topic has been the widely held assumption that elastin deforms as a dry rubber with the consequent validity of an 'incompressibility'\textsuperscript{4-33} assumption. The Poisson's ratio of dry rubbers is normally in the region of 0.5. With elastin, however, this is not the case. To maintain its rubbery condition at all elastin must be deformed both in vivo and in vitro in the diluted state. If a diluted rubber is deformed in the presence of diluent, then the bulk modulus is much lower than that of a dry rubber\textsuperscript{4-34} (typically $10^2$GN/m\textsuperscript{2}).

Dorrington\textsuperscript{4-34} has clearly explained the two situations -

'The reason for the difference between the bulk modulus of dry and diluted rubber is as follows. In the dry case, the volume can comply with the hydrostatic stress only by moving polymer molecules on average closer (or further apart); the forces opposing this are extremely high, which leads to negligible volumetric strains (Poissons ratio approximately 0.5). But if the rubber contains a diluent and is immersed in diluent then it may comply with an imposed hydrostatic tension by absorbing (or rejecting) diluent. For example, when natural rubber is swollen with petroleum ether and is then stretched while immersed in petroleum ether from 150\% of its
initial unstretched length to 250%, the swollen volume increases by 17%. (Gee G. Trans. Farad. Soc. 428 (1946) 33).

From these arguments it can be seen that to expect an uptake or release of solvent from a saturated specimen of elastin on the application of a tensile stress or strain is entirely reasonable, considerably more so than the opposite assumption.

This information can thus be considered in the light of the Abe and Prins equation (equation 4.1) which includes the term \( \frac{\partial N_1}{\partial l} P, T, eq \), a representation of the change in saturation with length at constant pressure, temperature and equilibrium swelling. From the above arguments, the sign of this term can be concluded to be positive.

As \( \frac{\partial N_1}{\partial T} P, l, eq \) has already been discussed (section 4.3.3.3), there remains in the equation one last term, the sign of which should be determined to complete the argument: this is the \( \frac{\partial F}{\partial T} P, l, T \) term, representing the change or force with saturation at constant pressure, length and equilibrium.

This term can be shown to be negative in two ways:

(i) By simply inverting the function to give \( \frac{\partial N_1}{\partial T} F, P, l, T \) (ie: equation 4.4) and reverting to the arguments in the initial part of this discussion, and

(ii) By performing the simple experiment described below.

A piece of saturated elastin was clamped in the jaws of an Instron tensile testing machine at constant length (approx. 30% strain) and temperature \( (20^\circ C \pm 1^\circ C) \) and allowed to slowly dry out. The experiment was repeated using ethanol/water and formamide diluent and the force vs drying curves qualitatively noted. In all cases they were found to be negative.
The Abe and Prins equation can thus be seen to support the re-interpretation of Weis-Fogh and Andersen's work.

That the swelling vs. temperature plots (x-plots) themselves were a valid measure of swelling was shown by the excellent agreement of the $\beta_v$ value obtained at 20°C with that of Mistrali et al.\textsuperscript{14-27}.

The most significant part of the argument, however, is the excellent linear correlation of the x-plot $\beta_v$ data with the heat output results of Weis-Fogh and Andersen (Fig. 4.6).

4.6.2 General Discussion

In the light of the new interpretation of the experimental data of Weis-Fogh and Andersen, it is interesting to put down precisely what information on porcine aortic elastin has been established in this chapter.

Firstly, Section 4.5 gave a qualitative indication of the composition of the composite in its water saturated condition. Two separate phases can be considered to be present:

1. A rubbery saturated water/elastin phase containing approximately 23\% vol molecular (dry) elastin, approximately 8\% vol associated molecular water, and

2. Free water lying outside the molecules unassociated with them in any chemical way, occupying inter-fibre and void areas where non-elastin constituents have been removed during the purification process. This free water constitutes approximately 69\% vol of the total water saturated volume at 20°C.

On raising or lowering the temperature of an elastin specimen immersed in a diluent, the saturated rubbery phase will imbibe or exude liquid out of the specimen into the surrounding free water, thus altering its liquid content to some new value depending upon the temperature. This change of saturation will create a volume change within the
fibres which will in turn cause a volume change in the bulk dimensions of the specimen, and cause it to take up or release some of its free water. The importance of the liquid uptake or release has been demonstrated in the data of section 4.3.3 and in the re-interpretation of the data of Weis-Fogh and Andersen.

The comparative swelling effects of some of the solvents were examined in experiment 4.3.4.2. The swelling in physiological saline was found to be virtually identical with that of distilled water, although the other solvents ranged in swelling efficiency from 30:70 v/v ethanediol/water to a maximum in formamide. It is interesting to note that in the case of the ethanol/water mixtures a minimum swelling was encountered in 20:80 ethanol/water, and this was similar in value to the results in the 30:70 ethanediol/water and 40:60 glycerol/water mixtures. The literature\textsuperscript{15-17} cites elastin in 30:70 v/v ethanediol/water as having zero swelling with temperature and 20:80 v/v ethanol/water has been shown to be similar from the work of this chapter. 40:60 glycerol/water also is shown in a later section of the thesis to be a mix giving zero swelling with temperature.

Elastin contains both polar and non-polar sites in its molecular structure. Water molecules will interact with the polar sites while the relatively less polar ethanol, ethanediol and glycerol will react with the non-polar parts of the chain. In solution together, ethanol (and ethanediol and glycerol) and water interact with each other exothermically to form molecular complexes. It is probable that 20:80 constitutes the optimum proportions of ethanol and water to fill all polar and non-polar interaction sites on the elastin molecules. If the proportion of ethanol is greater than 20 vol % excess, ethanol will be brought into regions of hydrophilic interaction where they will tend to be repulsed.
Conversely, in less than 20 vol % ethanol, excess water will be repulsed from the hydrophobic sites. Both these conditions will have a swelling effect upon the rubbery composite, an effect which increases as the ethanol/water composition moves away from 20:80 v/v (or the ethanediol from 30:70 or the glycerol/water from 40:60).

Kauzman has calculated that for every non-polar hydrophobic side-chain of a protein that is removed from an aqueous to a non-polar environment the protein gains an extra 4 kcal of free energy stabilization. The effect of supplying heat to the material will thus be to increase the non-polar activity of the hydrophobic groups which will have the effect of rejecting excess water from or accepting excess alcohol into the composite. As the water and ethanol in solution are closely associated, rejection of water from the molecules will at the same time remove associated ethanol, just as acceptance of ethanol will bring in associated water, thus resulting in the overall imbibing and exuding of liquid, depending upon the solvent composition.

At 20:80 v/v ethanol/water all hydrophobic and hydrophilic sites are exactly filled, so there is no excess unbound ethanol associated with the molecules to attract, nor any excess water to repel. This condition thus results in minimum swelling. These effects are observed in the results of experiment 4.3.4.2 where the 20:80 v/v ethanol/water solution is seen to give the minimum swelling. A similar argument will apply to the 30:70 ethanediol/water and 40:60 glycerol/water 'constant swelling' solutions.

The case of formamide, however, is significantly different. Formamide has a high dielectric constant, but according to Mukherjee this has a negligible effect on the elastin structure. Formamide is, however, known as being a strong denaturant of proteins and a swelling agent.
through its disrupting effect on both hydrogen and other secondary bonding mechanisms. Mukherjee claims that formamide, being a strong donor of protons, can interact with and break down both the stabilizing intrapeptide hydrogen bonds and hydrophobic interactions. Conceivably this has the effect of opening out the protein structure leaving bonding almost entirely to the covalent cross-links. The high affinity that elastin has for formamide is demonstrated in experiments 4.3.4.2 and 4.3.4.3. The swelling in formamide is approximately 2½ times greater than in water and 1¾ times greater than methanol or 0.1 M NaOH. On attempting to dry out formamide saturated elastin in dry air, it is observed in experiment 4.3.4.3 that after a certain initial weight drop the saturation seems to remain a constant level indefinitely. Even after two weeks lying in an open beaker in the laboratory the specimens still showed no significant saturation drop (and in fact registered a slight weight increase due probably to some take-up of moisture from the air), and retained their typical translucent-brown formamide saturated appearance. The water specimens on the other hand appeared to dry steadily down to only a few 1% above their vacuum dried values.

The initial drop in the formamide drying curves is undoubtedly due to evaporation of the microvoid formamide which appears to constitute a smaller proportion of the total swollen composite than the microvoid water in the water swollen case. In later sections of the thesis it is shown that elastin swollen with formamide conforms exactly to the physical requirements of an ideal rubber. For this to be the case, and with consideration of the high swelling, it seems likely that the formamide does in fact completely open out the secondary structure of the elastin to leave a system of randomly cross-linked molecular chains with little or no secondary bonding.
The motivation for experiment 4.3.4 arose from a desire for a method to be able to maintain constant saturation within a specimen over a range of temperatures and hence thermodynamically 'close' the system. It was considered that such a method could provide a conclusive decision on the contrasting interpretation of the mechano-calorimetric data of Weis-Fogh and Andersen using their own technique. Quantitative arguments show that for a strain of 30% and a specimen of 100 mg the weight change due to the absorption of water during straining is of the order of 1 mg. This is well outside the precision obtainable with the gravimetric techniques of experiment 4.3.3. However, the anticipated heat change, assuming a reasonable value of $\Delta H^{\Delta r} \approx -10 \text{ cal mol}^{-1}$ is approximately 0.5 cal. This quantity may be measured with considerable accuracy by micro-calorimetry. If the experiment is performed at equilibrium and then at constant saturation, the difference in the observed values of the heat outputs $\Delta Q$ will be a measure of the heat release due to strain induced absorption.

It was considered that the constant 'Nf' experiment could be achieved by immersing a water equilibrated specimen in mercury or a hydrophobic liquid which does not permeate elastin. The biological hazards of using mercury made it more desirable to use a hydrophobic liquid and consequently three such liquids were tried out in experiment 4.3.4.4, one of which, perfluorodecalin, gave the most successful results. Calorimetry, however, using this liquid as a specimen surrounding medium was not attempted for lack of equipment and this might be a useful topic for further work. The 'closed system' method proved useful in a subsequent section of the thesis (Chapter 5).

As a final comment to this discussion, the notable agreement between the general physical parameters of the purified porcine aorta specimens
and the purified bovine ligamentum nuchae specimens used by Mukherjee\textsuperscript{4-1}, Weis-Fogh and Andersen\textsuperscript{4-21} and Mistrali et al\textsuperscript{4-27} should be mentioned. The most striking example of this was the excellent agreement with Mistrali et al\textsuperscript{4-27} on the value of $\beta_v$ (water 20\%). The fact that these workers used ligamentum nuchae whereas the work of the thesis used porcine aorta represents added verification of the assumption that elastins from different locations and animal sources are the same fundamental material.

4.7 Conclusions

1. The liquid within specimens of swollen purified porcine aortic elastin can be divided into two classes:

   (i) Liquid 'dissolved' within and associated with the elastin molecules

   (ii) Free liquid trapped within the void spaces and between the elastin molecules and fibres. In the water-saturated case the dissolved water constitutes approximately 10 volume \%, the solid material approximately 20\% and the microvoid water 70\%.

2. Elastin saturated in solutions of water, ethanol/water and formamide alters its saturation with temperature, the amount of increase or decrease depending predominantly upon the sign and magnitudes of the partial molar heats of mixing of the solvent in the elastin.
3. The application of strain to a specimen of elastin in equilibrium with a diluent causes an uptake of liquid into the specimen resulting in the generation of a positive or negative heat of mixing. This heat generation effect was misinterpreted by Weis-Fogh and Andersen as being due to volume independent structural changes within the material.

4. The effect of different diluents in equilibrium with elastin is to swell the material by differing amounts. Of those solvents investigated, the greatest swelling at 20°C was given by formamide of 787.7% (α%) measurement and the least by 40:60 v/v glycerol/water at 217.5% (α%).

5. Formamide has a considerably greater affinity for elastin than water. Its effect is probably to disrupt the secondary bonding in the material and thus open out or 'unravel' its structure.

6. Density measurements carried out on the swollen elastin/liquid composites in the different solutions by two different methods indicate density values similar to, but not exactly equal to, the solutions with which they are saturated. In the case of ethanol/water mixture the decrease in density with increasing ethanol content is at a rate approximately half that of the density decrease of the mixtures themselves.

7. The swelling properties of elastin from adult bovine ligamentum nuchae and porcine aorta are similar.
5.1 Introduction

In the preceding chapter the hypothesis was advanced that the mechano-calorimetric results of Weis-Fogh and Andersen could be re-interpreted without the need to invoke the globular theory of elastin structure. Experiments to support this re-interpretation were provided by swelling studies on the purified material.

The object of this chapter is not to continue the attack on Weis-Fogh and Andersen's liquid drop model, but rather to examine the alternative assumption which is that elastin conforms to the classical theory of rubber elasticity.

If a polymer sample of unit cross-sectional area is subjected to a constant oscillatory strain, it is possible by measuring the stress in the material as a function of time to obtain a measure of the shear modulus of the material for any particular oscillation frequency and temperature. If the experiment is repeated at different temperatures, a constant-frequency shear modulus as a function of temperature for the polymer may be obtained.

For rubbers and linear amorphous polymers the types of behaviour most often observed in such tests are shown in Fig. 5.1. Four regions of behaviour are commonly observed. At low temperatures where the modulus is typically higher than 1 GN/m\(^2\) the polymer is hard and brittle; this is the glassy region. The glassy shear modulus is a slowly decreasing function of temperature and is a useful parameter to use in characterizing the polymeric behaviour of the material. In this glassy region
Fig. 5.1  Typical rubber modulus-temperature curve.
thermal energy is insufficient to surmount the potential barriers for rotational and translational motions of segments of the polymer molecules. The chain segments are essentially 'frozen' in fixed positions on the sites of a disordered quasi-lattice with their segments vibrating around these fixed positions, much like low molecular weight molecules in a molecular crystal. With increasing temperature the amplitude of vibrational motion becomes greater and eventually the thermal energy becomes roughly comparable to the potential energy barriers to segment rotation and translation. In this temperature region the polymer achieves its 'glass transition temperature' where short-range diffusional motions begin to occur. Segments are free to 'jump' from one lattice site to another; the brittle glass becomes a resilient 'leather'.

The glass transition phenomenon is accompanied by a catastrophic decrease in the modulus of several decades (usually about 3) as indicated in Fig. 5.1. The breadth of the transition region ranges from 5 to more than 20 degrees centigrade, depending on the nature of the polymer in question.

As the temperature is further increased, the modulus again reaches a plateau region, often termed the 'rubbery plateau'. Within this temperature interval the short-range diffusional motions of the polymer segments which initially give rise to the glass transition occur very rapidly. However, long range co-operative motions of chains which would result in translational motions of complete molecules are still greatly restricted by the presence of strong local interactions between neighbouring chains. In the case of a cross-linked rubber, these interactions consist of primary chemical bonds. In the linear polymer, however, the interactions are due to chain entanglements, the precise nature of which is not clear.
The viscoelastic response of linear and cross-linked rubbery polymers through the rubbery plateau region is essentially identical. As the temperature is further increased, however, differences between these two categories become evident (as shown in Fig. 5.1). In the case of the cross-linked 'rubber', as the temperature is increased, the cross-links consisting of primary chemical bonds remain intact, preventing the chains from translating relative to one another. Hence, although the modulus will change slightly with temperature in the rubbery plateau region of a cross-linked 'rubber', the effect is small compared with changes of the type exhibited during the glass transition. Thus, to a first approximation, the modulus will remain constant (or actually increase slightly) for a cross-linked rubber up to temperatures where chemical degradation begins to occur. The situation is quite different for the linear polymer. In this case increasing temperature causes molecular motions to become more large-scale until eventually whole polymer molecules begin to translate. When the temperature is sufficiently high, local chain interactions are no longer of high enough energy to prevent molecular flow, and further temperature increase will, barring chemical reaction, change the sample to a viscous liquid.

The 'rubbery plateau' region of a rubber where its modulus increases linearly with temperature is one of its most characteristic features. For elastin to exhibit such a region would greatly help to establish the validity of treating the material as a classical rubber. It was with this in mind that the work of Chapter 5 was undertaken.
The terms 'relaxation' and 'transition' in the literature are often used synonymously when applied to dispersive regions in the polymer spectrum, whether measured by mechanical means, such as static or dynamic creep or shear experiments, or by techniques such as nuclear magnetic resonance. Thermodynamically a first-order transition involves a discontinuous change in the volume or heat content of the sample, whereas a second-order transition involves a discontinuous change in the temperature derivatives of these qualities, i.e.: the volumetric expansion coefficient or the specific heat. For these reasons some authors refer the word 'transition' with its first-order characteristics, specifically to the glass transition and use the term 'relaxation' for all other dispersive processes.

As mentioned in section 5.1, the glass transition in amorphous polymers is well established, as the region in which the main molecular chains become free to make large-scale conformational rearrangements. Associated with this region is always a mechanical energy absorption or 'mechanical loss' maximum, measurement of which supplies a good definition of the actual glass transition temperature. In the glassy state, however, other secondary loss maxima are observed due to the motions of smaller molecular groups (such as side groups attached to the main molecular chain) or even to limited local motion of the chains themselves.

In semi-crystalline polymers, the assignment of an observed loss peak is greatly complicated by the presence of the crystalline regions. Molecular relaxation may occur either internally in the crystallites, in the amorphous regions, or even in the boundary regions between the two.
A number of contradictory theories have been advanced in recent years, each with the aim of relating these loss peaks to molecular relaxation processes. In most cases, however, the problems are still not fully resolved\(^5-6\).

It is customary to identify the various dispersive regions in the mechanical relaxation spectrum of a particular polymer by Greek alphabetic characters. Hence \(\alpha\) refers to the loss process observed at the highest temperature in low frequency dynamic tests or in long-time creep experiments, while \(\beta, \gamma, \delta, \epsilon\) refer to processes occurring at successively lower temperatures at constant frequency.

Temperature and frequency changes are inter-related. An increase in the measuring frequency or a reduction in the creep time causes a loss process to be observed at a higher temperature than before. Thus a process occurring at just below the melting point of a polymer at a low frequency or long creep time will eventually be shifted above the melting point as the frequency of the measurement is increased. \(\delta\) and \(\epsilon\) relaxations, however, are usually only observed in experiments performed below \(-196^\circ C\) at a frequency of approximately 1 Hz\(^5-2\).

In amorphous polymers the highest temperature relaxation observed is the glass-rubber relaxation, which is consequently labelled \(\alpha\)\(^5-6\). In the case of semi-crystalline polymers relaxations may be observed at both above and below the glass transition temperature (Tg) of the amorphous fraction\(^5-7\), since relaxation arising in the crystalline regions may occur at any temperature below the melting point of the crystallites. Hence the use of the same letters to label relaxations in semi-crystalline polymers and in amorphous polymers does not imply that the same molecular mechanisms are involved in each case. Indeed, the \(\alpha\)-process in crystalline polymers is usually associated with a crystal-related relaxation\(^5-2,5-5\).
Reviews of the literature on the relationship between molecular structure and loss peaks have been published for a wide range of polymers and temperatures and the reader is referred to these for further details.

Mechanical measurements on polymers usually consist of applying a pre-determined external stress to a small sample of the polymer and observing the time and temperature dependence of the resulting deformation. If a tensile stress $\sigma$ is applied, then the resulting strain as a function of time $\varepsilon(t)$ or 'creep' of the specimen can be measured to give the function known as the creep compliance.

$$D(t) = \frac{\varepsilon(t)}{\sigma}$$

where 't' is the creep time, measured from the instant at which the initial stress was applied.

An alternative method, however, is to apply a periodically varying (usually sinusoidal) stress and monitor the resulting deformation. The tangent of the phase difference ($\tan \delta$) between stress and strain can thus be obtained as a measure of mechanical loss in the specimen. In addition, the complex shear modulus $G^*$ may be obtained as well as its two components, the real or storage shear modulus $G'$ and the imaginary or loss shear modulus $G''$. These are related by the formula

$$G^* = G' + iG''$$

A free vibrational method may also be employed in which the specimen is excited by means of an 'instantaneous' torque pulse and the oscillations so induced are allowed to decay naturally. In this case a useful measure of the damping is the logarithmic decrement ($\Lambda$) of the oscillation peaks. Such methods include the torsion pendulum method.
where the specimen is fixed at its lower end while its upper end is attached by means of a connecting rod to a balanced T-piece. Application of a torque to the T-piece applies a shear force to the specimen, and if the torque is suddenly released, the T-piece will twist back and forth in decreasing cycles according to the viscoelastic condition of the specimen.

Another experiment which is frequently used is the resonance type where the specimen, in the shape of a thin reed, is caused to vibrate at its natural frequency \(5^{\text{-}10}\). Measurement of \(\tan \delta\) and of the modulus are achieved by measuring the width of the resonance curve. The disadvantage of this method is that the frequency of vibration changes as the modulus of the specimen varies with temperature and thus constant frequency data cannot be obtained from one specimen over a range of temperature.

Mechanical relaxations may be measured by the following parameters: \(G', G''\); \(E', E''\); \(J', J''\); \(D', D''\); \(\tan \delta\) and \(\mathcal{A}\), these being, respectively, the real and imaginary parts (ie: storage and loss) of the complex quantities; \(G^*\) the complex dynamic shear modulus; \(E^*\) the complex dynamic tensile modulus; \(J^*\) the complex dynamic shear compliance and \(D^*\) the complex dynamic tensile compliance. The parameters \(\tan \delta\) and \(\mathcal{A}\) have already been introduced. Relaxations are observed as peaks in the lossy parameters and as sigmoidal variations in the storage quantities; the latter increase as the temperature is reduced. Logarithmic decrement as measured in the torsion pendulum experiment has the great advantage that the value obtained is theoretically independent of the specimen dimensions \(5^{\text{-}2}\). Accurate values of \(\mathcal{A}\) may be obtained comparatively quickly.

Applications of dynamic mechanical testing methods to elastin, or indeed to any connective tissue, appear infrequently in the literature,
with one exception - studies of the arterial wall. In this case researchers have logically recognized the value of dynamic experiments on a tissue made to withstand dynamic working conditions in vivo. Dynamic mechanical studies have been undertaken on human\textsuperscript{5-11}, canine\textsuperscript{5-12} and bovine\textsuperscript{5-13,5-14} arteries. There is general agreement that the real dynamic tensile modulus $E'$ is not strongly frequency dependent above 2 - 4 Hz, but that it significantly increases from its static value at quite low frequencies. It also appears that the ratio $E'_{\text{dyn}}/E_{\text{static}}$ is higher in the more muscular arteries\textsuperscript{5-12}. The imaginary or loss component of the dynamic modulus is more complex than the real component. In the range 2 - 60 Hz it was found to increase non linearly with frequency\textsuperscript{5-12} and exhibited occasional maxima and minima\textsuperscript{5-14}. The use of sinusoidal stress oscillations have led to predictions on in vivo arterial elasticity and on wave velocity and attenuation which have generally been verified\textsuperscript{5-15,5-16}. Dynamic experiments on unpurified vessel material must, however, yield results dependent in some complex way on part or all of the various constituents present and, with the exception of collagen, little work has been done in investigating the mechanical relaxation spectra of each individually.

The kinetics of collagen denaturation were studied using dynamic mechanical methods\textsuperscript{5-17}, and torsion pendulum methods have also been used\textsuperscript{5-18,5-19} to characterize the viscoelastic behaviour of gelatin-water systems. The results of these investigations revealed the presence of two water-sensitive loss maxima at approximately -83°C and -13°C. Other attempts at characterizing the viscoelastic transitions in collagen and its amorphous form, gelatin, have been reviewed\textsuperscript{5-20}, although the majority of these rely on static stress relaxation and thermal measurements. Apparently the only torsion pendulum work specifically on collagen was
undertaken by Baer et al. who characterized the viscoelastic behaviour of human diaphragm tendon collagen in the region from -196°C to -33°C and compared the results with polyglycine-1 and nylon-6. Baer et al. observed three relaxation processes, which he labelled α, β and γ peaks. These strongly depended on the water content and age of the specimens. Increasing amounts of absorbed water intensified the relaxation strengths of all three loss maxima. However, whereas the α peak shifted to lower temperatures, the β peak remained constant and the γ peak shifted to higher temperatures. The height of the α, β and γ relaxations also diminished considerably with increasing age of the tendon donor. Comparison with data from specimens of polyglycine-1 and nylon-6 suggested to Baer et al. that internal friction maxima of all three materials originated from similar types of molecular motions.

Mechanical relaxation studies on elastin by low frequency torsion pendulum measurements appear to be limited to one paper - that of Gotte et al. Gotte measured the real shear modulus $G'$ and the loss tangent ($\tan \delta$) of samples of autoclave-purified ligamentum nuchae in the dry state and with a water content of 38% using a free oscillation torsion pendulum at about 1 Hz from -180°C to +200°C. For dry autoclaved elastin the loss tangent was found to be of the order 0.06 (below about 150°C), a value typical of amorphous or semi-crystalline polymers below the glass transition region. Above 170°C the loss tangent strongly increased, indicating the presence of a major transition near 180°C. At 220°C the elastin samples were leathery, were deformed easily under low stresses and rapidly started decomposing. Between -100°C and 0°C a secondary peak of $\tan \delta$ was observed and its frequency dependence studied by a bending vibration method at acoustic frequencies. The temperature $T_m$ at which $\tan \delta$ was a
maximum was found to change rapidly as a function of the frequency $\nu$ used in testing. From an Arhenius plot of $\ln \nu_m$ vs $1/T$ the activation energy of this transition was found to be 15 Kcal/mole, a value typical for secondary transitions in polymers.

5.3 The Torsion Pendulum Experiment

5.3.1 General and Theory

Dynamic mechanical testing methods can be divided into two types; those involving forced vibrations, and those involving vibrations at the resonant frequency of the specimen. The experimental devices associated with the latter class include, among others, the torsion pendulum and the suspended resonant rod. The results of all methods are essentially the same in the sense that all are sensitive to the same relaxation processes and yield approximately the same frequency-temperature relationship for each process. Because of its simplicity, however, the torsion pendulum is probably the most widely used of all these methods.

Basically, the apparatus consists of a horizontal rod known as the inertia rod or inertia bar, clamped by some method to one end of the specimen. The other end of the specimen is held rigidly. In some equipment the upper clamp is held rigidly and the lower clamp attached to the inertia arm. This arrangement, however, causes a tensile stress in the specimen which may cause elongation at high temperatures, so a preferable method is to clamp the lower end of the specimen and have the upper end and inertia bar suspended from one end of a lever/fulcrum arm, being balanced with weights.

The inertia bar is rotated slightly, twisting the specimen, and released. The decay of the oscillations and their frequency is then measured.
The system can be described by the following equation:

\[
\begin{align*}
\text{Inertial component} & \quad \text{Specimen component} \\
f(t) &= \frac{I\dot{\Theta}}{dt^2} + ZG^* \\
\end{align*}
\]

\[\Theta = \text{angle of twist}\]
\[f = \text{external force}\]
\[G^* = \text{complex shear modulus} = G' + iG''\]
\[Z = \text{a specimen dimension constant ('from factor')}\]
\[I = \text{moment of inertia of the oscillating system}\]

But the torsion pendulum works freely, ie: there is no external force, so

\[f(t) = 0\]

Thus

\[
\frac{I\dot{\Theta}}{dt^2} + Z(G' + iG'') = 0
\]

Assuming \(G'\) and \(G''\) to be independent of frequency (this is not always the case though for narrow frequency ranges it provides a good approximation\(^{5-10}\)), the solution is of the form:

\[
\Theta = \Theta_0 e^{-\alpha t}e^{i\omega t} = \Theta_0 e^{(i\omega - \alpha)t}
\]

\[\alpha = \text{an 'attenuation factor'}\]

Substituting into 5.2:

\[
I(\alpha^2 - \omega^2 - 2i\omega\alpha) + iZG'' + ZG' = 0
\]

And separating into real (storage) and imaginary (loss) parts:

\[G' = \frac{I}{Z} (\omega^2 - \alpha^2)\]
\[G'' = 2\alpha I\omega\]
The ratio of amplitudes $Q_1/Q_2$ for any two successive cycles in free decay is called the decrement. This is a reflection of the fractional dissipation per cycle of the torsional energy. In the ideal case where the decrement is unity, the amplitude does not change with time and no energy is dissipated; deformation is perfectly elastic.

In real situations, $\theta$ always decays exponentially and consequently it is easier to measure the logarithm of $Q_1/Q_2$ called the logarithmic decrement $\Lambda$.

Thus

$$\Lambda = \ln(Q_1/Q_2)$$  \hspace{1cm} (5.7)

But because the motion is sinusoidal, with period $p$

$$e^{i\omega t} = e^{i\omega(p+t)}$$

So from 5.3 and 5.7

$$\Lambda = \ln \left( \frac{Q_0 e^{-\alpha t} e^{i\omega t}}{Q_0 e^{-\alpha (t+p)} e^{i\omega (t+p)}} \right)$$ \hspace{1cm} (5.8)

The logarithmic decrement is obtained by measuring the amplitude of the $n$th oscillation $\theta_n$ and the $(n+1)$th, $\theta_{n+1}$. Random error can be reduced by measuring the decay of the amplitude over as many swings as possible.

Then

$$\Lambda = \frac{1}{k_n} \ln \left( \frac{\theta_n}{\theta_{n+1}} \right)$$  \hspace{1cm} (5.9)

where $k_n$ is the number of oscillations over which the measurement was taken.

The storage shear modulus $G'$ is obtained by observing the period of the oscillation, although it is also necessary to know the specimen geometry and the inertia of the system.
It may be shown\(^{5-25}\) that when a tensile stress \(t\) exists in a specimen of rectangular cross-section, the torque \(\Gamma\) produced by a twist through an angle \(\Theta\) is

\[
\Gamma = \frac{1}{3}G' \frac{\Theta}{\frac{a}{b}} \left[ (1 - 0.63\frac{b}{a}) + \frac{1}{120} \left( \frac{E'}{G'} \right) \left( \frac{a}{b} \right)^3 \Theta + \frac{t}{G'} \left( \frac{a}{b} \right)^2 \right] \quad 5.10
\]

where \(G'\) is the (storage) shear modulus, \(E'\) the (storage) Young's Modulus, \(a\) is the breadth, \(b\) the thickness \((a > 3b)\), and \(l\) the length.

In practice the angle of deflection \(\Theta\) is usually less than \(10^{-2}\) and consequently the terms in \(\Theta^2\) can be neglected. Also \((t/G)'\) is negligibly small for small specimens\(^{5-5}\).

So 5.10 is left as

\[
\Gamma = \frac{1}{3}G' \frac{\Theta}{\frac{a}{b}} \left( 1 - 0.63\frac{b}{a} \right) \quad 5.11
\]

\[
\Rightarrow G' = \frac{3\Gamma}{\Theta} \left( \frac{1}{\frac{a}{b}} \right) \quad 5.12
\]

Now let \(\frac{\Gamma}{\Theta} = \) torque per unit deflection = \(\Gamma'\).

Also let \(\frac{1}{3}ab^3(1 - 0.63\frac{b}{a}) = Z'\)

\[
\Rightarrow ab^3(1 - 0.63\frac{b}{a}) = 3Z' \quad 5.13
\]

Then \(G' = \frac{3\Gamma'1}{3Z'} = \frac{\Gamma'1}{Z'} \quad 5.15\)

If the moment of inertia of the pendulum around the central axis is \(I\) and the torque per unit deflection \(\Gamma'\), then the period \(t\) is given by\(^{5-5}\)

(Note: In the general proof leading to equation 5.13, periodic time was designated \(p\) to avoid confusion with general time \(t\). From now on in this chapter, however, \(t\) will be used to designate periodic time.)

\[
t = 2\pi \sqrt{\frac{1}{\Gamma'} \left[ 1 + \frac{A}{4\pi^2} \right]} \quad 5.16
\]
The (storage) shear modulus $G'$ is then obtained from 5.10 and 5.16 as follows:

$$t = 2\pi \sqrt{\frac{I}{I'}} \left( 1 + \frac{\mathcal{A}^2/4\pi^2}{I'} \right)$$

$$\Rightarrow \frac{t^2}{4\pi^2} = \frac{I + I \frac{\mathcal{A}^2/4\pi^2}{I'}}{I'}$$

$$\Rightarrow I' = \frac{4\pi^2 I + I4\pi^2 \mathcal{A}^2/4\pi^2}{t^2} \quad \text{5.17}$$

Substituting 5.17 into 5.15

$$\Rightarrow G' = \frac{1}{Z'} \left( \frac{4\pi^2 I + I \frac{\mathcal{A}^2}{t^2}}{Z'} \right)$$

$$= \frac{14\pi^2 I + I \mathcal{A}^2 I}{Z't^2}$$

$$\Rightarrow G' = \frac{4\pi^2 I}{Z't^2} \left( 1 + \frac{\mathcal{A}^2}{4\pi^2} \right)$$

Now a typical value for $\mathcal{A}$ is 0.2

So $\mathcal{A}^2 = 0.04$

And $\mathcal{A}^2/4\pi^2 \approx 0.001$

In comparison with 1 in the component $\left( 1 + \frac{\mathcal{A}^2}{4\pi^2} \right)$ this is negligibly small, so can be ignored.

Thus

$$G' = \frac{4\pi^2 I}{Z't^2} \quad \text{5.18}$$

where $Z' = \frac{1}{2}ab^2(1 - 0.63 \frac{b}{a}) \quad \text{5.19}$

the form factor depending entirely upon specimen geometry

5.3.2 The Test Rig

The tension pendulum test rig used in the experiments was based on the apparatus of Cooper$^{5-2}$ and is depicted in Fig. 5.2. The specimen (A) was attached either by clamping directly or by looping over two wire formers (B) held in the stainless steel clamps (C) to a stainless steel
Figure 5.2

Diagram of torsion pendulum apparatus with bath and water jacket for equilibrium saturation experiments.

Plate 5.1

Plate showing top half of torsion pendulum apparatus depicted in Fig. 5.2. Inertia arm, balance weights, mirror and electromagnets are clearly visible.

Plate 5.2

Plate showing water jacket and equilibrium swelling bath of torsion pendulum apparatus as depicted in lower half of Fig. 5.2. The three membered support column can be seen to the top right hand side, clear of the inner cavity for specimen attachment.
Fig. 5.2 Torsion pendulum apparatus.
connecting rod (D). This was attached at its top end via a second connecting rod (E) to the perspex (or Tufnol) inertia arm (F), which was suspended by means of nylon thread (G) to a brass balance arm (H) which lay over a steel knife edge (I). Equilibrium was maintained by adding weights to one of two positions on the balance arm, one (J) of distance from the fulcrum equivalent to the fulcrum - thread distance - and the other exactly twice (K) that distance (giving double the moment to be able to counteract with one weight the effect of adding two weights to the inertia arm); the lower end of the specimen was clamped to the bottom end of a three-membered, stainless steel support column (L), the top end of which was bolted to the baseplate (M) of the apparatus. At each end of the inertia bar protruded two soft iron pegs (N) to provide objects of attraction for the two electromagnets (O), whose purpose was to supply the initial twist to the inertia bar.

The base plate was mounted on the wall of the laboratory via three screw level adjusters (P) on an iron frame. The support column could thus be adjusted to obtain true vertical alignment of the specimen with the axis of the apparatus.

The recording system consisted of a 'Cambridge' spot-lamp producing a narrow pencil of light which was reflected by a silvered stainless steel concave mirror (R) attached to the upper connector rod, back on to a 'Graphispot' light-following chart recorder, which was placed 2 metres (the focal length of the mirror) from the mirror.

To perform an experiment, a series of short pulses of current were applied through the electromagnets by means of a hand-operated push-button until the amplitude was sufficiently large to give an approximate full scale deflection on the pen-recorder. The electromagnetic pulses
were then terminated and the torsional oscillations allowed to decay naturally, these being recorded on the pen-recorder.

Adjustment of the inertia of the whole torque generating system could be carried out by (a) using different sizes of inertia arm, or (b) placing pairs of solid steel spheres (ball bearings) (S) on the arm symmetrically on each side of the nylon suspension thread. These spheres were positively located in hemispherical depressions in the arm which had been accurately machined to form pairs of holes equi-distant from the point of suspension. The spheres available for use on this apparatus ranged in diameter from 10 mm to 50 mm, whilst the inertia arms were 381 mm (15") and 533.5 mm (21") (Perspex), and 228.5 mm (9") (Tufnol). Thus the inertia could be varied from approximately \(9 \times 10^{-5}\) kg m\(^2\) to more than \(9000 \times 10^{-5}\) kg m\(^2\), an interval of three decades. For each pair of spheres placed on the arm, a third was hung in a polythene bag (T) on the outermost (2 x moment) position of the balance arm to counter-balance the added weight. It was possible to change the inertia arm in use on the apparatus without disturbing the specimen by means of a clamp (U) which could support the connecting rod while the inertia arms and corresponding balancing weights were being changed, so preventing the accidental application of excess tensile or compressive loads on the specimen.

The support column was such that it could be inserted into a number of specially designed pieces of secondary apparatus, depending on the experiment being performed - these apparati are described in the appropriate experimental sections.
5.4 Experimental

5.4.1 $T > 0$, Equilibrium Saturation Experiment

5.4.1.1 Introduction and Theory

As mentioned in the introduction to this chapter, one of the prime objectives of utilizing the torsion pendulum was to examine whether the elastin specimens conformed to the theory of rubber elasticity under conditions similar to those employed by Weis-Fogh and Andersen.

Examination of rubber elasticity theory yields the following arguments:

Assume a rubber specimen of unswollen volume $V_{\text{dry}}$ is subsequently swollen by a diluent to a volume $V_s$ such that the volume fraction of polymer $v = V_{\text{dry}}/V_s$.

Let the dimensions of the specimen at temperatures $T_0$ and $T$ be $a_0$, $b_0$, $c_0$, and $a$, $b$, $c$ respectively. Let also the specimen swell isotropically, so that if $a = \gamma_T a_0$, then $b = \gamma_T b_0$ and $c = \gamma_T c_0$, in which $\gamma_T$ is a parameter which depends only upon temperature.

Recalling equation 5.18

$$G = \frac{4\pi I}{Z't^2} \quad t = \text{periodic time} \quad G = G' = \text{(storage) shear modulus}$$

For constant inertia experiment $I = \text{const}$

$$4\pi = \text{const}$$

$$\frac{1}{Z'} \propto V_{ST}$$

So

$$C_T \propto \left( \frac{1}{V_{ST}} \cdot \frac{1}{t^2} \right)$$

and

$$C_{T_0} \propto \left( \frac{1}{V_{ST_0}} \cdot \frac{1}{t_0^2} \right)$$

$$=> \quad \frac{C_T}{C_{T_0}} = \left( \frac{V_{ST}}{V_{ST_0}} \cdot \frac{t_0^2}{t^2} \right)$$

$V_{ST} = \text{vol swollen composite at temp } T$

$T = \text{temperature}$

$T_0 = \text{reference temperature}$
in which \( t \) and \( t_0 \) are the observed periods of oscillation of the
pendulum at temperatures \( T \) and \( T_0 \).

Now according to the prediction of the theory of rubber
elasticity,

\[
\frac{G_T}{G_{T_0}} = \frac{T}{T_0} \left( \frac{V_{sT_0}}{V_{sT}} \right)^{\frac{1}{2}} \cdot \frac{<r^2>_o{T_0}}{<r^2>_o{T}}
\]  

where \(<r^2>_o{T}\) is the mean-square end-to-end distance of the network
chains unrestricted by cross-links (at temperatures \( T \) and \( T_0 \) as
indicated).

Taking logs

\[
\Rightarrow \ln G_T - \ln G_{T_0} = \ln T - \ln T_0 + \frac{1}{2} V_{sT_0} - \frac{1}{2} V_{sT}
\]

\[
+ \ln <r^2>_o{T_0} - \ln <r^2>_o{T}
\]

And differentiating with respect to \( T \)

\[
\Rightarrow \frac{d\ln G_T}{dT} = \frac{1}{T} - \frac{1}{2} \left( \frac{d\ln V_{sT}}{dT} \right) - \left( \frac{d\ln <r^2>_o{T}}{dT} \right)
\]  

Now by definition

\[
\beta_V = \left( \frac{d\ln V_{sT}}{dT} \right)
\]

and

\[
\beta_<r^2>_o = \left( \frac{d\ln <r^2>_o{T}}{dT} \right)
\]

So substituting into 5.22 gives

\[
\frac{d\ln G}{dT} = \frac{1}{T} - \frac{1}{2} \beta_V - \beta_<r^2>_o
\]

in which \( \beta \) is the temperature coefficient of volume and of \(<r^2>_o\)
according to subscript.
For elastin in water at 20°C, with consideration of the errors involved in measuring $\beta_V$ (Chapter 4) and $\beta_{<\mathbf{F}^2>_0}$, $\beta_{<\mathbf{F}^2>_0}$ (0.5 x 10$^{-3}$)$^{5-27}$ is small in comparison with $\frac{1}{T}$ (3.14 x 10$^{-3}$) and $\beta_{V/3}$ (−4.5 x 10$^{-3}$)$^{5-27}$ and may be neglected. Since the value of $\beta_{<\mathbf{F}^2>_0}$ for water is apparently$^{5-27}$ unusually large, and in the absence of any evidence to the contrary, $\beta_{<\mathbf{F}^2>_0}$ can be assumed to be negligible for water/ethanol solutions, and for formamide.

Consequently, equation 5.21 can be re-written with

$$\frac{<\mathbf{F}^2>_{0T}}{<\mathbf{F}^2>_{0To}} = 1$$

and

$$\gamma_T = \left(\frac{V_{ST}}{V_{STo}}\right)^{1/3}$$

Thus

$$G_T\gamma_T = AT \quad 5.24$$

where $A = \text{const} = G_{To}/To$

From equation 5.20 the observed value of

$$G_T\gamma_T = B(\gamma_{tt})^{-2} = AT$$

where the constant $B = G_{To}t_o^2$.

Hence a specimen conforming to the rubber elastic model will exhibit a linear dependence between $(\gamma_{tt})^{-2}$ and $T$. If the transition from the glassy to the rubbery state is incomplete and the specimen is significantly viscoelastic, then a plot of $(\gamma_{tt})^{-2}$ vs $T$ will not be linear and will be of negative slope$^{5-5,5-6,5-28}$.

The experiments were thus performed, not to determine precisely the relative magnitudes of the magnitudes of the energy or entropy components of the force. The torsion pendulum is not well suited to the task. Nor was it the intention to determine exact modulus values for the specimen. The purpose was in fact much simpler: it was to determine whether or not the temperature dependence of $C_T\gamma_T$ was negative or positive.
5.4.1.2 Apparatus

The top half of the apparatus was the torsion pendulum rig as described in section 5.3.2 using the 15" perspex inertia bar without any spheres.

It was necessary, however, to surround the specimen in its diluent and be able to control its temperature or maintain it accurately constant if necessary. The apparatus employed for this purpose is shown in Fig. 5.2, Plates 5.1 and 5.2.

A double walled thermal water jacket (V) was used to form a central cavity into which the specimen column could be fitted, and which could be filled or emptied of liquid by means of a drain pipe (W). The temperature of the inner cavity could thus be varied by pumping water of the desired temperature through the thermal jacket from a pre-heated water-bath. This gave a useful method of providing accurate heating of the inner cavity liquid without significant generation of convection currents and stirring. The temperature of the inner cavity was monitored with a standard mercury in glass thermometer accurate to 0.5°C. Around the thermal jacket was arranged a further glass cylinder with drain pipe (X), the object of this being to collect any liquid overflow from the central cavity.

5.4.1.3 Method

Ring specimens were cut from region 2 of the purified porcine aortas of approximate dimensions 3 mm x 3 mm x 12 mm diameter and these put aside to equilibrate in glass distilled water.

The method used to attach the specimens to the clamps was by fabricating two 'staple' shaped wire pieces from 0.5 mm diameter stainless steel wire and looping the specimens over these (see Fig. 5.2). The ends of the staples were then clamped tight in the stainless steel clamps.
Sufficient counter-weights were then added to the balance arm to just remove the circular geometry of the specimen leaving it as two parallel lamellae with approximately 0.25 mm separation.

Before specimen mounting, the baseplate was carefully levelled with the aid of a plumb-line which was suspended from the point of attachment of the connector rod with the torsion bar and allowed to hang down the specimen column. This gave an indication of correct level by hanging exactly above the centre of the lower clamp in the level position. With the connector rod in position, further alignment could be effected by placing a mirror between the clamps and observing upwards to see if the rod was passing centrally through the hole in the baseplate.

In order to avoid problems of solvent disproportionation (ie: in the ethanol/water cases) at the higher temperatures, it was necessary to change the solvent in the inner cavity for fresh, pre-heated solvent shortly before an experiment was carried out. For this purpose several especially designed glass stoppered Pyrex cylinders were made up so that they could be inserted into the heated water-bath shortly before an experiment was to be performed to rapidly heat the solvent to the desired temperature. These tubes were designated 'McCrum tubes'. Samples could also be drawn from these tubes for density measurements to obtain the accurate solution composition.

To assemble the rig, the torsion bar, connector rod and upper clamp were carefully balanced by adding counter-weights to the inner hanging position of the balance arm and aligned with the bottom clamp using the methods already described. An equilibrated specimen was then attached and an extra 5 gm added to the inner balance pan to
'straighten' the specimen out and maintain positive contact between the wire holding frames and the specimen ends. In this way, the specimen approximated to a rectangular shape.

The thermal cavity was then filled with a fresh quantity of the appropriate diluent, raised around the specimen column, and the apparatus left for two hours to ensure complete specimen equilibration. At the end of this period, the water bath was filled with a crushed ice/water mix and the pump switched on to lower the temperature within the inner cavity. On reaching its minimum temperature the solvent was changed using a fresh mixture from a McCrum tube from the water bath and five minutes later the first experiment performed using the electromagnets to swing the torsion bar as previously described. The next temperature point was obtained by using a copper cooling coil through which was running tap water (at mains temperature - approximately 15°C). Again, and as at all other times, the McCrum tubes were used to change the solvent 3-5 minutes before the experiment.

Subsequent experiments were made at approximately 10°C intervals to approximately 60°C and then back again to room temperature. The whole cycle was then repeated to check for reproducibility.

For comparative purposes, the same specimen was used in each of the solvents in turn, these being

(i) Glass distilled water
(ii) 10:90 v/v ethanol/water
(iii) 20:80 v/v ethanol/water
(iv) 30:70 v/v ethanol/water
(v) 50:50 v/v ethanol/water
(vi) Formamide
After each solvent cycle the solvent was changed and the specimen allowed to equilibrate for at least 2 hours in the new mixture. During this time the temperature was raised to 70°C and back. The mixture was then changed again before the temperature cycle started.

5.4.1.4 Results and Correction for Swelling

As previously mentioned, the values of $t^2$ obtained had to be corrected by a factor $\gamma_T$ to allow for temperature dependent swelling effects and represent a true portrayal of rubber elasticity theory.

The values of $\gamma_T$ could be obtained from the density measurements of Chapter 4, section 4.4, and the plots of section 4.2 as follows:

On changing $T_0$ to $T$, we have

$$m_T - m_{T_0} = \bar{\rho}_V (V_{ST} - V_{S{T_0}})$$

$$= \bar{\rho}_V V_{ST} - \frac{m_T - m_{T_0}}{\bar{\rho}_v}$$

$$= \bar{\rho}_V V_{ST} \gamma_T^3 - \bar{\rho}_V V_{S{T_0}}$$

$$= \bar{\rho}_V V_{S{T_0}} (\gamma_T^3 - 1)$$

where $\bar{\rho}_V$ = mean liquid density between $T$ and $T_0$

$\bar{\rho}_S$ = mean saturated specimen density between $T$ and $T_0$

From 5.25

But $m_{T_0} = \bar{\rho}_S V_{S{T_0}}$

So

$$\frac{m_T - m_{T_0}}{m_{T_0}} = \frac{\bar{\rho}_V V_{T_0} (\gamma_T^3 - 1)}{\bar{\rho}_S V_{T_0}} = x \quad \text{(Chapter 4)}$$

$$\Rightarrow \gamma_T^3 = 1 + \left(\frac{\bar{\rho}_S}{\bar{\rho}_V}\right)x$$

$$\Rightarrow \gamma_T^{-2} = \left[1 + \left(\frac{\bar{\rho}_S}{\bar{\rho}_V}\right)x\right]^{-3}$$

5.28
From the experiments of section 4.3.3 and 4.3.1, the correction factor $\gamma_T^{-2}$ could be determined using the above theory. Although strictly the parameter required for the determination was the ratio $\rho_s/\rho_v$, that actually used was $\rho_{s20^\circ C}/\rho_{v20^\circ C}$. Due to the close association of the density of saturated elastin with that of the liquid with which it is saturated (eg: Fig. 4.11), the difference between these two ratios is likely to be very small. For example, from Fig. 4.11 as the density of the solvent drops from 1.00 kg $1^{-1}$ to 0.98 kg $1^{-1}$, there is a change in the ratio $\rho_s/\rho_v$ of only approximately 1%.

Plots of both $\gamma_T^{-2}$ and the corrected $(\gamma_T/\gamma_t)^{-2}$ parameters are shown in Figs. 5.3 to 5.8 for the various solvents used.

5.4.1.5 Discussion

From the plots is can be immediately seen that the elastin-formamide system exhibits a linear dependence between $(\gamma_T/\gamma_t)^{-2}$ and $T$ with a positive slope, in accord with the properties of a completely rubber elastic material as predicted by the theory of rubber elasticity.

The behaviour of elastin in the other solvents is typical of an amorphous polymer in the temperature region just below the rubber elastic state, namely in the transition region: the $(\gamma_T/\gamma_t)^{-2}$ vs $T$ plots are curved and of negative slope$^{5-5,5-6,5-28}$. It is well known$^{5-29,5-30}$ that amorphous polymers within the transition region tend to exhibit a Poissons ratio significantly below 0.5 or, more specifically, undergo appreciable volume increases on elongation. On the other hand, polymers in the rubbery region exhibit Poissons ratios close to 0.5$^{5-5,5-6,5-28}$ and show little volume change when elongated.

Further weight is thus added to the re-interpretation of the experimental results of Weis-Fogh and Andersen discussed in Chapter 4.
**Fig. 5.3**

Plots of $1/t^2$ and $1/(\gamma_T t)^2$ against temperature obtained from torsion pendulum experiment on saturated porcine aortic elastin. Solvent water.

**Fig. 5.4**

As Fig. 5.3, but with solvent mixture 10:90 v/v ethanol/water.

**Fig. 5.5**

As Fig. 5.3, but with solvent mixture 20:80 v/v ethanol/water.

**Fig. 5.6**

As Fig. 5.3, but with solvent mixture 30:70 v/v ethanol/water.

**Fig. 5.7**

As Fig. 5.3, but with solvent mixture 50:50 v/v ethanol/water.

**Fig. 5.8**

As Fig. 5.3, but with solvent formamide.
Figs. 5.3-5.8. \( T>0 \). Equilibrium torsion pendulum results.

5.3. Water.

![Graph for water showing \( \sec^2 x \times 10^2 \) vs. temperature.]

5.4. 10% ethanol

![Graph for 10% ethanol showing \( \sec^2 x \times 10^2 \) vs. temperature.]
5.5 20% ethanol.

5.6 30% ethanol.
5.7  50% ethanol.

![Graph showing temperature vs. certain parameter for 50% ethanol.]

5.8. Formamide.

![Graph showing temperature vs. certain parameter for formamide.]

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The observations of these workers are thus outlined below with the re-interpreted explanations of this thesis:

(1) In the cases of formamide and 20:80 v/v ethanol/water saturated elastins, Weis-Fogh and Andersen observed a heat release equivalent to the mechanical work put into the specimen.

**Explanation:** Elastin saturated with formamide behaves as a true rubber, deforming elastically with little or no volume change. Its Poisson's ratio is equal or close to 0.5. There is virtually no viscoelasticity.

Elastin saturated with 20:80 v/v ethanol/water behaves as an amorphous rubber within its glass transition zone. There is significant viscoelasticity and volume increase on deformation resulting in uptake of solvent from the surrounding medium. For 20:80 v/v ethanol/water, however, the heat of solution $\Delta H$ is zero, so there is no heat contribution to that generated by mechanical work with the specimen.

(2) In the cases of elastin saturated with ethanol/water solutions of less than 2 vol parts ethanol (to 8 parts water) Weis-Fogh and Andersen observed a heat release greater than the equivalent of mechanical work supplied to the specimen.

**Explanation:** In these solutions and in pure water a similar argument applies as to the 20:80 v/v ethanol/water saturated case, except that now the heats of solution $\Delta H$ are negative. The deformation generated solvent uptake thus induces a positive heat contribution in addition to that generated by mechanical work.

(3) In the cases of elastin saturated with ethanol/water solutions of greater than 2 parts ethanol (to 8 parts water) Weis-Fogh and Andersen observed a heat release less than the equivalent of the mechanical work supplied to the specimen.
Explanation: Again the material is in a viscoelastic condition with a resulting volume increase on deformation and liquid uptake. The heats of solution in these cases are, however, positive, resulting in a heat uptake which subtracts from the heat generated by mechanical work.

Elastin in its in vivo condition thus appears to exhibit typical transition-region viscoelastic properties, as opposed to true rubber-elastic properties. These findings are further examined in Chapter 6.

5.4.2 T>0, Varying Saturation Experiments

5.4.2.1 Introduction

In section 5.4.1 the modulus of the material was examined in relation to its temperature on specimens in their fully saturated (dissolved liquid + microvoid liquid) condition. The assumption in that section was made that the major contribution to the modulus arose from the elastin/dissolved liquid phase, any contribution from the microvoid liquid being negligible. In this section the validity of the assumption is experimentally examined.

An examination was also carried out of the shear modulus/temperature relationship of elastin at various water saturations to observe any effects that water has on the glass transition of the material.

5.4.2.2. Apparatus

The apparatus was essentially the same as that used in experiment 5.4.1 with the addition of a set of microbalances positioned near the apparatus.
5.4.2.3 Method

(a) Saturation dependence of shear modulus \(1/t^2\) at constant temperature (20°C)

This experiment was aimed at obtaining a plot of \(\alpha\%\) saturation (see Chapter 3) against \(1/t^2\) (proportional to shear modulus \(G\)). The effect of the swelling correction used in experiment 5.4.1 was simply to alter the magnitude of effects observed, not the temperature at which the effects occurred. Hence no such correction was necessary for this experiment. It was shown in Chapter 4 that full saturation of the solid elastin phase (dissolved water) occurred at approximately \(\alpha = 40\%\), the rest of the liquid consisting of microvoid water.

A sample of purified region 2 aorta was taken and two ring specimens cut directly adjacent to each other. Both were allowed to become saturated in the same water-bath at 20°C. A 0.5 mm pre-weighed stainless steel wire frame was then fabricated so that one of the ring specimens could be mounted with exactly the same deformation as the other, which was fixed, as described in section 5.4.1, in the torsion pendulum rig. The temperature control bath was not used and the specimen sections left in the air at 20°C ± 1°C room temperature.

The frame mounted specimen was used to obtain the \(\alpha\%\) values for the rig-mounted specimen. It was suspended with nylon thread within 30 mm of the clamps throughout the whole experiment, only being removed for weighing. At the end of the run it was vacuum dried to constant weight for the \(\alpha\%\) calculation.

Measurements were made at approximately 15 minute intervals on the rig-mounted specimen and each one of these accompanied by a weighing on the frame-mounted specimen.
(b) Shear modulus dependence on temperature at constant saturation levels

In this experiment, specimens were allowed to 'dry' to different saturation values before being tested at these values against temperature in the torsion pendulum rig. The saturation values were held constant using a surrounding 'hydrophobic liquid' perfluorodecalin. The method was developed in section 4.3.4.5.

Ring specimens were cut from a region 2 aorta section and allowed to equilibrate for 3 hours in glass-distilled water. They were then allowed to dry in a desiccator over P₂O₅ for various fixed periods of time. The drying curves of section 4.3.4.3 were used as a rough guideline to arrive at an approximate α% value. Following this, the specimens were accurately weighed and placed in separate beakers of perfluorodecalin (weighted down with wire hooks). Accurate α% values were obtained at the end of the experiment after vacuum drying the specimens.

The temperature control bath was filled with perfluorodecalin and the specimen column immersed in this. For the first two hours of the experiment the temperature was maintained at 20°C and measurements taken at 10 minute intervals to check for any modulus variation effects that the perfluorodecalin could itself have on the specimen. In no case, however, were any such effects noted. Similarly at every other temperature level a 2 hour period was allowed with constant monitoring to look for possible irreversible changes in the specimens; none were noted. Finally, after the temperature cycle had been completed, it was repeated to check for reproducibility.
5.4.2.4 Results

The results of experiments (a) and (b) are presented in graphs 5.9 and 5.10. They are discussed in the next section.

Owing to the large changes in $1/t^2$ over different runs of experiment (b), constant inertia could not be maintained over all the runs. It was, however, maintained over each individual run. Hence the $1/t^2$ magnitudes of the $y$ co-ordinates are arbitrary for each curve, simply being proportional to shear modulus in each case, but with the constant of proportionality varying from specimen to specimen.

5.4.2.5 Discussion

The results of experiment (a) showed that the hypothesis of the microvoid water having a zero effect on the dynamic torsional properties of the specimen was not entirely valid. If it were only the dissolved water/solid elastin phase which governed the properties, theoretically there should be no major modulus change until approximately $\alpha = 40\%$ when all the microvoid liquid would be removed. Instead, the material started shifting back further into the transition region at approximately $\alpha = 130\%$ which, although lower than its fully saturated state of $\alpha = 250-300\%$ was nevertheless significantly higher than the $\alpha = 40\%$ dissolved-water-only state.

A possible explanation to this is that the microvoid liquid itself can be divided into two classes:

(i) Liquid surrounding the elastin molecules closely associated with the 'dissolved water', though not directly bound to the molecules themselves - 'associated water'.

(ii) Free bulk water lying in between the elastin fibres free to flow in and out - 'free water'.

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Fig. 5.9

Plot of $1/t^2$ against water content. The shear modulus ($\alpha \, 1/t^2$) is seen to reach a minimum at $\alpha = 130\%$ and then to start rapidly increasing as further water is removed.

Fig. 5.10

Plots of $1/t^2$ against temperature for specimens held at different water contents.
Fig. 5.9. $t^2$ vs. water content.
Fig. 5.10  *Varying saturation\[\text{t}^{-2}\text{ vs temperature curves.}*

\[
\begin{align*}
\text{Fig. 5.10} & \quad \text{*Varying saturation } \\
& \quad \text{t}^{-2} \text{ vs temperature curves.} \\
& \quad \text{\[\text{°C.}\]} \\
\end{align*}
\]
The 'associated water' assumes the volume taken up largely by glycoproteins in the in vivo state, whereas the 'free water' occupies areas of other in vivo structural units such as collagen and smooth muscle.

The associated water is in equilibrium with the dissolved water so that as it begins to be removed by evaporation from the specimen, dissolved water is removed from the elastin molecules causing premature onset of glass transition modulus changes or at least significant de-swelling. From equations 5.17 and 5.18 it can be seen that among other parameters

\[ G \alpha \frac{1}{\delta_{ab}} \alpha \frac{L}{V_s} \]

so volumetric shrinkage will have the effect of an observed modulus increase. The magnitude of the change, however, suggests definite transition region changes.

Experiment (b) reinforces the conclusions of experiment (a). The \( \alpha = 130\% \) curve is very similar to the fully saturated water curve of experiment 5.4.1 (corrected for \( \gamma_T \)) with the material apparently just at the upper end of the transition region. Reducing the saturation to \( \alpha = 60\% \) however, pushes the specimen well into the transition region with a \( T_g \) (glass transition temperature) of approximately 55°C. Further reduction to \( \alpha = 20\% \) takes the material to a stage just at the beginning of the transition region.

5.4.3 Low Temperature Dynamic Tests

5.4.3.1 Introduction and Theory

The previous experiments of this chapter demonstrated the effects of temperatures above water freezing point and saturation on the dynamic viscoelastic condition of elastin at low frequencies.
Whereas the water and alcohol/water mixtures placed the material in the viscoelastic transition region, formamide appeared to act as a plasticizer pushing the elastin well into a rubber elastic state. Without undertaking experiments at lower temperatures, however, it is impossible to discover exactly the temperature of the glass transition of elastin saturated in formamide or investigate any secondary relaxations which occur in the material. Consequently it was decided to perform a series of low temperature mechanical experiments on the saturated material. The two solvents chosen were water and formamide, as these generated the two mechanical states of interest without resorting to multi-component systems which could suffer problems of rubber and viscoelastic freezing disproportionate.

In the previous experiments only the storage shear modulus $G'$ was investigated. This was because it was considered that any measurements of damping would be hindered by the changing viscosity of the solvent surrounding the specimen. To observe secondary relaxations, however, a damping parameter is desirable as well as $G'$, and that chosen was the logarithmic decrement ($\eta$) (see section 5.3.1).

5.4.3.2 Apparatus

The low temperature apparatus designed for fitting around the specimen column is shown in Fig. 5.11.

Operation was effected by passing the coolant (liquid or gaseous nitrogen) from a self-pressurising Dewar vessel through a tight-fitting coil of copper tubing (A) soldered to the outside of the copper specimen chamber (B), into which the specimen column of the torsion pendulum rig snugly fitted. This coil and tube assembly was thermally insulated by the surrounding 'cold box' (C) which consisted
Fig. 5.11. 'Cold box' for torsion pendulum rig.
of a sheet metal enclosure filled with polyurethane foam. The ends of the box were ¼" Tufnol sheet (D) and the whole apparatus could be raised to surround the specimen column by means of a hoist arrangement attached firmly to the laboratory wall.

The temperature in the specimen chamber was controlled by regulating the flow of coolant by means of a pair of solenoid operated valves (E) connected in series as an insurance against the failure of either. The operation of the valves was governed by a 'Transtat' (F) type BP7 control box (Control Instruments Ltd.), the operating principle of which was to compare the voltage from a chrome-alumel thermocouple fixed in the cold box near to the specimen (G), with a reference voltage determined by an adjustable potentiometer in the control box. Then by variation of its basic cyclic switching pattern the cold box temperature was caused to change until the thermocouple voltage and the reference voltage were equalized.

The temperature in the region of the specimen was measured by three copper constantan thermocouples all mounted approximately 10 mm from the specimen in different locations. The output from any one of these could be recorded on a 'Servoscribe' multivolt chart recorder (Smiths Industries Ltd.).

5.4.3.3 Method

Region 2 purified ring aorta specimens were cut and allowed to equilibrate for 3 hours in beakers of glass distilled water and fresh formamide respectively. The formamide equilibrated specimens were then transferred for two further 30 minute plus one final 5 minute period to fresh solvent before experimenting. One was then taken and allowed to dry in a desiccator to approximately α=140% saturation.
(water) or a 400% saturation (formamide). The drying vs time curves
of Chapter 4 were used as guidelines to determine approximately the
correct time. This was done to try to prevent large regions of
bulk frozen solvent in the free-water areas which could influence
the true properties of the elastin/water composite.

Mounting the apparatus was as described in previous sections,
but on this occasion the initial tension was increased slightly to
10 gms to ensure minimum distortion on freezing.

The cold box (at room temperature) was then raised around the
specimen and minimum temperature immediately selected on the con­
troller (liquid nitrogen through the copper coils). Cooling to
approximately -190°C took about 4 minutes although half way through
this process, at about -100°C, the cold box was gently lowered and
the specimen examined for condition.

On achieving minimum temperature (approximately -190°C) the
specimen was left for one hour before any experiments made. Experi­
ments were then carried out at approximately 10°C intervals with
constant inertia of the torsion bar. Half an hour at the desired
temperature was allowed in each case before an experiment was made.
A 10 minute period was then allowed before the experiment was
repeated to check for reproducibility.

Values of \( \Lambda \) could most accurately be obtained from each
experiment by plotting the logarithm of amplitude of each sinusoidal
peak of the oscillation (obtained from the pen recorder) against the
number of the particular peak during the oscillatory decay; a line
could then be drawn through those points, the slope of which gave
the logarithmic decrement .
Each run was continued until the periodic time had increased so much that tests were no longer feasible with that particular inertia on the torsion bar. At this point the temperature was lowered again to -190°C and the whole run repeated to check for reproducibility.

To check for purity and freedom from dissolved water, the formamide from the bottle in which the specimens had been equilibrated was tested by measuring its freezing point.

5.4.3.4 Results

The results are shown in Figs. 5.12 and 5.13.

5.4.3.5 Discussion

Measurements of $\Delta_\alpha_0$ on the frozen water and formamide saturated specimens indicated three damping peaks - $\alpha$ (the glass transition), $\beta$ and $\gamma$. In the case of the water saturated specimen, however, apart from the glass transition the $\gamma$ peak only was visible whereas, conversely, for the formamide case the $\beta$ peak only was visible.

As predicted by experiment 5.4.1 the glass transition of the formamide saturated specimen was observed to lie at a lower temperature (approximately -62°C) than that of the water saturated specimen.

The study of mechanical relaxations in polymers is a vast one (eg: ref. 5-5) and many theories have been put forward to explain the various relaxations observed in different polymers. A limited study on elastin such as this cannot hope to achieve any more than tentative suggestions for the causes of the various relaxations in the material. As previously mentioned, its real aim was to examine for agreement with the random coil model of elastin by demonstrating a decreased glass transition temperature in the formamide saturated specimens and also to show the presence of any further relaxations.
Fig. 5.12

Plot of torsion pendulum logarithmic decrement ($\Delta$) against temperature for water and formamide saturated elastins below $0^\circ$C.

Fig. 5.13

Corresponding plot of $1/t^2$ against temperature for water and formamide saturated elastins below $0^\circ$C.
Fig. 5.12  Low temperature mechanical damping curves.
Fig. 5.13  Low temperature $t^2$ curves.
Further study of the secondary relaxations in elastin in various degrees of saturation in various solvents would probably prove a highly fruitful topic for further research.

5.5 General Discussion

The work of this chapter on the whole substantiates the interpretation of the structure of elastin according to the theory of rubber elasticity. It is, however, only when the material is saturated with formamide under typical room temperature conditions that it is a rubber in its true sense. In water and ethanol/water solutions at typical room and physiological temperatures the material is nearly, but not quite out of its glass transition region and into the rubbery plateau of the modulus/temperature curve. In a sense, formamide thus has the effect of shifting the coordinates of such a curve along the temperature axis in the x direction from the water or ethanol/water saturated situation. It hence acts as a plasticizing as well as a swelling agent.

Water itself also acts as a plasticizing agent if the material is considered in its dry state. This was shown in the experiments of 5.4.2 where the glass transition was demonstrated to be a function of water content as well as temperature. A similar situation is observed in the case of polymethyl methacrylate which can absorb up to about 2% by weight of water owing to the presence of hydrophilic ester groups. In this case it can be observed that in addition to the plasticizing effect of water in lowering the temperature of the $\alpha$ relaxation, absorbed moisture also gives rise to an additional relaxation region which is not found in the dry polymer. A 'water peak' occurs in the region of $-100^\circ\text{C}$ to $-150^\circ\text{C}$. 

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It is hence possible that it is such a 'water peak' which is responsible for the γ peak observed predominantly in the water saturated elastin specimens and which is invisible in the formamide saturated dry specimens.\(^5\)\(^-\)\(^22\).

The β relaxation peak can probably be attributed to side chain relaxations of the polymer chain. In the water saturated case, however, it cannot be observed, which at first sight seems an anomaly. Again, however, it has a parallel case of polymethyl methacrylate (in its isotactic form)\(^5\)\(^-\)\(^33\). The peak does in fact exist, but merges with the much larger α peak, the latter having been shifted by a relatively large amount to lower temperatures (about \(50\,^{\circ}\text{C}\)) due to isotacticity.

Not enough is known of the structure of elastin to state whether it is atactic, syndiotactic or isotactic. It is, however, conceivable that the structure-disrupting and secondary bond breaking effect of formamide converts the amino acid chains from isotactic to amorphous atactic or syndiotactic forms with the resultant change in the damping curve.

The α relaxation or glass transition of elastin thus appears to be shifted down by water content from about \(200\,^{\circ}\text{C}\)\(^5\)\(^-\)\(^22\) to its observed value of \(0\,^{\circ}\text{C}\) for its fully water saturated state, and still further by saturation with formamide. Unlike the formamide saturated state, however, the glass transition in the water saturated state occurs at its solvents own phase transition temperature; ice to water. The strong association of water with the various polar side groups of the elastin molecules has already been discussed (Chapter 4) just as the tendency for repulsion from hydrophobic groupings. As the entropy of the water structure decreases to become ice it is likely that the hydrophobic side groups become surrounded by ordered molecular cage-like structures which act to prevent mobility of the side groups. Thus the α peak is shifted upwards in temperature to the melting point of ice, whence the whole chain gains mobility.
The effect of the formamide is to restructure the material by disrupting secondary bonding. In itself it has no particular tendency for chemical association with or dis-association from hydrophilic or hydrophobic groups and hence its swelling temperature has no effect on the relaxation of the side chains (β relaxation) or the main chains (glass transition). There is no tendency for the β or α peaks to be pulled up towards the melting point of the formamide.

It is possible that the γ peak has the same origins as a similar peak observed in doped, polycrystalline ice. Studies of the mechanical damping in various types of ice have been performed⁵⁻³⁴ and yield α, β and γ transitions down to approximately -180°C. A β peak at about -20°C to -60°C is attributed to the reorientation of the protons in the ice while a sharp rise approaching 0°C in polycrystalline ice is attributed to grain boundary relaxation. The γ type relaxation in the doped polycrystalline ice, however, is attributed to the presence of the doping impurities. Fluorine atoms which probably occupy interstitial positions in the ice lattice produce a very small peak, but larger polar molecular impurities which form clusters in ice, such as NaCl, HCl and NaOH give rise to much larger peaks. Similar polar side chain clustering effects may well be present in elastin. The temperature at which the peak due to impurities occurs is found to be independent of frequency, although the amplitude of the peak may vary.

Discussion of the origins of secondary relaxations in elastin must of necessity concern the molecular structure of the material. Yet it is here where difficulty arises, for it is precisely the structure which is as yet unknown. In spite of this, however, a certain amount of information and deduction may be obtained by consideration of the amino acids of the material which have been accurately determined by a number of researchers (see Chapter 1).
Of the total number of amino acids occurring in elastin, approximately 93% (by number) are of non-polar, hydrophobic nature, and approximately 6% hydrophilic with 1% being covalent cross-linking agents. This gives an indication of the extreme hydrophobicity of the protein. The segregation of hydrophobic side chains is a powerful factor in stabilizing a protein molecule in aqueous solution and leads in many cases to the formation of globular proteins. Such globular proteins consist of polypeptide chains bundled so that most of their non-polar groups lie inside the bundle and their polar groups outside.

Why should this not be so with elastin?

It is possible that the answer lies in the closer examination of the amino acid content of elastin. Of the total number of hydrophobic groups, 32% (by number) consist of glycine and 22% alanine. These amino acids have side chains so small that the 'non-polar in, polar out' rule is found not necessarily to apply; glycine and alanine can be accommodated in the interior or on the surface with equal ease.

The remaining quantity of hydrophobic amino acids consists predominantly of valine and proline. These have been found in close proximity with each other in frequently repeating tetrapeptides, pentapeptide and hexapeptide units. There appears to be a considerable degree of segregation of these amino acids from the others. If a rough calculation is made (accepting Mukherjee's (Chapter 4) calculation of the molecular weight between cross-links of elastin in its most rubber-like (formamide saturated) form to be approximately 7000), and calculating approximate mean molecular weight of constituent amino acids (~ 100 Daltons), it can be seen that about 70 amino acids might be expected between cross-links. Approximately one-third, or 23, of these might be expected to have large hydrophobic side chains, and of these segregation occurs into groups of
two or three at a time. Hence the 70 amino acid long inter-cross-link chain might only have 7 or 8 areas containing amino acids with large hydrophobic side groups, a number which is unlikely to globularize the chain, even in a non-cross-linked condition.

The presence of large-side-chained polar amino acids was recognised by Grey et al (see Chapter 1) and incorporated into their oiled coil model. In this model these amino acids were segregated into \( \beta \) helical regions of structure and \( \alpha \) helical regions consisting primarily of glycines and alanines. Desmosine cross-links occur only between the \( \alpha \) helices which act as connecting links between the \( \beta \) helices. The model is useful in that it explains the \( \alpha \) helix content of elastin, experimentally observed in solutions of dissolved elastin\(^{5-38}\), at the same time removing the necessity to incorporate proline into such helices. Proline, with its large hydrophobic side chain is the only commonly found amino acid whose side chain loops back to re-attach itself to the main chain. It has the property of forcing a bend in the main chain and of disrupting \( \alpha \) helices\(^{5-36}\). By incorporating it into \( \beta \) turns the model overcomes such objections.

The oiled coil model, however, proposes a structure which as yet has no backing from X-ray diffraction data\(^{5-39}\) and continues to rely on a 'liquid drop' type mechanism to explain the retractive force in the material.

The dynamic mechanical experiments performed in this chapter entirely justify the treatment of elastin as an amorphous polymer. No other postulated structure is necessary to explain the data observed. Water-elastin interactions, however, do have some effect on the mechanical relaxation spectrum of the material in its water-saturated condition.
5.6 Conclusions

(1) The real dynamic shear modulus of elastin at approximately 1 Hz in a constant inertia torsion pendulum experiment when examined as a function of temperature, in water, alcohol/water mixes and formamide and yielded curves:
   (i) In water and ethanol/water mixes typical of an amorphous polymeric material approaching the higher temperature end of its glass transition zone,
   (ii) In formamide, typical of a fully rubber elastic material.

(2) Observation of the real dynamic shear modulus parameter as a function of water content, and temperature at different water contents demonstrated the elastin/water composite to have a glass transition temperature highly dependent on water content, providing the water content was less than the water/dry elastin volume ratio of approximately 1:3.

(3) Measurement of the mechanical damping in elastin saturated with water and formamide at temperatures below 0°C yielded:
   (i) In its water saturated state a glass transition temperature closely associated with the melting point of ice and a β relaxation peak at -130°C;
   (ii) In its formamide saturated state a glass transition temperature at -60°C, unassociated with the melting point of solid formamide and a γ relaxation peak at -90°C.

(4) The results of this chapter are in complete agreement with the interpretation of the properties of elastin in accordance with the theory of rubber elasticity.
6.1 Introduction

The experimental findings of Chapter 5 were of considerable importance to the thesis. The conclusion that water saturated elastin behaves as a viscoelastic material at temperatures which include the mammalian physiological range of 35-40°C is of significance. In the fields of arterial research it is very common to find the assumption that 'the wall behaves as a perfectly elastic incompressible rubber' (eg: 6-1, 6-2). It was thus considered essential to attempt to obtain further support for these conclusions from some other reliable experimental technique.

In general, the field of mechanical testing of polymers falls into two categories:

(i) dynamic mechanical experiments where, as in Chapter 5, an oscillating stress or strain is applied to the material and its mechanical response measured, and

(ii) static mechanical, or step-function experiments where a set stress or strain 'step' is applied nearly instantaneously to the material and the resulting material creep or stress relaxation effects measured.

The work of Chapter 5 having fallen within the dynamic category, it was determined to employ an experimental method from the second group described above. One of the most useful and versatile pieces of manufactured apparatus available for static testing of materials is the 'Instron' tensile testing machine which employs a mechanically or hydraulically driven beam to apply strain to a specimen under test. The other
end of the specimen is connected via a connecting rod to an electronic load cell of considerably greater stiffness than the specimen. This machine is almost ideal for stress relaxation experiments where the beam can be used to apply a high-speed step extension to the specimen and the load cell used to accurately monitor the resulting time dependent axial force generated by the specimen.

A second application of the apparatus is in the determination of isometric force-temperature curves, the traditional method for establishing the relative, entropic \( (f_s) \) and energetic contributions \( (f_e) \) to the retractive force \( (f) \) in a polymeric material. The theory of such determination is described in detail in the subsequent sections of this chapter. To conform to the classical rubber type structure, the material should have a ratio of energetically generated force to total force \( (f_e/f) \) of less than 0.1 to 0.2. The determination of this ratio for elastin was thus considered a highly desirable objective and one which could establish beyond doubt the justification or otherwise of treating elastin as a classical rubber.

6.2 Background and Literature Review

The most common application force-extension measurements in experiments on rubber is in the determination of the respective energy and entropy contributions \( (f_e) \) and \( (f_s) \) to the total retractive force \( (f) \) as derived from simple thermodynamics

\[
f = \left( \frac{\delta f}{\delta l} \right)_{T,V} = \left( \frac{\delta u}{\delta l} \right)_{T,V} - T \left( \frac{\delta s}{\delta l} \right)_{T,V} = \left( \frac{\delta u}{\delta l} \right)_{T,V} + T \left( \frac{\delta f}{\delta T} \right)_{T,V,1} = f_e + f_s
\]  \hspace{1cm} 6.1
Where $F$ is Helmholtz free energy, $U$ is internal energy, $S$ is entropy, $V$ is the volume of the system, $l$ is the length of the sample and $T$ is the absolute temperature.

For 'ideal' rubbers, the internal energy term $f_e$ is zero, and the rubber elasticity can be attributed to entropy change only$^{6-8}$. However, in a similar manner to gases, the truly ideal state never completely exists and consequently there is inevitably a small energy term contributing to the rubbery retractive force of the material$^{6-4,6-5}$. Advanced rubber elasticity theory predicts that this internal energy term is essentially related to the internal rotation of polymeric chains$^{6-6}$.

For the investigation of energy and entropy contribution to rubber elasticity, it is necessary to determine the coefficient $(\partial f/\partial T)_{V,l}$ in equation 6.2, the temperature variation of retractive force at constant length and volume. This is, however, very difficult to measure by conventional experimental technique - a pressure chamber would be necessary to maintain constant volume. For this reason several thermodynamic approximations have been established to obtain $(\partial f/\partial T)_{V,l}$ from the corresponding coefficient at constant (atmospheric) pressure $(\partial f/\partial T)_{P,l}$ which can be easily determined under ordinary laboratory conditions.

The earliest studies of rubber elasticity were carried out by Meyer et al.$^{6-7,6-8}$ who used the simple approximation

$$\frac{\partial f}{\partial T} \approx \frac{\partial f}{\partial T}$$ 6.4

So

$$f_e = \left( \frac{\partial U}{\partial l} \right)_{T,V} = f - T \left( \frac{\partial f}{\partial T} \right)_{V,l}$$ 6.2

$$f_s = -T \left( \frac{\partial S}{\partial l} \right)_{T,V} = T \left( \frac{\partial f}{\partial T} \right)_{V,l}$$ 6.3
By using this equation, the internal energy term \( fe \) could be calculated immediately from the temperature coefficient of the retractive force at constant length and pressure. Meyer et al's results were the first to indicate that rubber elasticity was attributable predominantly to changes in the configurational entropy of randomly coiled polymer chains.

The accurate thermodynamic relationship, however, for relating

\[
\left( \frac{\partial f}{\partial T} \right)_{V,1} \quad \text{and} \quad \left( \frac{\partial f}{\partial T} \right)_{P,1}
\]

is\(^6,9\):  

\[
\left( \frac{\partial f}{\partial T} \right)_{V,1} = \left( \frac{\partial f}{\partial T} \right)_{P,1} - \left( \frac{\partial V}{\partial T} \right)_{T,P} \left( \frac{\partial P}{\partial T} \right)_{V,1}
\]

and the approximation of 6.4 is not always adequate since the second term in equation 6.5 is missed. Although \( (\partial V/\partial T)_{T,P} \) is generally very small for a perfectly rubbery material, \( (\partial V/\partial T)_{V,1} \) is usually large\(^6,9\) and can generate considerable error. As a consequence, another approximation has been found to be preferable\(^6,9,6,10,6,11,6,12\):

\[
\left( \frac{\partial f}{\partial T} \right)_{V,1} \cong \left( \frac{\partial f}{\partial T} \right)_{P,\alpha}
\]

where \( \alpha \) is the extension ratio defined by \( \alpha = l/lo \), \( lo \) being the length of the specimen in the unstretched state. This approximation is valid, however, only when \( \alpha \) is sufficiently small\(^6,9\).

Systems in swelling equilibrium with a diluent or mixture of diluents have added complications. Consideration must be given to the molar values \( N_i \) of the \( i \) diluent components present in the polymer phase. In this case equation 6.1 becomes in the most general case\(^6,9\):

\[
f = \left( \frac{\partial U}{\partial T} \right)_{V,T,N} = \left( \frac{\partial f}{\partial T} \right)_{V,1,N}
\]

where the subscript \( N \) signifies constancy of each of the molar values except \( N_i \).
As in the non-swollen case, the term \( \frac{\partial f}{\partial T} y^j \) cannot be measured without considerable difficulty, and so the equivalent relationship at constant pressure must be used instead. The relationship between the two has been provided as\(^{6-13}\):

\[
\begin{align*}
\left( \frac{\partial f}{\partial T} \right)_{P,1,eq} - \left( \frac{\partial f}{\partial T} \right)_{V,1,N} &= V\beta_v \left( \frac{\partial f}{\partial V} \right)_{T,1,N} + \sum_i \left( \frac{\partial f}{\partial N_i} \right)_{T,V,1,N} \left( \frac{\partial N_i}{\partial T} \right)_{P,1,eq}
\end{align*}
\]

where the subscript eq represents equilibrium swelling of the specimen in its diluent and

\[
\beta_v = \frac{1}{V} \left( \frac{\partial V}{\partial T} \right)_{P,1,eq}
\]

The last term in equation 6.8 is difficult to evaluate experimentally. It is dependent upon such parameters as the thermal expansion of the diluent components, the degree to which interactions between the diluent components and the molecules of the polymer network alter the molecular conformation, and the degree of disproportionation within the polymer phase.

The penultimate term \( V\beta_v \left( \frac{\partial f}{\partial T} \right)_{T,1,N} \) is also beset with experimental difficulties in its evaluation. Unlike the last term, which need only be evaluated in the case of an open system, it must be evaluated for both open and closed systems. As has been previously mentioned, an experimental system to provide the condition of constant volume for the \( \left( \frac{\partial V}{\partial T} \right)_{T,1,N} \) term would require the application of high pressures and extremely precise monitoring, so it is desirable to experimentally approach this term in some other manner such as by using a 'constant swelling' diluent to reduce \( \beta_v \) and hence the whole of the penultimate term to zero\(^{6-14}\). Alternatively
an equation of state may be invoked to calculate the correction term\textsuperscript{6-15}, an approach used by Mistrali et al. These workers performed experiments on purified ligamentum nuchae specimens in single component diluents where no multi-component disproportionation could occur, and selective influences on protein conformation were likely to be very much less dominant. It has been pointed out\textsuperscript{6-16} however, that Mistrali et al’s method is liable to some inaccuracy as the correction terms used were in some cases more than an order of magnitude greater than their largest stated values of $\frac{f e}{f}$.

An ingenious approach for obtaining the required correction has been derived by K.L. Dorrington\textsuperscript{6-16} and is utilized in Section 6.4.2 of this chapter. It is based on the fact that whether for pure shear, or simple elongation in which the volume ratio $\frac{V_{SD}}{V_{SU}}$ is regarded\textsuperscript{6-17} primarily as a function of $\alpha$ the extension ratio ($V_{SD}$ = the swollen deformed volume of polymer and $V_{SU}$ = the volume of the saturated material in its undeformed state), the theory of rubber elasticity predicts for a given sample an expression in the form of a product of a function of temperature and a function of relative elongation.

$$f = A(T)B(\alpha)$$

(6.9)

The theory of rubber elasticity\textsuperscript{6-9} associates $A(T)$ with $\frac{\nu k T}{\eta} \eta^{-\frac{3}{2}}$

where

$$\eta = \frac{\langle r^2 \rangle_1}{\langle r^2 \rangle_0}$$

$\langle r^2 \rangle_1$ is the mean square length of the $v$ cross-linked chains for the unswollen polymer, and $\langle r^2 \rangle_0$ the mean square length of the unperturbed chains at temperature $T$ and in the same solvent as exists in the polymer phase when under an equilibrium tensile force $f$:

$$\nu = \frac{V}{V_S} \quad \gamma = \text{volume of unswollen polymer}$$
Hence
\[ f = \frac{\nu k T}{l} \eta v^{-2/3} C(\alpha) \] 6.10

where \( C \) is a constant.

The need to consider the nature of dependence of 6.10 on relative elongation is eliminated\(^{6-13} \) by differentiation at constant \( \alpha \). Thus:
\[
\left( \frac{\partial \ln(f/T)}{\partial T} \right)_{P, c, eq} = -\frac{\partial \ln(r^2)_{\alpha}}{\partial T} + \frac{2}{3} \beta_v - \lambda \] 6.11

where \( \beta_v = \left( \frac{\partial \ln V_s}{\partial T} \right)_{P, eq} \) and \( \lambda = \left( \frac{\partial \ln l}{\partial T} \right)_{P, eq} \).

For isotropic swelling \( \beta_v = 3\lambda \) and so, using this and combining equations 6.2, 6.10 and 6.11:
\[
\frac{f_e}{f} = -T \left( \frac{\partial \ln(f/T)}{\partial T} \right)_{P, c, eq} + T\beta_v/3 \] 6.12

Dorrington\(^{6-16} \) has pointed out the two-fold advantages of this equation over that containing the force/temperature coefficient at constant length used by Mistrali et al.\(^{6-15} \). Firstly the analysis makes no assumption about the form of the extension ratio dependence of the load isotherm, and secondly the resulting swelling correction term does not become extremely large, tending to infinity as the extension ratio \( \alpha \) tends to unity as occurs for the correction term used in the analysis of Mistrali et al.

Dorrington's analysis provides a useful method for an estimation of the energy contribution to retractive force (\( f_e/f \)) for the water saturated elastin specimens as can be seen in Section 6.4.2.

The above theory applies to perfectly rubbery materials in their equilibrium condition. The work of Chapter 5, however, established elastin as being significantly viscoelastic when saturated in water and ethanol/water mixtures under small, dynamic shear stresses. Viscoelasticity in
mammalian elastic tissues is not only a recently recognized phenomenon. Roy in 1880\textsuperscript{6-10} described the effect in arteries as 'elasticity after action' and recognized its importance in the analysis of their mechanical behaviour. Since then, however, there has been rather a tendency to overlook viscoelastic effects for the sake of analytical simplification. Burton's comprehensive review of the structure and function of the blood vessel\textsuperscript{6-19} wall discusses the considerable viscoelasticity of its smooth muscle component, but makes no mention of any such properties in its elastin. More recent papers\textsuperscript{6-1,6-2} already mentioned are found to invoke the assumption of incompressibility resulting from the premise that the material of perfect rubber demonstrates complete in vivo elasticity.

Equilibrium force/temperature data can be obtained for the estimation of the energetic and entropic force contributions from rubbery materials in their transition region to the glassy state, but certain experimental precautions must be taken. The specimen must be allowed to relax, after the application of strain at the highest temperature of the range in which the retractive force is to be measured. The highest temperature is specified because it is invariably found that the distribution of relaxation times for a viscoelastic solid shows a temperature dependence giving a rapid decrease in times as the temperature is increased. Following relaxation, the temperature is cycled to lower values and up again. The degree of reproducibility of the curve can be taken as a measure of the extent to which pure equilibrium has been approached. Such methods have been discussed in detail by Tanaka et al\textsuperscript{6-20} who showed the marked variation on the $fe/f$ ratio generated by simple variations in experimental technique.
6.3 The Test Rig

The experimental apparatus consisted of a standard Instron Model TT/L floor-standing testing machine with mechanically driven loading beam, which could be raised or lowered at a wide range of speeds from 1 mm/minute to 50 mm/second.

Although the machine was basically designed to use the beam below the specimen and moving downwards in its tensile mode, another arrangement was found to be more suitable because of the necessity of retaining the specimen immersed in duluent. This was to use the beam (A) above the specimen. A hole through the centre of the beam permitted the passage of an invar connecting rod (B) which could hang freely with ample clearance from the sides of the hole.

A three-membered invar specimen column (C) of slightly larger proportions than the one used on the torsion pendulum rig in Chapter 5 was constructed, bolted at its top end to a plate (D) which could then be bolted into bolt holes on the under side of the beam. The connecting rod could thus hang down the centre of this column and baths of diluents could be raised around them when desired. The clamp attachments at the bottom end of the connector rod and base-plate of specimen column were designed so that two types of system could be fitted: a clamp system for strip specimens and a 'roller' system for aorta ring specimens.

The clamp system consisted of two sets of stainless steel clamps approximately 20 mm square and with blunt serrations on their inner surfaces to ensure adequate grip without tearing. They could be tightened or loosened by two small stainless steel bolts. Fitting into the connector rod was by a 'peg and pin' method; the top of the upper clamp was so
Diagram of essential features of apparatus based around Instron TT/L tensile testing machine used for step-function and static mechanical tests on purified porcine aortic elastin.

Plate 6.1
Plate of water-jacket and solvent bath surrounding specimen - as depicted in Fig. 6.1.

Plate 6.2
Plate of temperature control apparatus consisting of two water baths with pumps and thermostatic heater/stirrer units. Each bath also contains a 'McCrum tube' of fresh solvent for insertion around the specimen. An auxiliary heating element can also be seen in the left hand bath to provide an increased heating rate.
Fig 6.1. Instron test rig.
designed that it could be inserted into a cylindrical hole drilled into the bottom of the connector rod and when in place, held with a stainless steel pin driven through a horizontally drilled hole. The lower clamp was screwed directly into a central threaded hole in the base plate of the column.

The roller system consisted of two pairs of hook shaped stainless steel flanges (E) designed to occupy the top and bottom clamp positions respectively. Between each pair of flanges a small stainless steel 'roller' 2 mm diameter could be positioned and held in place against the bottom of the hooks by the specimen's own retractive force pulling on them. The space between the hook pieces was 5 mm, giving a roller width of that same distance.

The stiffness of the specimen support system (~ 10 GN/m) was considerably greater than that of the elastin (~ 300 kN/m) and the forces exerted during the experiment, low - typically less than 2N. Under these loads the extension in the connecting rod (the thinnest structural member) would be of the order 0.5 x 10^{-2} mm which, compared with the extension of the elastin (~ 5 mm), was clearly negligible.

The apparatus is outlined in Fig. 6.1.

The solvent bath and temperature control system were essentially the same as that used in the torsion pendulum experiment of Chapter 5. In this case, however, two temperature control water baths were used (see Plate 6.2) and flow could be changed rapidly from one to the other by a system of connecting brass pipes and taps. This was useful for the purpose of 'annealing' the specimen between applications of strain: to relax out the effects of previous strain cycles before each test one bath could be kept at the high annealing temperature and the other at the
temperature under study. The long process of raising and lowering the
temperature of a single water bath before each set of readings could
thus be avoided.

The double walled glass container was again used to surround the
specimen with solvent, this time, however, with no outer glass spill-trap,
as this was found to be obstrucrive and served little function if care
was taken in filling and emptying the inner cavity. McCrum tubes were
used for pre-heating the solvent with minimum evaporation.

The load cell of the Instron (F) could be accurately calibrated with
the aid of a set of calibration weights supplied with the machine. Cal-
libration was always checked before any specimens were mounted.

6.4 Experimental

6.4.1 Load Relaxation Curves at Varying T

6.4.1.1 Introduction and Theory

This experiment was designed solely to investigate further the
conclusion of Chapter 5 that elastin saturated with water and water/
ethanol mixtures behaves viscoelastically under similar experimental
conditions to the microcalorimetric experiments of Weis-Fogh and
Andersen and in a completely rubbery fashion when saturated with
formamide.

The viscoelastic condition of a polymer can be assessed by the
investigation of its time dependent stress response to an imposed
strain. On application of the strain the load will decay from its
initial peak at a rate dependent upon the temperature and distribu-
tion of relaxation times within the material. The phenomena of
relaxation processes can be thought of in terms of the effect of thermal motion on the orientation of the polymer molecules. When a mechanical stress is applied to a polymer, introducing deformations of the chains, the entropy of the system decreases as less probable conformations are taken up. The free energy correspondingly increases. If the sample is kept in the deformed state, stress relaxation takes place as a result of thermal motions of the chains, the molecular deformations are obliterated and the excess free energy is dissipated as heat.

The exact form of the relaxation process depends upon the multiplicity of ways in which the polymer molecules can regain their most probable conformations through thermal motion. The complex motions of a polymer molecule can be expressed as a series of characteristic modes requiring various degrees of long-range co-operation among the segments of the chain. Thus the first mode corresponds to translation of the entire molecule, requiring maximum co-operation, the second corresponds to motion of the ends of the chain in opposite directions, requiring somewhat less co-operation, and so on. With each of these modes is associated a characteristic relaxation time, but there are so many modes that over most of the time scale the spectrum of relaxation times can usually be approximated by a continuous distribution.

As the temperature is increased, so the thermal motion within the specimen is increased and the distribution of relaxation times is found to become shorter and shorter. In the rubbery region, however, the motions of the molecules are long-range, involving motions of units of the order of the length of the molecule itself. In this case thermal motions at any temperature within the region are sufficient
to allow the molecules to immediately adopt new lower entropy equilibrium positions without restriction. Virtually no relaxation effects are therefore exhibited, and the rubber will immediately present its theoretically predicted linear force-temperature curve, almost completely independent of time.

It is generally found that, in order to obtain reproducible results with creep and stress relaxation experiments, some form of conditioning procedure is required before each experiment to eliminate any non-linear viscoelastic effects and enable the material to 'forget' its past loading history\textsuperscript{6-21}. Such a procedure can generally be suitably carried out by 'annealing' the material for a period of time before each step-strain application, at some temperature equal to or higher than the maximum temperature of the experimental temperature cycle. This has the effect of removing any residual stresses of the previous step-strain application, hence presenting the material in a completely relaxed condition.

A further complication is added in the case of polymers (such as elastin) in swelling equilibrium with a diluent and this is the variation of swelling with temperature. The problem has already been mentioned several times during the thesis and was the main reason why many researchers\textsuperscript{6-14,6-15} ran their mechanical tests in constant swelling solutions such as 30:70 v/v ethanediol/water\textsuperscript{6-14} and water above 50°C\textsuperscript{6-15}. In any type of experiment where force is to be measured as a function of temperature at constant length, changes in swelling will artificially alter the true force generated by the material in response to the temperature. A constant swelling solution will hence correct this effect.
Three 'constant swelling' solutions for swollen elastin (excluding Mistrali et al's water between 50°C and 70°C6-15) were known to the author, two of which were discovered during the work of this thesis. The three are:

(i) 30:70 v/v Ethanediol/water, the solution used by Hoeve and Florey6-14.
(ii) 20:80 v/v Ethanol/water, 'discovered' during the work described in Chapter 4.
(iii) 40:60 v/v Glycerol/water, 'discovered' while investigating the glycerol/water system for similarities to the ethanol/water system using the apparatus of Chapter 4.

It was determined to use each of these three systems as well as water in the relaxation tests.

6.4.1.2 Apparatus

The apparatus used for this experiment was that described in section 6.3. As aorta ring specimens were to be tested, the 'rollers' were used instead of the clamps.

Temperatures were measured with standard mercury in glass thermometers and the water baths maintained at the required temperature to an accuracy of ± 0.2°C.

The magnitude of the strain-step could be pre-set on a pair of built-in mechanical gauges on the Instron. Initially a cathetometer was used to check the reproducibility of this strain-step, although this was discontinued after it had become apparent that the strain was being reproduced on each occasion to within an accuracy of ± 1%.

The loads measured by the load cell could be read off against time on a variable speed chart recorder.
6.4.1.3 Method

Ring specimens of purified porcine aorta were cut from a sample of region 3 aorta. Their dimensions were approximately 4 mm wide x 3 mm thick x 13 mm diameter. Large quantities of the solutions were made up of the appropriate diluents and their compositions adjusted with the aid of density measurements. These solutions were stored in clean, well-stoppered Winchester bottles until needed.

The Instron was calibrated by hanging calibration weights directly on to the load cell and adjusting electrically until the appropriate full scale deflection was achieved. The connector rod was then attached and the diluent equilibrated specimen looped over the rollers. The rollers were slipped on to their appropriate flanges and held in place while the inter-roller distance was increased slightly to ensure positive location. The inner cavity of diluent maintained at 20°C was then raised up around the specimen.

At this point the combined effects of the weight of the connector rod, specimen and rollers, and upthrust on specimen etc., were balanced out of the load cell response using the electrical balance control. The inter-clamp distance was then slowly widened until 10 gm load was registered on the chart recorder. This had the effect of straightening out the ring into two lamellae and applying a slight pre-load on the specimen to establish a clearly defined 'zero load' point. Using the cathetometer, the inter-roller distance was measured and from this result a suitable increase calculated to yield a 30% strain in the specimen (approximately 7 mm). The experimental procedure was then performed as follows:

One temperature control bath was heated to the 'annealing temperature' of 80°C and the other filled with a mixture of crushed ice and
water. Circulation from the annealing bath was allowed through the specimen water jacket for one hour before switching to the low temperature bath. At the same time the inner cavity mixture was replaced with fresh diluent from a McCrum tube from the cold bath. Five minutes were allowed for temperature equilibration before the chart drive-motor was switched on and a strain-step applied to the specimen at a speed of 20 mm/second. Relaxation was allowed to continue for 200 seconds. This was measured from the time of initial strain application and recorded on the chart recorder. During this period the temperature of the inner-cavity mixture was carefully monitored with a mercury in glass thermometer accurate to 0.2%. After 200 seconds the strain was removed.

The 'cold' or 'experiment' bath was then switched out of the circulation circuit and the annealing bath switched back in for a further hour. During this time the temperature of the experimental bath was raised to its next measurement level.

As before, the annealing bath was then switched out of, and the experimental bath pump switched into the circuit, the inner cavity solution was changed, and five minutes allowed for temperature equilibration before the second strain-step was applied and the specimen allowed to relax for a further 200 seconds.

The same procedure was thus carried out up the temperature scale at approximately 10°C intervals to around 60°C and then back again to 20°C. The whole cycle was then repeated to check for reproducibility.

At the end of this second cycle the specimen was left under strain at the annealing temperature for two hours. The temperature was then lowered and further measurements made of the force (fe) generated in this pseudo relaxed condition at various temperatures.
down to about 1°C. Following reproducibility checks on this curve, the experiment was terminated and the apparatus prepared for exactly the same complete experimental procedure with the next solvent.

Runs were thus carried out on the systems 20% ethanol/80% water, 30% ethanediol/70% water, 40% glycerol/60% water, pure water and pure formamide. The results and their analyses are presented in the next section.

6.4.1.4 Results

The results obtained from the experiment were in the form of force vs time, \((f_t \text{ vs } t)\), curves drawn by the chart recorder. From these, readings of \(f_t\) were taken at 2 seconds, 5 seconds, 10 seconds, 60 seconds and 190 seconds, the instant of strain application being taken as 'zero' time. These \(f_t\) readings were plotted against temperature \(T\) to obtain the load relaxation curves shown in Figs. 6.5 to 6.9. On the same graphs are plotted the \(f_R\) data points.

6.4.1.5 Discussion

Reference to graphs 6.5 to 6.9 shows that over the temperature range tested in all solvents except formamide the material exhibited significant viscoelasticity.

In the case of the ethanol, ethanediol and glycerol solutions, interpretation of the curves was particularly straightforward, since the \(\beta_v\) values as mentioned in Chapter 5 were close to unity in the measured temperature range of 0-60°C. The observed behaviour was in complete accord with the torsion pendulum experiments of Chapter 5. The temperature dependence of \(f_t\) (negative and curved) and the time dependence (greatest at lowest temperature) was typical of an amorphous polymer in the viscoelastic transitional zone. Furthermore, the \(f_R\) vs \(T\) plots of the final phase of each experiment made clear the
Typical $f_t$ curve produced by Instron in stress relaxation experiments of section 6.4.1. Data from these curves used to obtain curves of figs 6.5-6.9.
Fig. 6.5

Plots of time dependent force ($f_t$) and force at pseudo-infinite time ($f_\infty$) against temperature for step strain experiments on purified porcine aorta saturated with 20:80 v/v ethanol/water.

Fig. 6.6

As Fig. 6.5, but with 30:70 v/v ethanediol/water solvent mixture.

Fig. 6.7

As Fig. 6.5, but with 40:60 v/v glycerol/water solvent mixture.

Fig. 6.8

As Fig. 6.5, but with water alone as solvent.

Fig. 6.9

As Fig. 6.5, but with formamide alone as solvent.
Fig 6.5. $f_t \& f_R$ vs. $T^\circ C$ in 20/80 ethanol-water mixture.
Fig. 6.6 $f_t$ & $f_R$ vs. T°C in 30/70 ethanediol/water mixture.

![Graph showing force as a function of temperature for different time intervals (25s, 55s, 10s, 60s, 120s).]
Fig. 6.7 $f_t$ & $f_R$ vs. $T^\circ C$ in 40/60 glycerol/water mixture.
Fig. 6.8. \( f_R & f_t \) vs. \( T \degree C \) in water.
Fig. 5.9 $f_R$ & $f_t$ vs. T°C in formamide.
relationship of the determined values of $f_R$ to force-temperature observations obtained in conventional thermoelastic experiments on rubbers. The observed curves were all of positive slope at low temperatures and essentially zero slope above 50°C.

The usual thermoelastic procedure for studying elastomers is to deform at the highest temperature for some time (for example overnight $^{6-15}$) and then to observe the temperature dependence of force at constant length. The resulting force temperature plot is analogous to the $f_R-T$ plots observed in the solvent/water equilibrated systems. This experiment, however, does not measure the mechanical state of the material under the conditions described by Weis-Fogh and Andersen. The essential difference is one of time: short time ($10$ to $10^3$ seconds) in the case of Weis-Fogh and Andersen and pseudo-infinite time in the determination of $f_R$. In the case of the elastin/formamide system of Fig. 6.9 this difference of time scale is not significant; the observations show that the $f_T$ and $f_R$ vs temperature plots are of positive slope and essentially parallel, and it follows that for this highly swollen system the theory of rubber elasticity is directly applicable. For the less swollen elastin-water and elastin/solvent/water systems, the time dependent $f_T$ will depend on other parameters in addition to $f_R$, notably the distribution of relaxation times for the relaxation process.

The curves of the elastin-pure water system also show the viscoelasticity of the elastin solvent/water systems. In this case, however, results are complicated by the extreme swelling effects; $\beta_v$ is not zero, which is represented by the sharp drop off in $f_R$ and $f_T$ values at the lower end of the experimental temperature range.
6.4.2 $f_e/f$ Assessment of Water Swollen Elastin

6.4.2.1 Introduction and Theory

This method has already been partially discussed in section 6.2 of the chapter. In conception it is attributable to K.L. Dorrington, and the following experiments of this section were performed in conjunction with him on the test rig described in section 6.3.

The object of the experiment was examination of the $f_e/f$ ratio of the material without having to resort to the use of either multi-component diluents or special temperature regions to obtain the constant swelling condition or equations of state employing large correction. Such methods have been outlined already in section 6.2.

Dorrington's method permits an assessment of the $f_e/f$ ratio for the material from force-temperature results obtained in water, utilizing only a comparatively small correction term.

The importance of knowing the ratio $f_e/f$ for the material must (at the risk of repetition) be stressed. It is the predominant contribution to elastic force generated in the material from entropy which is the distinguishing factor of elastomeric solids. Their recoverability from comparatively large mechanical strains is an outcome of this fact, but not necessarily a requisite requirement for a material to be classified as a rubber. A metal spring, for example, has equal ability to a strip of rubber to recover elastically from a large extension, yet its retractive force is energy generated arising from the distortion of inter-atomic forces in its crystal lattice.

Assessment of a value of $f_e/f$ for elastin in conditions similar to its physiological state (water equilibrated) gives a clear affirmation or refutation of the treatment of the material as a rubber, according to the classical theory of rubber elasticity. Hence
Dorrington's method and the experiments of this section might be described as one of the most important mechanical tests described in this thesis.

6.4.2.2 Apparatus

The apparatus was identical to that used in the experiments of section 6.4.1 with the one exception that the clamps were used instead of the rollers. This was because the experiment was to be performed on a strip specimen to minimize possible end effects.

6.4.2.3 Method

A thick aortic ring specimen was taken from a piece of region 3 porcine aorta and one side cut across to allow the ring to be opened out into a strip of approximate dimensions, 30 mm length x 17 mm width x 2 mm thick. This specimen was equilibrated in water and mounted in the clamps of the Instron test rig for testing.

The temperature of the inner cavity water was raised to 72.9°C and a step-strain of approximately 30% applied to the specimen. Following this it was allowed to relax at that temperature for 2 hours. The temperature was then varied between 0-70°C and force measurements ($f_R$) recorded over this range. Before each reading of load the temperature was maintained constant for 30 minutes. Upon re-attaining the high temperature, the length of the specimen was decreased and the specimen relaxed for a further hour before repeating the procedure. Plots of $f_R$ against temperature were thus obtained for successively decreasing lengths of specimen down to zero load. After all the plots had been completed, the specimen was extended at the high temperature to a length near the maximum used for the first curve and allowed to relax for 2 hours. The $f_R$ value derived from this was taken as a
check of overall reproducibility. It was found to be within 2.5%
of the value suggested by interpolation of the main spectrum.

6.4.2.4 Results

The force vs. T curves obtained directly from the experiment are
shown in Fig. 6.10. From these curves it was possible to obtain
constant temperature plots of force against length (Fig. 6.11).
Extrapolation of these curves to zero load yielded the undeformed
swollen length as a function of temperature (Fig. 6.12), and from
this curve logarithmic differentiation provided the linear swelling
coefficient (Fig. 6.13) in the direction of retraction. This co-
efficient $\lambda$ could be taken as a close approximation to $\beta_V/3$ as
discussed in section 6.2 and shown in Fig. 6.17, where $\beta_V$ values
from the data of Chapter 4 were used to plot $\beta_V/3$ against $\lambda$ values
calculated from the data of this chapter.

Hence, with the linear swelling of the unloaded specimen known,
it was possible to plot constant temperature curves of retractive
force against relative elongation ($\alpha$) (Fig. 6.14).

Fig. 6.14 shows the degree to which the results conform to
equation 6.9. For the separation of variables to be valid, it is
necessary for the different $f_R$ vs. $\alpha$ isotherms to bear the same ratio
to each other for all relative elongations. Conformation to the
separation of variables was demonstrated quantitively (Fig. 6.15)
where normalised force values, obtained by calculating $(f_R/f_{70^\circ C})_\alpha$
for five different $\alpha$ values in the range 1.0-1.25, were plotted against
temperature. For perfect separation of the variables, the curves for
different $\alpha$ would be coincident and in Fig. 6.15 the scatter is within
5%. When data for $\alpha = 1.05$ was neglected, for which the analysis was
particularly difficult owing to the narrow load range, then the scatter was well within 2%. Hence it could be concluded that the assumption of equation 6.9 was valid for the experimental conditions used.

Equation 6.12 could therefore be used, re-arranged in terms of the normalised force $f_N$:

$$f_e/f = -T \left[ \frac{\partial \ln(f_N/T)}{\partial T} \right]_{p,eq} + \frac{T \beta_v}{3}$$  

6.13

As it has been shown that the derivative is essentially independent of $\alpha$, the subscript $\alpha$ is no longer written in the above equation.

Fig. 6.16 shows the plot of $\ln(f_{N}/T)$ against temperature for data obtained directly from $f_{R} = \text{length isotherms of Fig. 6.11}$. $f_{N}$ represents the average of the normalised force over the five $\alpha$ values in the range 1.0 - 1.25. For comparison are plotted curves for $f_e/f$ values of 0 and 1 which could be obtained by re-arranging equation 6.13:

$$\left( \frac{\partial \ln(f_N/T)}{\partial T} \right)_{p,eq} = \frac{T \beta_v}{3} - \left( \frac{f_e/f}{T} \right)$$  

6.14

or remembering $\beta_v/3 = \lambda = \frac{d \ln l}{d T}$

$$\Rightarrow \left( \frac{\partial \ln(f_N/T)}{\partial T} \right)_{p,eq} = \frac{d \ln l}{d T} - \frac{f_e}{f} \cdot \frac{1}{T}$$  

6.15

And hence, substituting the values 0 and 1 for $f_e/f$ in 6.15 and integrating between $T^0K$ and $343^0K (70^0C)$, the theoretical $\ln(f_N/T)$ against temperature curves for $f_e/f = 0$ and $f_e/f = 1$ could be calculated.

Of necessity, all the data corresponds for $T = 70^0C$ by reason of the definition of $f_N$. Scatter is greater at low temperatures, but
Plots of force against temperature from water saturated porcine aortic elastin specimen held at varying lengths after time dependent stress relaxation effects allowed to decay to insignificance.

Fig. 6.11

Isothermal plots of force against length obtained from data of Fig. 6.10. Extrapolation of these isotherms to zero force enables a plot of length (due to swelling and thermal expansion) against temperature to be obtained. This is shown in Fig. 6.12.
Fig. 6.10. \( f_R \) vs. \( T \) curves in water.
Fig. 6.11. $f_R$ vs. length isotherms. [from fig. 6.10.]
Fig. 6.12. Length vs. temp. at zero force.
(from fig. 6.11)
Plot of the linear swelling coefficient $\lambda$ (in the direction of retraction) against temperature from the data of Fig. 6.12. Knowledge of the value of the linear swelling coefficient can then be applied to the force-length data of Fig. 6.11 to obtain the force-extension ratio ($\alpha$) isotherms of Fig. 6.14. The observation of the close identity of these isotherms justifies the use of equation 6.12.

With the data of the previous figures, it is possible to obtain a true value (without swelling effects) of the elastic force generated by the material and this is plotted in Fig. 6.15 (normalized as indicated) against temperature.
Fig. 6.3  Swelling in direction of retraction.
Fig. 6.14. $f_R$ vs $\alpha$ isotherms.
\( f_N = \text{Normalized force} = \left( \frac{f_{R|0^\circ C}}{f_{R|70^\circ C}} \right) \) at constant \( \alpha \).

\( \bar{f}_N = \text{Mean } f_N \text{ of } \alpha \text{ values; } 1.05, 1.10, 1.15, 1.20, 1.25. \)
Finally it is possible to plot $\ln(F_N/T)$ against temperature and compare the experimentally derived results with those predicted for $f_e/f$ ratios of 0 and 1 respectively. An indication of the energetic contribution to the elastic rettractive force of porcine aortic elastin is thus obtained.

**Fig. 6.16**

Plot of values of the volume expansion coefficient ($\beta_V$) derived from liquid uptake studies of Chapter 4 against those derived from the linear expansion coefficient (ie: $3\lambda$) obtained experimentally from section 6.4.2.
Fig. 6.16. $\ln(\frac{f_N}{T})$ vs. $T$. 

- Theoretical curves based on swelling data.
- Data from $f_e$ and $f'_e$ length.
- The $f_e/f = 1$ and $f_e/f = 0$ conditions are indicated.

Temperature [°C] vs. $\ln(\frac{f_N}{T})$. 

- The graph shows a plot of temperature against the natural logarithm of the ratio $\frac{f_N}{T}$.
Fig. 6.17  $\beta_x$ vs. $3\lambda$
this is predictable from the limited isotherm lengths calculable from the raw data of Fig. 6.10 for these temperatures.

The results indicate a value of \( f_e/f \) for the system of \( 0.0 \pm 0.1 \).

6.4.2.5 Discussion

The results of the experiment thus produced an assessment of the \( f_e/f \) ratio for elastin equilibrated in water. The result is typical of an elastomeric material and is in full agreement with the treatment of elastin as a classical rubber.

One particular advantage of the method was its reduction of errors resulting from non-uniformity of loading from uneven specimen support. This was because the equation used (6.13) was valid for all behaviour corresponding to equation 6.10. Thus, whatever load condition the specimen was under, so long as the two variables of equation 6.10 could be split, equation 6.13 was valid. This was particularly fortunate, as for a highly swollen gel it is virtually impossible to obtain a uniformly retracting sample. For a sample which is particularly broad in relation to length (for this experiment the ratio was approximately 0.5) the restraint at the clamps is likely to produce non-uniformity due to lateral contraction, the effect being most noticeable at large extensions. Shear will thus be present in such a system.

Another source of non-uniaxiality of load is due to the fact that the specimen was cut from the wall of a nearly cylindrical vessel, and as a consequence has an in-built tendency to curve naturally if left unsupported. The straightening action of the clamps will thus introduce additional stresses due to bending.
These two factors hence introduce a small measure of triaxiality into the system. Nevertheless, if firstly the load isotherms show a functional relationship in which the variables $T$ and $\alpha$ may be separated, and secondly if the temperature dependence predicted by the theory of rubber elasticity remains valid even for the possibly non-affine deformations encountered in the experiment, the analysis can be considered valid.

The first of these two qualifications has already been considered. The second, however, is more difficult to assess. In consideration of the problem, Dorrington\textsuperscript{6-16} has written:

'To state a pre-supposition for looking at the temperature dependence of the load isotherms, we must specify a value and temperature dependence of $f_0/f$; and yet this is the parameter we set out to determine. We can only conclude from the analysis that assuming, with some good reasons for doing so, dependence of retractive load given by the equation (6.10), we observe the value of the parameter $f_0/f$ to be very much less than unity and to be sensibly constant. In that the latter observation is to be expected for a moderately small temperature range, it is an important one on which to judge the theory.'

Another possible source of error which must be considered is the effect of any swelling anisotropy within the specimen. Mistrali et al\textsuperscript{6-15} noted qualitatively some degree of anisotropy of swelling in purified elastin, although later quantitative measurements showed its overall effect on the volume swelling coefficient $\beta_v$ not to be large enough to significantly alter the final value of the $f_0/f$ ratio. Hence, taking into account that the work of Mistrali et al was with purified ligamentum nuchae which is likely to be considerably more anisotropic than purified aorta, the assumption of $\beta_v = 3\lambda$ seems reasonable.
6.5 General Discussion

The work of Chapter 6 confirms the hypothesis derived from the experiments of the earlier chapters that elastin can be classed as a classical rubber. The fact that it is not completely through its transition zone in the presence of water and various solvent water mixes, at physiological and typical ambient temperatures, does not prevent it from being predominantly entropy elastic.

The experiments of 6.4.1 showed that in order to perform meaningful force temperature plots for $f_e/f$ analysis on water and solvent/water saturated specimens, it is necessary to allow time for the specimen to relax to a pseudo-infinitely relaxed condition. A simple experiment showed that to reach the pseudo-infinitively relaxed state reached after 2 hours at 30% strain in water at 70°C, approximately 50 days would be required at 1.0°C. It was with this knowledge that the $f_e/f$ assessment experiment of section 6.4.2 could be successfully planned and undertaken without providing erroneous non-repeatable force values resulting from insufficient relaxation.

Indication that the pseudo-infinite 2 hour 70°C relaxation period was itself sufficient was gained from the reproducibility point taken at the end of the experiment which showed an upward shift of 2.5%. Any significant further relaxation would have shown the opposite effect as would other possible error sources such as slippage in the clamps or tearing within the specimen itself.

One interesting result to arise from experiment 6.4.2 is a comparison of the one dimensional swelling coefficient ($\lambda$) shown in Fig. 6.13 and the swelling coefficient ($\beta_w$) obtained from the water uptake data of Chapter 4. These parameters are compared in Fig. 6.17, the curve of which shows the
excellent correlation of 0.985. This thus gives an important confirmation of the method employed in the x-plot determination.

Finally, one other point which must be considered is the deviation of the ethanol/water, ethanediol/water and glycerol/water curves from direct proportionality between equilibrium force and absolute temperature (as in the formamide case). Such proportionality is predicted$^{6-13}$ for a system in which:

(i) the internal energy contribution to the total retractive force is small ($f_e/f << 1$).

(ii) disproportionation of solvent within the polymer phase is negligible;

(iii) equilibrium swelling is independent of temperature.

Considering, therefore, these three points:

(i) has been shown$^1$ to apply to water and formamide saturated elastin and it is thus reasonable to assume that it extends to the other systems;

(ii) as a contributory factor remains in dispute, with Hoeve and Flory$^{6-13}$ claiming invalidity of the criticism of their two component diluent method by Oplatka et al$^{6-22}$, but significant temperature dependent disproportionation actually claiming to have been measured in the 30:70 v/v ethanediol/water system by Mistrali et al$^{6-15}$;

(iii) has been examined in the liquid uptake studies of Chapter 5; the work shows that although $\gamma_T$ varies almost imperceptibly with temperature and the temperature dependence, nevertheless has a magnitude large enough to modify the slope of the $f_R$ curve.

Hence, taking these considerations into account, the experiment shows no discrepancy between the form of the $f_R$ vs temperature curves obtained and the claim of near ideal elasticity for swollen elastin in the equilibrium state.
(1) Elastin saturated in solutions of water, 20:80 v/v ethanol/water, 30:70 v/v ethanediol/water and 40:60 v/v glycerol/water exhibits significant viscoelasticity between the temperatures 0°C and 70°C but near ideal rubber elasticity in its equilibrium state.

(2) Elastin saturated in formamide demonstrates typical equilibrium rubber elastic properties between the temperatures of 0°C and 70°C.

(3) The ratio of $f_0/f$ for water saturated elastin is of the order $0 \pm 0.1$.

(4) The experimental results fully agree with the conclusions of the previous chapters and the re-interpretation by this thesis of the results of Weis-Fogh and Andersen in terms of classical rubber elasticity theory.

6.7 Acknowledgement

The work of section 6.4.2 of this chapter was performed in conjunction with K.L. Dorrington, who formulated the bulk of the relevant theory described in sections 6.2 and 6.4.1.1. Its inclusion in this chapter is in consideration of its high degree of relevance to the arguments of the thesis and with the full permission of K.L. Dorrington. For this permission the author expresses his gratitude.
Chapter 7

ELECTRON MICROSCOPY

7.1 Introduction

Experimental studies of the structure of polymeric and other material can be roughly divided into two groups: indirect and direct methods. Indirect methods include the bulk of the experimental work described in Chapters 3 to 5 of this thesis, and consist basically of examining the response of the specimen to some form of applied energy (i.e., heat or mechanical). The results can then be compared with predictions based on theoretical models of material structure.

The somewhat less subtle 'direct' techniques rely on the modification in some manner of an energy source applied directly to the specimen. Such energy sources include electron, neutron or photon beams, X- or \( \gamma \)-rays, magnetic and electric fields. In each case the applied energy pattern is modified by the chemical or crystal structure of the material, and the modified pattern displayed visually on screen or recorded on film, chart, tape, etc. One of the most common of the group of direct methods available is the technique of electron microscopy. Electron microscopes have been applied to the study of elastin structure with varying degrees of success.

In the indirect experiments used in previous chapters of this thesis, an argument was established for a random molecular chain structure of elastin, akin to that of a classical rubber. This, however, appears in the literature to be contradicted by a number of electron microscopic investigations. These works are described in the following section and, with their results in mind, it was thus considered appropriate to
re-examine the material by electron microscopy to investigate some of
the elastin structures previously claimed to exist.

7.2 Background and Literature Review

The full background and history of the development of electron
microscopy will not be included. The techniques are now too well estab-
lished to warrant any special review of their development and are
excellently covered elsewhere\textsuperscript{7-1,7-2}. The section will be limited rather
to electron microscopic studies of the connective tissue and in particular
to elastic tissue and elastin as well as to pertinent specimen preparation
techniques. Except where mentioned, the following text refers to trans-
mission electron microscopy, scanning having been already reviewed in
Chapter 3.

The first reports of the use of electron microscopy for studies of
elastic tissues were in the 1940's by Wolpers\textsuperscript{7-3} and Gross\textsuperscript{7-4} respectively.
Wolpers used a partial digestion treatment of ligamentum nuchae with
pepsin in acid for 24 hours before examination in the microscope. He
reported large branching amorphous fibres varying in width from 250 nm
to 20 nm, the very smallest observed being 8 nm. No axial periodicity
was noted though fixation in osmic acid appeared to result in a fine
longitudinal fibrillation which was ascribed to the action of the fixative
and not intrinsic to the actual structure. Orcein, a stain considered
relatively specific for elastic fibres, was observed to deposit on these
fibres in the form of small flakes. Elastin from mouse aorta, after acid
pepsin digestion, osmic acid fixation and sonic fragmentation, appeared
as thin fenestrated laminae with numerous short, stubby fibres protruding
from the surface.
Gross's observations\(^7\text{-}^4\) were made on fragmented elastic tissue from fish swim bladder, bovine ligamentum nuchae and aortas of various mammals including man. Boiling in dilute acid was found to destroy associated collagen, but not 'obviously alter' the elastic tissue. Digestion in crystalline trypsin of either boiled or unheated tissue from any of the above mentioned sources caused release of thin threads ranging in length from 100 nm to many microns. A large proportion of these consisted of evenly and tightly coiled double helices formed from at least two interlacing filaments and measuring about 12 nm in diameter. Raising the pH of a neutral suspension of threads from ligamentum nuchae, it was found that the ratio of threads to uncoiled filaments was lowered, whereas lowering the pH with acetic acid resulted in clumping of threads with complete loss of identity at about pH 3.6. Gross was the first to suggest that the elastic fibre was a two component system composed of bundles of trypsin resistant threads of characteristic form and size plus a trypsin sensitive heat-resistant 'amorphous' binding matrix.

Far more recently the two phase elastic fibre has been demonstrated by a number of workers\(^7\text{-}^5,7\text{-}^6,7\text{-}^7\), culminating in the complete enzymatic study of Ross and Bornstein\(^7\text{-}^8,7\text{-}^9\). These workers studied each of the two components separately by electron microscopy and amino acid analysis and concluded that the threadlike or microfibrillar component consisted of a glycoprotein, rich in polar amino acids and cystine, but relatively poor in basic and neutral amino acids and lacking hydroxyproline, hydroxylysine and the desmosines. The seemingly amorphous component appeared to be the 'true elastin', being rich in non-polar amino acids, containing desmosine and isodesmosine and being of general amino acid content identical to that of the material which formed the major proportion of the elastic fibre in the adult mammal.
Electron microscopy has been used in specific studies of each of those two components although probably to a somewhat greater extent in the case of the 'amorphous' elastin than the microfibrillar phase. Microfibrils from elastic tissue were, however, studied electron-microscopically by Haust\textsuperscript{7-10} who concluded that they were, in fact, identical to the fine microfibrils of the general extracellular space. Fibrils observed in relation to various basement membranes appeared to be of the same nature, and the observations strongly supported the concept that there was one organized precursor 'the microfibril' common to both collagen and elastic tissue. Haust also found that the 'periodicity' of the microfibrils seemed to vary. In the material examined which included a number of locations and animal species (including human) it ranged from 7 nm to 14 nm, while the diameter of the microfibrils varied from less than 4 nm to as much as 14 nm. Thus, while the width of certain microfibrils was comparable in several tissues, their periodicity was not necessarily identical. The structure proposed to account for these observations\textsuperscript{7-10} was that of either a helical- or coiled-coil type. It was claimed that either of those configurations (provided there were no rigid cross-linkages) could account for the variation in the width and periodicity of the microfibrils.

Haust's work\textsuperscript{7-10} showed that the fine microfibril of extracellular space was a common structural denominator in collagenic and elastic tissues and in basement membranes. It is, however, the amorphous component, or elastin, (as defined in Chapter 1 of the thesis), which is relevant to this work. It has been demonstrated that the elastin/microfibril ratio increases markedly with age\textsuperscript{7-5}. In rat foetuses aged 15-18 days, most elastic fibres consist almost entirely of microfibrils, the elastin only appearing in later development. By the time the animals reach adulthood
the elastic fibre consists predominantly of elastin with microfibrils forming only a relatively small proportion of their mass. It is thus the elastin which is the important structural element in the elastic fibre of the adult mammal, not the microfibril.

In order to study the structural geometry of isolated connective tissues such as the molecular architecture of elastin, high instrumental magnifications are required to produce the necessary resolution as well as effective image contrast enhancement techniques such as staining. Early electron microscope workers were, without exception, limited by the instruments and techniques available to them.

Attempts, however, were made to improve image contrast by new methods of electron-dense staining. Usuku made use of a potassium permanganate stain developed by Luft to observe the structure of ultra-thin sections of elastase purified ligamentum nuchae. His conclusions were that the sections were constituted of a reticular network of beaded fibrils which lacked axial periodicity, measured 10 - 20 nm in width and were composed of 'beads' of a diameter of equal dimension to the width. It was assumed that those beaded fibrils were an elementary unit of the elastic fibre. Further studies by Usuku and co-workers using the same technique, showed similar beaded microfibrils in trypsin and α-amylase treated ligamentum nuchae and fixed but untreated rabbit internal elastic lamina.

Probably one of the most significant advances of the last two decades in biological ultrastructural research was the development of a technique of a routine negative staining method for electron microscopy. This was largely due to Horne and Brenner. The positive staining techniques used in most previous elastin electron microscopic studies worked by the principle of contrasting the structure by combining some heavy
metal fixative or stain of high atomic number with the material. This procedure preserved the three-dimensional structure and the fixed and stained particles appeared as electron dense objects against a relatively transparent background. In the negative staining technique, however, the reverse principle is used, namely that of surrounding or 'embedding' the particles or fibrils of the structure with an electron-dense material. The structure thus appears bright against a dark background.

The earliest examples of the principle of negative staining were in light microscopy in the studies of bacteria. It was not until the mid 1950's however, that the first reports of reverse contrast effects in the electron microscope were published.

During studies on tobacco mosaic virus rods, Huxley showed that the electron-dense phosphotungstic used by him as a stain penetrated along the 4 nm diameter axial hole known to exist in the tobacco mosaic virus rods from previous X-ray analysis. Moreover, areas of rods were seen to be surrounded by the phosphotungstic acid. It was this work which stimulated Brenner and Horne to develop their method.

The original purpose of Brenner and Horne's method was to enable large numbers of specimens prepared by other physical and chemical methods to be examined in the electron microscope. Consequently, simplicity was of major importance. Their original procedure was to prepare a 2% solution of phosphotungstic acid (PTA) in water or ammonium acetate and adjust the pH to a neutral value between 6.8 and 7.4 by adding small drops of 1M-NaOH. The potassium phosphotungstate was then added to a suspension of the specimen (in this case a virus) and the mixture sprayed on to carbon filmed supports. Droplet patterns were formed by the electron-dense phosphotungstate enclosing or surrounding the virus particles and thus produced a reversal of contrast seen in the final image.
Phosphotungstic acid salts have been found not to be the only materials effective as negative stains. The merits of silver nitrate, uranyl nitrate, sodium tungstate, cadmium iodide have also been considered\(^7\textsuperscript{-25}\). Uranyl acetate, however, has been shown to produce the highest resolution in examinations of the tobacco mosaic virus\(^7\textsuperscript{-26}\).

The first report of the technique of negative staining applied to elastin appears to have been by Gotte\(^7\textsuperscript{-27}\) and co-workers\(^7\textsuperscript{-28},7\textsuperscript{-29}\) during his numerous electron microscopic and other studies on the structure of elastin in the early 1960's. From these it was claimed that if elastin was sonicated with ultrasound to fragment its structure, an 'ultimate' filament was finally produced with a diameter variously stated as 10 nm, 8 nm and 3 nm. These filaments were claimed to be arranged in a roughly parallel manner within the whole fibres.

In 1966 two unrelated papers were published reporting use of negative staining techniques in electron microscopic studies of elastin\(^7\textsuperscript{-30},7\textsuperscript{-31}\). Both studies used solutions of phosphotungstic acid, although in one\(^7\textsuperscript{-30}\), lithium tungstate was also used. Specimens included foetal, adult and old adult human aorta\(^7\textsuperscript{-31}\), three and four week old normal, copper-deficient and \(\beta\)-aminopropionitrile (BAPN) fed chick aorta, bovine ligamentum nuchae and ear cartilage\(^7\textsuperscript{-30}\). Extraction was by autoclaving, hot alkali\(^7\textsuperscript{-30},7\textsuperscript{-31}\) and formic acid treatments\(^7\textsuperscript{-30}\). The results of these investigations were in marked disagreement. One\(^7\textsuperscript{-30}\) reported a three-dimensional network of amorphous fibrils and the other\(^7\textsuperscript{-31}\) a fairly regular filament structure of constant minimum filament diameter 2.5 nm. Both works, however, had one important aspect in common, namely, use of a homogenization process of specimen preparation as opposed to one utilizing the ultramicrotome for thin sectioning purposes.
Ultramicrotome sectioning, a standard biological electron microscope preparation technique, is a method which has seemed to meet with little success in elastin ultrastructural studies, although it was the method used predominantly by the early electron-histologists. Fibrillar networks do not lend themselves naturally for high resolution electron microscopic studies via sectioning techniques. Even with the most efficient ultra-microtomes it is difficult to obtain sections below around 50 nm thick, and it is usually necessary to use some form of polymeric embedding agent in which to conduct the sectioning. The setting of these embedding agents can chemically or physically damage the fine micro-architecture of the material, or themselves demonstrate structures under high magnification which can confuse analysis of results. The action of the sectioning blade also can produce artifacts and specimen damage, and will rarely section exactly in the plane of greatest interest (in this case parallel with the axis of the fibre). It is thus probably true to say that ultrathin sectioning techniques are best suited to low resolution 'topographical type' studies where the diameter of the object under study is of the same order as the thickness of the specimen, and not to high resolution studies of tissue ultrastructure.

Nevertheless, one of the more successful studies of elastin preparation by ultrathin sectioning was that of Cox and Little who combined electron microscopy with wide-angle X-ray diffraction studies on aorta specimens from rabbit, sheep, goat and man. Stereoscopic photographs were taken using the method of Little to produce a clearer view of the elastic tissue texture by means of three-dimensional images. Their results indicated that all the elastic tissue from the various sites of the human and animal body possessed the same fine structure. The texture was homogeneous with some variation in internal density, and frequently
with a rather more opaque surface zone. The X-ray diffraction patterns obtained were those of a non-crystalline polymer, and so a truly amorphous molecular structure was concluded.

If one aspect has emerged from electron microscopic studies of elastin over the last three decades, it is that a considerable amount of disagreement has been generated about its structure. In the late 1960's the situation was further complicated by the proposal of the globular model elastin structure, described in Chapter 1.

In support of the globular structure, a series of electron micrographs were presented purporting to demonstrate the proposed arrays of linked globules (see Fig. 1.3). It was recognized that during the drying of elastin some collapse of the structure was likely, and an attempt was made to avoid this by impregnation with a polyglycol. This was found to produce a structure with a significantly different appearance to dry elastin, as well as to dry, precipitated α-elastin films. It was suggested that previous observations of fibrillar type structures could be the result of the ultrasonication to which the specimens had been subjected during their preparation process.

Gotte et al studied negatively-stained specimens of purified ligamentum nuchae prepared both with and without ultrasonication. Observations showed the elastin to be composed of a network of branched bundles possessing a wide range of diameters. High resolution micrographs revealed that the fibres consisted of slender filaments of about 3 - 4 nm diameter, arranged roughly parallel to the fibre long axis. A regular periodicity of 4 nm was resolved which suggested a helical array. To establish that the results were not due to any contamination by collagen fibres, differences were pointed out between the periodicities of the observed material and collagen. It was also stressed that the results
could not be due to contamination by the microfibrillar glycoprotein component\textsuperscript{7-8} for several reasons. Firstly, an amino acid analysis clearly indicated that no component other than elastin was present in the homogenized specimen suspensions, and secondly, glycoproteins are positively stained in the presence of the uranyl acetate stain used, whereas the filaments shown in the results appeared negatively stained. (This different affinity towards cationic dyes at low pH of the two components can be explained on the basis of the different amino acid compositions of the two substrates\textsuperscript{7-9}. Interaction with negative staining materials tends to occur on the polar glutamyl and aspartyl residues, which in the glycoprotein number approximately 200 residues per 1,000\textsuperscript{7-8,7-36}. In the elastin, however, the number per 1,000 is approximately 10 times less and too few to allow any significant interaction between the stain and the filaments.)

Two further reports on electron microscope ultrastructural studies of elastin appeared at the time of writing this thesis. One of these was an extension of Gotte et al's 1974 paper, and the other a similar study by Serafini-Fracassini et al\textsuperscript{7-38}. The essential difference between the two studies was the method used for specimen preparation. Gotte et al relied on their traditional alkali purification method, while Serafini-Fracassini et al employed an enzymatic method. This latter group also carried out low-angle X-ray diffraction and optical polarization analyses to investigate anisotropy within the specimens. Results obtained agreed with those of Gotte et al\textsuperscript{7-35} with the material being resolved into parallel arrays of primary filaments of indefinite length. Again, the presence of such filaments was observed in specimens without ultrasonication, but prepared by homogenization and negative staining. The wide-angle X-ray diffraction patterns obtained from both relaxed and stretched specimens
was almost identical to the amorphous pattern obtained from keratins, which are devoid of regular secondary structure. This suggested that the segments of polypeptide chains located between adjacent cross-links adopt a disordered conformation.

The most recent paper of Gotte et al reports the application of a technique of optical diffraction to results obtained by their previously reported method. The technique of optical diffraction represents one of the more important advances to have been made in the field of ultrastructural research over the last few years. It employs the principle of using laser light to produce diffraction patterns from periodically repeating images on photographic plate. Thus, suspected periodicity can be resolved via the optical diffraction pattern where it would be extremely difficult to identify from the direct image. Using this technique, Gotte et al observed an elastin structure consisting of filaments of diameter 3 - 4 nm with an average centre-to-centre distance of 5 nm. A regular periodicity of about 4 - 4.5 nm was resolved along the individual filaments, with evidence of cross-bridging spaced at about 3 - 3.5 nm. The final conclusion was that the filaments consisted of a rope-like structure of 3.5 - 4 nm diameter and composed of paired sub-filaments of about 1.5 nm diameter.

The above text has referred solely to transmission electron microscopic structures of elastin. Somewhat surprisingly, reports of scanning electron microscope studies have appeared in the literature to a lesser extent. A review of such studies has already been presented in Chapter 3, so will not be reiterated. The only study aimed directly at elastin structure, as opposed to unpurified tissues, was that of Gotte et al who made observations on purified ligamentum nuchae at both low and high resolutions. Apart from commenting on the apparent anisotopy of the material, no other significant structural observations were reported.

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7.3 Experimental

7.3.1 Scanning Electron Microscopy

7.3.1.1 Introduction

The technique of scanning electron microscopy was utilized in Chapter 3 as a method of examining specimen purification. In that chapter a method was described of specimen preparation employing freeze-drying, which appeared to give preferable results to methods of drying the specimens in air at room temperature.

Due to its range of working magnifications, scanning electron microscopy is commonly classified as lying between optical microscopy and transmission electron microscopy. In many ways, however, this represents a misinterpretation, as unlike the transmission electron microscope it is basically a topographical method. (Although 'scanning transmission' electron microscopes have been developed, these have yet to become standard laboratory tools.) Perhaps a more appropriate classification would thus be to place scanning electron microscopy with reflected light microscopy and transmission electron microscopy with transmission light microscopy. In the former case the applied energy interacts only with the surface layers of the material to produce resolvable information by secondary emissions, whereas in the latter case information is obtained by modification of the applied energy by the internal structure of the material. It is important to bear these considerations in mind when applying the technique to ultrastructural studies of connective tissues as well as the fact that the resolving power of the instrument is typically of order five times less than that of the transmission electron microscope.
Thus for observations on elastin and other connective tissues the limitations of the technique must be realized and experimental work planned accordingly to avoid the inherent disadvantages of the instrument.

The practical working range of a typical laboratory scanning electron microscope (Cambridge Stereoscan Mk.IIA) for biological specimens is approximately x20 to x50,000 although detail begins to deteriorate significantly above x20,000. A resolution of approximately 25 - 50 nm is thus obtainable, which is insufficient for direct examination at the level where current ultrastructural controversy centres. This is around the tertiary molecular structure of the material which is reported to exist at around 1 to 4 nm.

The technique, nevertheless, will permit observations in a range from the micro-elastic fibre level (see Fig. 1.1) to the largest fibres, and with its high depth of field permit an excellent three-dimensional survey of the fibrillar architecture of the material far exceeding any obtainable by any other technique.

In this respect, one of the most interesting aspects if the relationship between elastic fibres and other components of elastin tissue in the unpurified state. The study of isolated components is only a first step in understanding the complete structure/function relationships between the complete tissue composite in its in vivo state: the second step is to examine the structural relationships between the various tissues present. Although such studies progress ultimately beyond the scope of the thesis, a preliminary scanning electron microscopic study was considered pertinent in that it would perhaps outline a direction in which any further work resulting from this thesis should go. In addition, it was considered that another
interesting study which should be undertaken would be an examination of the effect of formamide on the material. Such a reagent was shown in previous chapters to induce near ideal classical rubber elasticity to the material at room temperatures, cause significant swelling and impart to the material a translucent appearance. Such effects have been attributed to structural re-organization of the molecular conformations of the material (see Chapter 2) and it was consequently considered relevant to examine what effects, if any, this has on fibre structure of the purified material.

Hence, with these considerations in mind, further scanning electron microscopic studies were undertaken. The details of these are reported in the following sections. In each of these sections the method, results and discussion are presented together in order not to destroy the continuity of the text.

7.3.1.2 Observations on Unpurified Aorta

(a) Method: Specimens of fresh (unfrozen) unpurified porcine aorta 10 mm square were cut from all four regions of several aortas. Orientation of the specimens with respect to the host vessel were carefully noted and to avoid confusion two small notches were cut in each specimen, one on the 'top' edge and one on the right-hand side when viewing the adventitial surface with bloodflow passing from 'top' to 'bottom' of the specimen. From each of these specimens, three sub-specimens were taken for viewing. One of these was a section of innermost (intimal) surface, the second a section of outermost (adventitial) surface, and the third a surface obtained by carefully pulling the specimen in two as in the 'split mounting' method described in Chapter 3. The split was made as accurately down the centre of the edge as possible. This sub-specimen thus gave a view
of the medial regions of the vessel. The adventitial surface sub-specimen was found to be fairly heavily coated with a layer of fatty tissue. This was scraped from half of the specimen with a scalpel blade but the other half left for viewing.

Preparation for the s.e.m. examination was according to the freeze-drying method described in Chapter 3. The specimens were equilibrated in water before freezing to avoid possible saline concentration effects. Viewing was carried out at 45° tilt in the back-scattered electron mode of a Cambridge Stereoscan Mk.IIA scanning electron microscope.

(b) Results and Discussion: Plates 7.1 to 7.10 show typical observations obtained.

Plate 7.1 shows a view of the intimal surface of the vessel. Such a surface was noted to be similar in all the four regions observed and was seen to consist of an array of longitudinally (parallel with bloodflow) orientated elastic fibres well sheathed and interconnected with collagen. An equivalent area is shown under somewhat higher magnification in plate 7.2. If the size of the detail on the surface is compared with the size of a typical red blood cell (~ 7.8 micrometers diameter), it can be seen that they are of the same order of magnitude. Thus it would be expected that if the delicate red blood cells were to flow across this surface, considerable damage would result. In vivo, however, a thin endothelial layer of cells is present which will smooth out this surface. This layer appeared to have somehow been removed during the preparation process (probably by osmotic damage when the specimens were immersed in water, from saline). If this is the case, it demonstrates just how easily the endothelial layer can be damaged to expose 'rough'
sub-endothelial structure, and it is interesting to note that it is just such damage to localised areas which is commonly accepted as the first stage in atherosclerotic plaque formation. The fact that the surface below the endothelial layer apparently stimulates blood clot formation is possibly due to this 'roughness'.

Typical views of the adventitial surface are shown in plates 7.3 to 7.6. Plates 7.3 and 7.4 show the surface in its 'as received' form covered with a layer of fatty tissue. This fatty tissue was found to adhere very strongly to the surface of the vessel, and considerable scraping was necessary to remove it. When this had been achieved, however, typical views were those shown in plates 7.5 and 7.6 where a rather disorganised structure can be seen of elastic fibers of varying shapes and sizes sheathed in collagen. Collagen and collagen-coated elastic fibres appear to bridge the larger fibres and fuse and split to create a semi-filled composite. The presence of the collagen caused difficulty in the measurement of fibre dimensions, but typical average sized collagen-coated fibres appeared to have diameters of approximately 5 micrometers, ranging down to approximately 1 micrometer, or even less. This on average was slightly larger than the typical dimensions of 2 to 3 micrometers observed in the intimal regions.

The medial regions displayed a somewhat less fibrous appearance than the intimal and adventitial sections (Figs. 7.7 to 7.10), due to the almost complete filling of void spaces by collagen and smooth muscle. Smooth muscle cells could be clearly seen protruding from the surface (Fig. 7.7), the largest being approximately 3.5 micrometers in diameter and the smallest approximately 0.8 micrometers. Sites of other cells, presumably extracted during the splitting process, were
also in evidence as small craters in the material. Occasionally part of an elastic fibre was seen to protrude from the mass of other material, and such is shown in plate 7.9. A higher magnification view of a similar area is also shown (Fig. 7.10) to demonstrate the apparent closeness of adhesion between the elastic fibres and their collagen sheathing. The collagen appears completely adhered to, and virtually fused with the fibre, and no line of demarcation is visible where the two phases meet. Indeed, it is interesting to speculate just what the bond is between the two materials, elastin and collagen. Smooth muscle cells have been shown in the aortas of rats to be the synthesizers of both elastin and collagen\textsuperscript{7-41}. Could it thus also be possible to synthesize a continuous bridge between the two? The topic presents an interesting subject for further research and one of crucial importance for any accurate modelling of the aorta as a multi-phase composite.

The complexity of the structure in all three of the areas under study is notable and must bring into question the validity of such simple two-phase parallel fibre type models which have been proposed\textsuperscript{7-42} for the material. Only when an accurate and quantitative three-dimensional mapping of the material has been fully completed will any modelling become fully accurate. Such an investigation could possibly be accomplished by multiple sectioning complete with standard histological techniques.

7.3.1.3 Observations on Purified Aorta and Ligamentum Nuchae

(a) Method: The method adopted for specimen preparation of purified specimens was the freeze-drying method as described in Chapter 3. The aorta specimens examined were sectioned and split in the same manner as the unpurified specimens of the previous section.
Plate 7.1
Intimal surface of water-rinsed but unpurified porcine aorta.
Blood flow from lower left to upper right side of plate.  
Magn. x 570

Plate 7.2
As Plate 7.1  
Magn. x 1100

Plate 7.3
Adventitial surface of unpurified but water-rinsed porcine aorta.
Surface is coated with adherent fatty tissue.  
Magn. x 26

Plate 7.4
As Plate 7.3  
Magn. x 650

Plate 7.5
Scalpel scraped adventitial surface of unpurified but water-rinsed porcine aorta.  
Magn. x 500

Plate 7.6
As Plate 7.5  
Magn. x 1200

Plate 7.7
View of medial surface of a section of unpurified porcine aorta split down the centre of the wall.  
Magn. x 1400

Plate 7.8
As Plate 7.7  
Magn. x 2400

Plate 7.9
Unpurified elastic fibre of tunica media of porcine aorta.  
Magn. x 5100

Plate 7.10
Collagen sheathed elastic fibre of tunica media of unpurified porcine aorta specimen.  
Magn. x 12,000

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Specimens of ligamentum nuchae were also taken for high resolution observations, due to the fact that generally their preparations gave somewhat more resolvable fibres than the aorta. This was because their larger size and generally greater simplicity of structure provided a better coating surface for deposition of gold/palladium conductive film.

Views of the intimal and innermost region are best shown in plates 3.9 and 3.10 of Chapter 3, which demonstrate the structure of delicate elastic fibres.

For fibre measurement, micrographs were taken using the 'derivative process' function of the microscope which enhances changes of density from one region to another, but flattens out constant density levels. The consequent effect on images of the elastic fibre structure is to sharpen the edges of the fibres and produce an image which simplifies the measuring process. A typical derivative process micrograph is shown in plate 7.11. Measurements for such micrographs indicated mean fibre diameter of 1.4 micrometers with a standard deviation of 0.48 micrometers. The fibres appeared to be generally oriented parallel to the bloodflow and form a roughly directional network, joining and dividing in many separate places. Measurement of the fibre areas and void areas indicated a 30% to 40% volume occupied by voids.

A typical view of the adventitial region is shown in plate 7.12. The fibres appear much less ordered than those of the intimal surface and show little preferential orientation in any direction. Measurements indicated a mean fibre diameter of 3.5 micrometers (with a standard deviation of 1.1 micrometers), which showed the fibres to be almost twice as coarse as those on the innermost surface of the vessel.
A view typical of medial regions is shown in plate 7.13. Fig. 7.13 demonstrates clearly the lamella structure of the material (see also plates 3.1, 3.2 and 3.9), each layer containing fibres of orientation helically wound around the axis of the vessel. The wind angle of these helices could be measured from the 'derivative process' micrographs (eg: plate 7.14) and was found to be in the region of 17° to the radius of the vessel. Each separate layer appeared to be approximately one large fibre diameter thick (a distance of approximately 4 to 5 micrometers), and the 'handedness' of the helices appeared to change alternately with each lamella. The mean fibre diameter was measured to be only slightly less than that of the adventitial region, approximately 3.2 micrometers with a standard deviation of 0.9 micrometers.

The low pitch helical winding pattern observed is in agreement with the work of Wolinsky and Glagov 7-43 who studied the inter-relation of the structural components of the rabbit aorta by light and transmission electron microscopy fixed at various distending pressures. These researchers found that the total interlamellar space comprises about 50% of the wall thickness (NB: the author found approximately 60% in Chapter 3), with the large elastic fibre lamellae accounting for the remainder of the wall thickness. Although Wolinsky and Glagov only reported the elastin networks to be 'oriented circumferentially', the collagen network was found to adopt a helical arrangement 'of small pitch'. It was suggested that both collagen and elastin were deposited along lines of tension during development of the tissue, such a mechanism having been experimentally shown to be a possibility by Weiss7-44.
Plates 7.15 to 7.18 show views of elastic fibres approaching the limits of resolution of the technique on purified medial aorta (plates 7.15 and 7.16) and purified ligamentum nuchae (plates 7.17 and 7.18) respectively. In both cases there is suggestion that the fibres are constituted of a mass of smaller fibres by longitudinal grooves in the surface which define the edges of these smaller micro-fibre units. In the smallest cases these microfibres appear to have diameter of approximately 150 nm, a value which agrees well with the low magnification transmission electron microscopic results of Ross and Bornstein\(^7-9\). In their micrographs (Figs. 7.4, 7.5, 7.6 and 7.7) presented in reference 7-9 cross sections of ligamentum nuchae fibres are shown, and in these, large circular and oval cross-sectioned fibres of diameters ranging from 1 to 6 micrometers can be seen to be made up of closely packed bundles of micro-fibres ranging from approximately 150 nm to 500 nm. The micro-fibres appear to be so closely associated with each other that in many places no distinction can be seen between one border and the next. No structure can be identified between the borders of the micro-fibres, the elastin appearing completely amorphous in these regions. The edges of the fibres in Ross and Bornstein's micrographs also display a very ragged appearance, which would correspond to the heavily pitted appearance of the surfaces of some of the fibres in plates 7.15 to 7.18. Such pitting is no doubt a result of the close relationship between the collagen phase before removal and the elastin, and if nothing else, would imply a strong bond by mechanical interlock between the two components.
Plate 7.11
'Derivative process' micrograph of intimal surface of purified porcine aorta. Blood flow direction bottom to top of plate. Magn. x 1100

Plate 7.12
View of adventitial surface of purified porcine aorta. Magn. x 1100

Plate 7.13
View of medial surface of purified porcine aorta. Blood flow direction top to bottom of plate. Lamella structure can be clearly seen. Magn. x 980

Plate 7.14
'Derivative process' micrograph of medial region of purified porcine aorta. Blood flow direction top to bottom of plate. Magn. x 460

Plate 7.15
High magnification view of purified porcine aorta elastic fibre from tunica media. Magn. x 20,000

Plate 7.16
As Plate 7.15 Magn. x 19,000

Plate 7.17
High magnification view of purified elastic fibre from bovine ligamentum nuchae. Magn. x 21,000

Plate 7.18
As Plate 7.17 Magn. x 21,000
7.3.1.4 Observations on Formamide Equilibrated Elastin

(a) **Method:** Specimens of elastin were taken from region 2 of a sample of purified porcine aorta and equilibrated for two hours in a beaker of fresh formamide. The solution was then changed and the samples left for a further two hours to ensure complete equilibration. Following this, the specimens were split and an attempt was made to freeze-dry them according to the standardised method undertaken for the water-saturated specimens. After 24 hours at 0.025 torr and -50°C, no drying appeared to have taken place. Because of this, the temperature of the unit was raised to 20°C and a further 10 hours allowed to attempt to simply dry the material in vacuo at room temperature. After this period the material was still in a highly rubbery condition, demonstrating the considerable plasticizing effect of the formamide on the mechanical state of the material at room temperature. The preparation process was nevertheless continued successfully and no loss of vacuum was experienced either during the vacuum coating process or under microscopic examination.

(b) **Results:** Typical results are shown in plates 7.19 to 7.22. In general, the effect of the formamide appeared to impart to the fibres a 'sinuey' appearance, accentuating the impression that each fibre was made up of a number of smaller units intertwined together. Rather surprisingly, mean fibre diameter appeared to have decreased quite considerably (medial region) to a new value of 2.5 micrometers. A similar decrease in the standard deviation obtained in deriving this value for the mean (s.d. = 0.6) however, indicated that the range of fibre diameters had also decreased. At the same time there appeared to be an increase of the number of fibres per unit surface area examined over the water equilibrated specimens.
The probable explanation for these facts was that the formamide, apart from swelling the elastic fibres themselves, also had the effect of breaking up the larger fibres into multiples of their constituent microfibres. This would imply either some form of chemical attack on the inter-micro-fibre bonds of the larger fibres or a simple physical tearing away effect caused by geometrical dimension changes experienced by the material. Such action would account for the generally disturbed, kinked and sinuey appearance of the individual fibres.

An interesting indication of the gel-like texture of the formamide impregnated elastin is supplied in plates 7.21 and 7.22. These plates show similar areas cut across the wall of the aorta. Plate 7.21, however, shows a surface cut before the specimens were equilibrated with formamide, whereas plate 7.22 shows a similar area cut after full equilibration with formamide. Both sections were cut with new scalpel blades. In plate 7.21 the cut fibres of the individual lamellae have swollen out over the sectioned surface to show a structure of troughs and swollen distorted ridges lying in approximately parallel array. Where such a swollen surface has been cut, in plate 7.22, the fibres give the appearance of having a soft, perhaps jelly-like nature leaving smooth cross-sectioned views where the blade has passed through them and almost appearing in many places to have been 'smeared' and flattened out to some extent over the surface. Such surfaces were not seen in similar cut areas of water-saturated specimens (eg: plate 3.2) and therefore can be attributed to the effect of the formamide.
Plate 7.19

Formamide saturated purified porcine aorta, medial region.
Magn. x 1000

Plate 7.20

As Plate 7.19
Magn. x 5000

Plate 7.21

Formamide saturated section across purified aortic wall. Section cut before saturation with formamide.
Magn. x 1050

Plate 7.22

As Plate 7.21, but with section cut after saturation with formamide.
Magn. x 980
7.3.2 Transmission Electron Microscopy

7.3.2.1 Introduction and Objectives

Although the scanning electron microscopy of the previous section and Chapter 3 served the useful purpose of supplying a survey of the architecture of the purified elastic tissues of interest to this thesis at magnification levels between the visible and approximately 100 nm, it served little function in the examination of the major area of controversy, namely the structure of the elastin at molecular levels. It is at these molecular ultrastructural levels that the material becomes most difficult to examine and analyse, for it falls at the very limits of direct examination techniques such as X-ray diffraction or transmission electron microscopy, yet requires a level of information from the material unobtainable by chemical analytical techniques such as chromatography or spectroscopy.

In essence, the problem is not difficult to formulate. Simply stated it is to determine what structure is adopted by the amino acid chains of the so-called 'amorphous' elastin component of the elastic micro-fibres. Such micro-fibres have been shown in the previous section and literature\(^7\)\(^-\)\(^9\) to range down to approximately 150 nm diameter. The diameter of a peptide acid chain is in the region of 0.3 - 0.5 nm and so if these were arranged in an array parallel to the micro-fibre axis, some 2,500 of them would be able to fit into the volume of a typical micro-fibre, while a random coil type structure would be far less densely packed.

To date there have been a number of structural investigations undertaken using the transmission electron microscope at its maximum resolving power. These were reviewed in section 7.2 of this chapter and have reported results of varied and conflicting nature from a
host of different specimen preparation techniques. Almost without exception, they suffer from one particular criticism, namely the methods used to dry out the specimens for microscopic examination. Invariably such methods consist of one of two types: straightforward drying down in air or under vacuum, or water extraction using solvent/water baths of ever increasing solvent concentration. Both methods are liable to generate specimen shrinkage, swelling or distortion during the process which can introduce considerable artifacts through the introduction of stresses at sub-microscopic levels. In addition, the aspect of molecular structure responsible for the production of rubber-like elasticity has already been shown to be critically dependent upon the liquid content of the material. Elastin becomes completely glassy when dry. It was thus considered quite possible that many of the fibrillar type structures previously reported could have been artifacts due to the drying processes employed. Because of this it was considered that an extension of the freeze-drying process developed for scanning electron microscopy of the thesis might be able to overcome any drying artifact problems as well as being able to avoid the introduction of solvents into water-saturated specimens.

As a starting point it was decided to adopt the method of Gotte et al. to examine specimens of aorta and ligamentum nuchae to check for confirmation of his results on both these elastin sources. The work of Gotte et al. only used bovine ligamentum nuchae and so it was considered that a confirmation of their findings on porcine aortic elastin would help establish their results as being valid for all types of dry elastin.
7.3.2.2 Examination of 'Air dried' Elastin Specimens

(a) Method: Specimens of purified aorta and ligamentum nuchae were each taken and a further stage of purification carried out on them to ensure complete and total removal of the glycoprotein microfibrillar component. This was achieved by treating the specimens with hot 0.1M NaOH at 98°C for 45 minutes, followed by boiling for three one-hour periods in fresh glass-distilled water.

The specimens were then frozen in liquid nitrogen, mixed with approximately ten times their volume of frozen CO$_2$ and individually pulverized in the dry stage of a standard rotary homogenizer for four three-minute periods. Fresh frozen CO$_2$ was added after each three-minute period. At the end of the pulverization process single, spatula load samples of the frozen powders were placed in clean test tubes containing 10 ml glass-distilled water at room temperature and equilibration allowed to occur. Transmission electron microscope copper specimen grids (6 squares per mm) were carbon coated by a standard method of scooping thin vacuum deposited films of carbon from the surface of a liquid bath. These were used to mount the specimens as follows:

A Pasteur pipette was taken and its dropping end thinned to the finest point possible by drawing in a Bunsen flame. This was used to place one drop of the specimen suspensions on each of the carbon coated grids. These were then left for 20 minutes before being removed by touching with filter paper.

Two types of negative stain were prepared:
(i) a 2% aqueous potassium phosphotungstic solution at pH 6.8, and
(ii) a 2% aqueous uranyl acetate solution at pH 4.
Large drops of those solutions were placed on a clean microscope slide and the specimens stained by floating the grids, specimen sides downwards on these drops, for periods of 1, 2, 5 and 10 minutes. Subsequent results, however, showed the time for optimum results to be 5 minutes, and so later preparations adopted this time as standard. Following staining, the grids were allowed to dry in a desiccator over P₂O₅ for not less than three hours before examination.

The electron microscope used was a JEOL J.E.M. 100B transmission electron microscope operating up to accelerating voltages of 100 kV. To attempt to avoid excessive radiation damage and heating effects during examination, the accelerating voltage normally used was 80 kV. Results were recorded on photographic plate and are presented below.

(b) Results and Discussion: Considerable searching was necessary to find any areas of specimen at all on each grid and when they were found they were often too thick for electron transmission. A few examples of the type of fibrillar–appearing structure reported by Gotte et al 7−35, 7−37 and Serafini-Fracassini et al 7−38, however, were observed and examples of this are shown in plates 7.23 and 7.24. Plate 7.23 shows the structure stained with phosphotungstate and plate 7.24 with uranyl acetate. As can be seen, the uranyl acetate gave considerably sharper results than the phosphotungstate, an observation also made by Gotte et al 7−35. It must therefore be questioned whether the acidic conditions of the uranyl acetate stain have some form of including effect on what might otherwise be a random coil type structure.

Similar fibrillar structures were observed in elastin from both the aorta (plates 7.25, 7.26) and the ligamentum nuchae (plates 7.27, 7.28) specimens. The mean fibril diameter was measured to be 3.8 nm and the mean centre to centre inter-fibril distance 3.5 nm.
Plate 7.23

High resolution transmission electron micrograph of purified elastin from bovine ligamentum nuchae elastin. Stained with potassium phosphotungstate.
Magnification as indicated on plate.

Plate 7.24

As Plate 7.23, but specimen stained with uranyl acetate.
Magnification as indicated on plate.

Plates 7.25 and 7.26

High resolution transmission electron micrographs of purified porcine aortic elastin, stained with uranyl acetate.
Magnifications as indicated on plates.

Plates 7.27 and 7.28

High resolution transmission electron micrographs of purified elastin from bovine ligamentum nuchae elastin, stained with uranyl acetate.
Magnifications as indicated on plates.
Also observed were the much larger micro-elastic fibres mentioned in section 7.3.1.2. In many cases these were seen to exist in pairs of closely coiled filaments (plate 7.32) each filament having a diameter of some 150 - 200 nm. Within these large filaments no sub-structure could be distinguished.

It was hoped to be able to observe a branch area or an area at the edge of a micro-elastic fibre leading into an area demonstrating the fibrils of Gotte et al. This would have identified the micro-fibrillar structure as being part of a definite elastic fibre. Such an area, however, was not found, and consequently there still remains no conclusive evidence to connect the observed 'Gotte fibrils' with the micro-elastic fibres.

It is interesting to consider these and the literature findings in the light of some of the more recent morphological theories of amorphous polymers. Geil has suggested that the whole concept of amorphous polymers both in the glassy state and above the glass transition temperature (Tg) consisting of randomly coiled entangled chains with no local order, should be modified. In its place he proposes amorphous polymers to consist of small (3 to 10 nm) domains in which there is a local ordering or alignment of neighbouring segments. These liquid crystal-like domains would mostly, but not always, contain molecules passing from one domain to another. In the glassy state, the structure is frozen, whereas above Tg there is a continuous re-distribution of segments along the domains, individual domains forming and disappearing continuously. Geil has shown that the effect of stresses on such structure would be to develop a fibrillar type of structure due to alignment of molecular segments within the domains. This would offer a possible explanation for not
only the Gotte type fibrils observed in elastin, but also the
electron microscopic results of Fitton-Jackson et al\textsuperscript{7-34} which
showed a globular type structure of globule diameter 5 nm in
elastin specimens where drying collapse was prevented by means of
a filling agent. It is conceivable that such globules could repre­
sent the domains of the structure demonstrated by Geil. On drying
without suitable precaution against shrinkage, molecular segments
within the domains could experience stress-induced alignment effects,
resulting in the observed fibril structure.

7.3.2.3 TEM Observations on Formamide Saturated Elastin

(a) Method: Basically the method was similar to that of the
water saturated specimens of section 7.3.2.1, although in this case
specimens of ligamentum nuchae only were used to reduce chances of
any stray impurities being present. The specimens were soaked in
fresh formamide for three hours, the formamide being changed after
each hourly period. Freezing was then carried out by direct immersion
in liquid nitrogen followed by four three-minute pulverization
periods in the rotary homogenizer. Spatula loads of the resulting
powder were then placed in test tubes containing 10 ml formamide
and these allowed to stand, well stoppered, for a minimum of three
hours for the larger saturated particles to settle. A clean, Bunsen-
drawn Pasteur pipette was then again used to place fine drops of
the test tube solution on carbonized specimen grids, and these drops
allowed to stand for 20 minutes in a dry atmosphere before being
dried by touching with filter paper.

The negative staining agent again used was the uranyl acetate
at pH 4.0. Even though this was an aqueous and not a formamide based
solution, it was considered that the action of the formamide was
sufficiently irreversible and the staining process too rapid to
induce any significant return to an elastin/water structure. Stain-
ing was thus carried out in similar manner to the water-saturated
specimens, namely by floating the grids, specimens down on large
drops of negative stain for a period of five minutes followed by
storage in dry air in a desiccator over \( \text{P}_2\text{O}_5 \) for not less than three
hours.

(b) Results and Discussion: With the formamide-saturated
specimens it was even more difficult than with the water-saturated
specimens to obtain a fibril demonstrating any internal structure.
After examining some twenty prepared grids, only three examples were
found, and one of these is shown in plate 7.29. Again, a structure
of 'Gotte fibrils' of fibril size and inter-fibrillar diameter,
almost identical to the water-saturated case, was discovered.

The fact that such a structure was found also in the formamide-
saturated specimen, provides strong evidence for the observation of
'Gotte fibrils' to be due to some form of artefact. Although it
still might perhaps be possible to dispute the acceptance of the
elastin/water system as a classical amorphous elastomer, it is very
difficult to do in the case of the formamide/elastin system which
exhibits behaviour so near that of an ideal swollen amorphous
elastomer as to be virtually a 'textbook' case (see Chapters 5 and 6).
A complete structure of 'Gotte fibrils' would be far too ordered a
system to allow the significant entropy changes necessary to generate
the elasticity demonstrated by the bulk material. It is therefore
apparent that the 'Gotte fibril' structure does not represent the
structure of bulk elastin in its in vivo state.
Plate 7.29

High resolution transmission electron micrograph of purified elastin from bovine ligamentum nuchae, saturated with formamide. Stained with uranyl acetate. Magnification as indicated on plate.
7.29.
7.3.2.4 T.E.M. Observation of Elastin prepared by a Method of Freeze Drying

(a) Method: A considerable number of methods were attempted before one was found to yield satisfactory results and even this method as described below was found difficult to perform satisfactorily. Nevertheless, a number of observations were made by the method.

One of the central problems of the method was found to be in producing specimens freeze-dried in their negatively stained condition. If the specimens were dropped on to the specimen grids, stained and the grids subsequently frozen and freeze-dried, inevitable destruction of the carbon film occurred during the rapid freezing process. If, however, the specimens were freeze-dried as powders, then applied to the grids, they were in an unstained condition and subsequent application of the aqueous stain destroyed all the advantages gained by the freeze-drying. The final solution to the problem was found by freezing the specimens, pulverizing, mixing a measured amount of the powder into a dilute solution of the negative stain, then re-freezing and pulverizing. In the meanwhile the carbon coated grids were gradually frozen down as the freezing platform of the freeze drying unit and a delicate sprinkling of the pulverized mix allowed to drop on to them by brushing gently across the surface of the mix container with a fine camel-hair brush. Great care was found to be necessary during this operation not to apply too much powder, as the grid surfaces would then contain too many fibres. Speed was also desirable to reduce the risk of dust contamination from the air.

Initially it was feared that such a pre-staining method would meet with failure as the stain was being applied to the specimens.
directly and not being allowed to spread out over a backing of carbon film. This fear, however, seemed to be unfounded when the results described below were obtained.

The freeze-drying process was carried out at -50°C and 0.025 torr for four hours, and the specimens then transferred immediately to a desiccator over P₂O₅ for storage, which in no case was for longer than one week.

A summary of the preparation process is thus as follows:

Water-equilibrated aorta and ligamentum nuchae specimens were frozen by immersion in liquid N₂, mixed with approximately ten times their volume solid CO₂ and pulverized for four three-minute periods in the rotary homogenizer. One spatula load (approximately 125 mm³) of the powder was then placed in 10 ml of 0.5% uranyl acetate at pH 4 and left freezing for one minute. This was carried out by pouring the mixture over solid CO₂ in the pulverization chamber whence four more three-minute pulverizations were carried out with addition of fresh amounts of solid CO₂ inbetween to maintain volume. The remainder of the process has already been described.

(b) Results: As with the 'air-dried' specimens, observations were made under 80 kV in the JEM 100B transmission electron microscope. It became immediately apparent, however, that the freeze-dried specimens were far more prone to radiation damage than the air-dried specimens. On many of the micro-fibres the effect of the electron beam was found to cause the fibres to shift position, buckle and distort. In addition, if the beam was focussed down on one particular area, rather a curious phenomenon would often be observed: the whole fibre in the region of the beam would be seen to 'blow up', creating a hole in its centre randomly crossed by small 'bridges' of seemingly amorphous material.

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The main observations of interest were areas of what appeared to be partially decomposed micro-elastic fibres of approximately 100 nm (plates 7.30 and 7.31) diameter. Off these could be observed randomly branching smaller fibrillar units of diameter ranging down to approximately 40 - 50 nm. Such structure is shown in plates 7.30 and 7.31, and should be compared with the air-dried specimen plates 7.23 to 7.28. In the air-dried case, the structure within the fibres appears to be entirely fibrillar, while in the freeze-dried case such fibrillar structure appears to be only partially formed, and where the fibrils are found they appear to be of somewhat larger diameter than in the air-dried specimens, having a measured diameter of approximately 8 - 9 nm.

In the areas of no fibrillar structure, no internal structure of any type at all was apparent. Furthermore, those regions were of slightly larger area than the fibrillar areas, demonstrating that lateral contraction could have occurred as part of the fibril forming process. The fact that the amorphous-appearing regions were part of the fibre, and not simply gaps or holes in the material, was demonstrated by the continuity of the stain from region to region, and its comparative uniformity of density within the two regions. Also visible in plate 7.30 were a number of more sharply outlined 'Gotte fibrils'.
Plate 7.30
High resolution transmission electron micrograph of elastin specimen from purified bovine ligamentum nuchae stained with uranyl acetate and prepared by the method of freeze-drying. Magnification as indicated on plate.

Plate 7.31
High resolution transmission electron micrograph of elastin specimen from purified porcine aorta stained with uranyl acetate and prepared by the method of freeze-drying. Magnification as indicated on plate.
Plate 7.32
Transmission electron micrograph of two negatively stained (uranyl acetate) micro-elastic fibres (see Fig. 1.1, Chapter 1), coiled together to form a larger unit. Elastin from bovine ligamentum nuchae, prepared by freeze-drying process. Magnification as indicated on plate.

Plate 7.33
Transmission electron micrograph of several arrays of aligned tubular microfibrils (each of diameter approximately 10 nm) (see Fig. 1.1, Chapter 1), remaining unaffected by purification process. Specimen negatively stained with uranyl acetate, from bovine ligamentum nuchae, prepared by freeze-drying process. Magnification as indicated on plate.
Central to this thesis is the question of the validity of treating saturated elastin as a 'classical' amorphous rubber. Such structures consist of networks of random molecular chains cross-linked covalently at regular intervals.

According to the theories of polymer physics, with reference to polymer chain statistics, if each chain in a network structure has n links each of length L, then the fully extended length of the chain $R_c$ is, (ie: ref. 4-8):

$$R_c = nL_c$$

It is, however, more meaningful to consider an average size of the chain such as the mean-square vectorial end-to-end distance $\bar{R}_c^2$. An analysis in terms of $\bar{R}_c^2$ can be performed (eg: ref. 7-47), and yields

$$\bar{R}_c^2 = nL_c^2$$

Hence, in order to estimate the mean-square end-to-end distance (cross-link to cross-link) it is necessary to estimate the number of links or segments between cross-links and the mean segment to segment separation.

If the typical elastin amino acid analysis is examined (eg: ref. 7-9), an average molecular weight per amino acid can be calculated. This value is close to 100. Now, assuming that this value holds also for a typical inter-cross-link amino acid distribution, an estimate of the molecular weight between cross-links $M_c$ divided by the above value will yield n. Such estimates\textsuperscript{7-48,7-49} made on the basis that all cross-links are inter-chain and unite two chains, have yielded a value of 6700. Hence it is possible to estimate

$$n = 67$$
In order to obtain $L_c^2$ it is necessary to examine the dimensions of an amino-acid chain backbone; such a structure will consist of a chain of units:

![Diagram of amino-acid chain units]

The dimensions of and angles between such units were established by early molecular biologists\textsuperscript{7-50,7-51} and calculation from their data yields an $\alpha$-carbon to $\alpha$-carbon vector of approximately 0.38 nm. This is thus the value $L_c$.

Hence: $R_c^2 = 67 \times 0.38^2 = 3.1$ nm

Using these values the length of the fully extended chain between cross-links would therefore be:

$R_c = 67 \times 0.38$

$= 25.5$ nm

Thus, elastin in its solvated, rubbery state might be expected to consist of a network of peptide chains of length approximately 25.5 nm between covalent cross-links, but with an average inter-cross-link distance of 3.1 nm.

Also of importance is an estimate of the width of such chains. This can be made by examining a typical amino acid chain, which consists basically of a backbone of C - C - N units with the main bodies of each amino acid branching out at various intervals. Some of these branches, such as the single hydrogen atom branches of glycine, will not protrude very far from the backbone, whereas others will protrude considerably further.

The predominant amino acids in elastin are glycine and alanine. Both these, however, have their side groups near their $\alpha$-carbon (backbone) molecule (ie: furthest point less than 0.2 nm away), so will not contribute
significantly to the diameter of the chain. Undoubtedly the 'longest' side-chains present in elastin belong to the basic hydrophilic groups of lysine and arginine which together account for about 1.3% of the total number of amino acids present. The total length of a lysine molecule is some 1.2 nm and arginine is of similar dimension. Neither, however, branch out at exactly 90° to the axis of the backbone, but rather at an angle of approximately 34°. This gives their ends an effective distance from the backbone of about 0.7 nm, with a resulting chain diameter (ie: two lysines 120° opposed to each other), of around 1.4 - 1.5 nm.

It is very interesting to compare this estimate with the conclusions of Gotte et al7-37 who write:

'From the experimental evidence reported here (7-37) we are inclined to conclude that elastin results from an assembly of rope-like structures each with an overall diameter of 3.5 - 4.0 nm of paired elements of infinite length, each 1.5 nm in width and periodically linked longitudinally at a distance capable of varying 3.5 - 4.5 nm according to the degree of stretching.'

The 'individual elements' of Gotte et al could thus be individual amino acid chains, these linking together in pairs by means of increasing hydrophobic interactions as the material dries out. Any extra length of chain could be taken up during this process by the formation of β-spiral sequences which could then stack together according to the model of Gray et al7-52 in a modified cross-β-structure.

The above estimates thus give an indication of the dimensions of the random molecular coil structure which may exist in water-saturated elastin; molecular amino-acid chains of effective diameter approximately 1.4 - 1.5 nm covalency cross-linked at intervals along each chain of approximately 25 nm, but with a mean vectorial inter-cross-link distance of approximately 3 nm.
The production of a specimen of such a structure on a carbon film sufficiently thin and contrasted for transmission electron microscopy is, for reasons described below, virtually an impossibility. Firstly, a true thin section of such a three-dimensional structure would have a very low chance of capturing any significant length of molecular coil on its surface; it would require an ability to be able to 'pin down' a length of molecular chain on one plane, a chain which above its glass transition temperature will be in continuous thermal activity in all three dimensions. Then, even if this were possible, and the structure was frozen without the introduction of artefacts and a section laid on a carbonized grid, the main bulk of this cage-like structure would be away from the surface of the carbon where it would be unaffected by the negative stain. Such molecules, which were contrasted by the stain, would be virtually unresolvable as their size places them beyond the resolution limits of the technique (approximately 2 nm).

The type of structure seen in plates 7.23 to 7.28 and published by Gotte et al. and Serafini-Fracassini et al. is one presenting itself well to the negative-staining technique where the closely aligned fibrils will provide grooves and fissures to trap the stain in regions surrounding the fibrillar structure. Hence the fact that their structure has been the only type consistently observed in elastin, by no means qualifies it as necessarily representing the true structure of rubbery solvated elastin.

It is thus the author's view that from the findings of this chapter and others that the 'amorphous elastin' phase of elastic fibres in their 'in vivo' rubbery condition is, in the polymeric sense, truly amorphous, consisting of a network of randomly oriented coiled amino-acid chains cross-linked at various intervals. The cross-linking is carried out.
after formation and ejection of the chains from the cells. The material is then organized according to the proposed method of Ross and Bornstein\(^7\)-\(^9\) along the previously laid down parallel tubular micro-fibrillar phase (plate 7.33) to form micro-elastic fibres of around 100 nm or larger. These micro-elastic fibres coil together (plate 7.32) to form larger fibrillar units (S.E.M. plates) which create a network, oriented to meet the particular stress-field of the tissue of which the elastic network is part. The other components of the elastic tissue composite then fill up the inter-fibre voids and cavities and are laid down along the surface of the elastic fibres possibly 'guided' also by the micro-fibrillar phase\(^7\)-\(^10\), and eventually the complete composite tissue created.

7.5 Conclusions

1. Scanning electron microscopy was used to study:
   (i) freeze-dried unpurified porcine aortic tissue;
   (ii) freeze-dried purified porcine aortic and bovine nuchael ligament tissue;
   (iii) purified aortic tissue subjected to treatment with formamide.

The results demonstrated:
   (a) Oriented network structures of elastic fibres ranging in diameter from approximately 0.5 to 10 micrometers, depending on the tissue and the location within the animal.
   (b) Under high resolution a structure of smaller fibrillar units (150 nm) was indicated within the fibres.
   (c) Disruption of the larger fibres by the formamide to smaller (2.5 micrometers) somewhat more disturbed sub-units.
2. Transmission electron microscopy was carried out on negatively stained specimens of highly purified porcine aorta and bovine ligamentum nuchae prepared by air-drying and freeze-drying respectively. Specimens treated with formamide were also examined. The results demonstrated:

(a) With the air-dried specimens a structure of oriented micro-fibrils similar to results previously obtained in the literature. The negative stain, aqueous uranyl acetate, at pH 3 was found to give better results for the fibrillar structure than aqueous potassium phosphotungstate at pH 6.8.

(b) With the freeze-dried specimens a semi-fibrillar, semi-amorphous structure.

(c) With the formamide-treated specimens, a fibrillar structure similar to that seen in (a).

The results, particularly of (c), indicated that the fibrillar structures observed by these and other methods were not representative of elastin in its 'in vivo' rubbery condition.
The work of this thesis has been aimed at gleaning some insight into the structure of elastin. The methods which have been employed have been taken from a wide span of diverse disciplines, but this was deemed necessary in a field where controversy still rages and conclusions tend to be drawn from single experimental procedures. Generally the discussions have been concentrated within the area where disagreement exists, with particular attention to the merits or otherwise of the Globular, Fibrillar and Random Coil theories of elastin. The question must be asked, nevertheless, whether elastin structure conforms to some other pattern, yet unproposed in the literature? Could it possibly be akin to some of the structurally more well understood connective tissues? Such possibilities are discussed in this chapter, as are the major theories of polymer structure which led to our current understanding of the subject. These theories demonstrate the considerable degree of thought which investigators have put into the subject during its development.

Consideration is also given in the chapter to the results of techniques not previously discussed but relevant to elastin structure. These include the techniques of low and wide angle X-ray diffraction and nuclear magnetic resonance (n.m.r.).

Finally, a discussion is made of the possible implications of the conclusions of this thesis on the role of elastin 'in vivo'.

8.1 Introduction
8.2 Development of Ideas of Polymer Morphology

The fine structure of polymeric materials can range from the wholly amorphous to the wholly crystalline. Between these two extremes, however, there range various combinations of crystalline and non-crystalline regions, which may be aggregated into fibrils or other supra-molecular structures. Understanding of this fine structure acquired over the last forty years is derived from a variety of sources including X-ray diffraction, electron microscopy, atomic absorption and other techniques. Current ideas, however, on the structure of fibrous polymeric materials originated a century ago with the theory of Nageli that starch grains and cell membranes in plants are composed of sub-microscopic crystalline particles, termed micelles. Between the micelles there is some intermicellar substance. Solutions of the material were supposed to consist of colloidal dispersions of the micelles. Later Nageli's theory was brought into wide publicity by the development of X-ray diffraction methods which showed conclusively that there were, in fact, crystalline particles present in many polymeric fibres.

By 1930 the molecular formula of cellulose had been established, but the lengths of the molecules had not been accurately determined. On the assumption that the X-ray diffraction patterns were due to the presence of discrete, minute but perfect crystals, it was calculated that the micelles in ramie were about 60 nm long by 5 nm wide, and in viscose rayon about 30 nm long by 4 nm wide. This corresponded with the views on molecular length prevalent at that time.

A completely different view, however, was taken by Staudinger who deduced from measurements of the viscosities of polymer solutions that the molecules were much longer. Staudinger's opinion was that while low
molecular weight polymers could form separate crystallites, crystalline high polymers must consist of a continuous, imperfect crystal in which the end groups of the molecules appear as local distortions. He suggested that in amorphous high polymers there was a continuous network of irregularly distributed chains, and was supported in this theory by a number of other workers. In later years, Staudinger's views on molecular length were shown to be substantially correct, and it is now known for example that molecular length in native celluloses is of the order of 1 micrometer, and 200 nm to 400 nm in regenerated celluloses respectively.

Conflict also centred around the view that fibres were made up of discrete crystallites held together by some non-crystalline material and the alternative view that their fine structure was continuous and homogeneous down to the level of the molecules themselves. The establishment of the fact that the molecules were about ten times as long as the micelles meant that the old micellar theory had to be abandoned, but the exponents of the micellar theory then took the idea of the fringed micelle which had previously been suggested for collagen and gelatin in 1930. It was assumed that the same molecules must pass through more than one micelle, and the 'inter micellar substance' was a region of non-crystalline material linking the structure together in a continuous molecular network. The fringed micellar theory of polymer structure hence incorporated the most important aspects of both the micellar and the continuous theories of structure and was the offspring of the two conflicting views held in the early 1930's. According to the theory, amorphous polymers could be regarded as the limiting case in which crystalline content is zero.

The fringed micelle concept proved very successful in explaining many observed polymer properties. Variations in moisture absorption, uptake of dyes, and reactions with other chemicals could be explained if the
non-crystalline regions were more accessible to penetration by foreign molecules than the crystalline regions. The stability of the crystalline regions would cause the material to show limited swelling in water. Differences in density could be attributed to closer packing in crystalline regions compared with that in non-crystalline regions. Mechanical, electrical and optical properties were all explained in terms of the theory. Orientation, its cause and effects, could be explained by a lining up of the crystallites and the individual molecules in the non-crystalline regions. Infra-red absorption and nuclear magnetic resonance studies confirmed the division between crystalline and non-crystalline materials. In addition, several electron microscope observations seemed to confirm the micellar shape of the crystalline regions with a size of about 50 nm long by 5 nm wide. For these reasons the fringed micelle theory has remained the working theory of most polymer scientists to this day.

It is interesting to note the striking similarity between the 5 nm width of the micelles and the 4.6 nm diameter of the 'Gotte fibrils' of elastin discussed in Chapter 7. The question must thus be raised whether there are in elastin parallel arrays of micelles within a predominantly amorphous structure? If, for example, as with other semi-crystalline polymers such as the polyamides, water only enters the amorphous regions of the material and the micellar volume is small in relation to the amorphous areas, the crystallinity in the material could remain virtually undetected by many of the physical test methods such as mechanical relaxation or swelling experiments. Furthermore, it is conceivable that the existence of the amorphous phase is entirely dependent upon the presence of liquid within the specimen, complete removal of the liquid resulting in a marked increase of 'dry' crystalline material. If this were true,
saturated elastin would be very predominantly amorphous, whereas the opposite would be true with dry elastin. An explanation would thus be provided for the fibrillar structures of the transmission electron microscope observations discussed in the previous chapter as well as for the saturated amorphous rubber characteristics exhibited in the solvated state.

In both biological and synthetic polymers, the barrier between the ordered 'crystalline' state and the 'amorphous' state is narrow. Frequently, only small changes in physical conditions are necessary to produce change from, for example, an entropically favourable random molecular structure to an energetically favourable crystalline structure. Examples of this are the reversible stress induced crystallization of many rubbers, or the glass transition of many crystalline polymers where the crystalline regions 'melt' into an amorphous condition on raising the temperature\textsuperscript{8-9}.

In the biological field there are many similarities: the crystalline connective tissue, collagen, for example, becomes highly rubbery on heat or calcium chloride denaturation\textsuperscript{8-9}, and exhibits at X-ray diffraction pattern very similar to that of elastin\textsuperscript{8-9}. Under controlled conditions, however, it can recrystallize back into its fibrillar structure. Dermatan sulphate and other connective tissue mucopolysaccharides present within organisms in a form of jelly for many years were thought to be uncrystallizable. Yet recently, using combinations of strain and heating, dermatan sulphate has, under laboratory conditions\textsuperscript{8-11}, been crystallized. In effect there has been a gradual tendency for materials scientists to discover crystallinity and order where none was previously thought to exist. The process occurred first with metals (originally thought to be amorphous) and then continued with polymers. Now, certain biopolymers, previously thought to be amorphous, are also producing results suggesting structural
order. The situation is well summed up by Keller\textsuperscript{8-11} in describing his past researches into polymer crystallinity:

'I set out in search of order in a world where only disorder and randomness was believed to reign. What I found was more order, clarity and precision than I had even dared to hope . . . '

Although it has generally remained the model from which others have been copied, the fringed micelle structure has not been the only model of polymeric fibre crystallinity which has been advanced. One of the more novel was suggested by a group of Russian workers\textsuperscript{8-12,8-13} who put forward the theory that order can exist within a system without crystallization. They acknowledged\textsuperscript{8-12} that amorphous and crystalline polymers undoubtedly existed, but that only the extreme representatives of these groups were widely different in their properties. They pointed out that it was difficult at the same time to find properties which the representatives of both groups did not possess, and thus concluded:

'While the crystalline polymers differ from the ordinary crystals by a lesser degree of order, it is by the high degree of order in their molecules that the amorphous polymers differ from low molecular weight liquids and from amorphous bodies. The presence of long molecular chains prevents the growth of crystals and favours the formation of well-ordered clusters and oriented structures, and this is the reason for relating the properties of those systems. The appearance of well-ordered amorphous systems often leads to a misunderstanding based on the fact that the amorphous polymers (or amorphous regions) are considered to be a system of chains irregularly interwoven, and the crystallization of the polymers is meant to be the only source for the introduction of order.'

Another theory which has been put forward\textsuperscript{8-14}, and perhaps bears relevance to the structure of elastin, is the fringed fibril theory, a
major offshoot of the fringed micelle theory. In this theory the assumption implicit in the fringed micelle theory that all the molecules in a crystalline region diverse from one another at the same position, giving rise to discrete crystallites, is abandoned. Instead, the crystalline regions are regarded as continuous 'fringed fibrils' composed of molecules diverging from the fibrils at different positions along their lengths (Fig. 8.1). It is assumed that some distortion of the crystal lattice may occur so that a slight curvature of the fibrils is permissible. It is also possible that there may be some branching of the crystalline fibrils.

The fringed fibril theory represents a move backwards towards the ideas of continuous structure held by Staudinger, but at the same time incorporates the most important feature of the fringed micelle theory. It discards only the concept of micelles of limited length which derived from Nageli's original idea. In reality it is illogical to assume in the case of either biological or synthetic polymers that crystallization must cease abruptly for all the component molecules along a certain line at the end of a crystallite; the process is more likely to continue indefinitely.

Hearle has suggested the possible formation mechanism for a fringed fibrillar structure within synthetic fibres:

'An example of the conditions in which it is reasonable to suppose that a structure of this sort will be formed is the crystallization of a filament as it is spun. At a certain distance beyond the point of extrusion the molecules will be ready to crystallize, but the portion immediately ahead of this will already have been crystallized and will therefore provide the nuclei for the continued crystallization. This will lead directly to a fibrillar structure. However, owing to the tangled and disordered arrangement of the molecules it will not be possible for a
Fig. 8.1 Fringed fibril structure

Free molecular chain.

Fibril.
given molecule to be incorporated indefinitely in the same crystal fibril: it will diverge from the fibril and pass through non-crystalline regions before being incorporated in another crystalline fibril. There may be places where the fibres come to an end because all their components have broken away, and other places where a new fibril starts by the chance approach of two molecules in positions appropriate to crystallization, but those events should be infrequent.'

It is conceivable that a similar mechanism could occur during the drying of elastin. As the solvent departs crystallization may extend from the few already crystallized areas or from the inter-chain cross-link regions. However, it would, in similar manner to the mechanism described above, not be possible for a given peptide chain to be incorporated indefinitely into the same crystal fibre owing to the tangled and disordered arrangement of the molecules. It would hence pass from crystalline to non-crystalline regions. The tendency for crystallization would increase as solvent continued to leave the structure.

The fringed micelle and fringed fibril conceptions are by no means the only or even the newest models to have been formulated to explain crystallization. In recent years polymer crystallographers have turned to yet another concept - that of chain folding. By this mechanism a polymer chain left to its own devices will repeatedly fold back on itself to form a platelet approximately 10 nm thick. That such a structure can exist in one specimen together with fringed micelles has been demonstrated. Chain folding mechanisms, however, are probably more relevant to the comparatively simple synthetic polymer chains than the complex peptide units of biopolymers and so will not be further discussed.

The ordered structures which polymer chains can adopt to produce crystallinity within the bulk polymer still remain centres of intensive
study and controversy. The brief descriptions of those provided in the foregoing text include only a selected few. They do, however, illustrate that the subject is by no means one of clearly defined fact but remains subdivided into schools of thought in much the same way as the study of elastin ultra-structure.

8.3 Other Connective Tissue Structures

Viewed from a biological angle, elastin is part of the general group of biological structural tissues. Hence it is pertinent to discuss the structures of some of these tissues, and examine for any marked similarities with elastin which might provide a clue to its structure.

Generally the connective tissue proteins are found to be built up from three main structures: the α-helix, the antiparallel β pleated sheet, and the triple helix. The keratins are mostly α-helical, although the feathers of birds and some of the stiffer parts of non-mammals consist of a complicated form of β-sheet. Myosin, epidermin and fibrinogen are also α-helical while the best examples of the β sheet are found in the silks. The collagens use a characteristic triple helix.

Elastin is invariably found in close association with collagen; indeed to such an extent that it has been suggested that elastin itself is in fact a form of collagen⁸⁻¹⁰. The mechanical properties of the two tissues, however, differ widely. The basic mechanical characteristics of collagen are rigidity and resistance to stretching, while elastin is of low modulus and rubbery. Collagen is found where mechanical force must be transmitted without loss and in general network strengthening.

The fundamental structural unit of collagen is a 'tropocollagen' molecule 1.5 nm diameter and 280 nm long with a molecular weight of
about 300,000. Tropocollagen molecules are thought to pack with a displacement of one quarter of their length to form an overlapping collagen fibril having a characteristic banding pattern every 64 nm. These fibrils are typically about 500 nm diameter. Each tropocollagen molecule consists of a triple-helix, a third of which is made up of glycine and another 25% proline and hydroxyproline. The great strength of the collagen triple-chain coiled coil arises from the considerable inter-chain hydrogen bonding. These also add an element of rigidity to the complete molecule, the individual collagen molecules collapsing if these bonds are broken. The fact that the tropocollagen molecules line up in the fibril with just the right quarter-length displacement indicate that there must be some long-range regularity in amino-acid sequence beyond that for the creation of the triple-helix structure. What this sequence is, however, remains a centre of controversy.

Perhaps the most familiar of all the fibrous proteins are the keratins which form the protective coverings of all land vertebrates; skin, fur, hair, wool, claws, nails, hooves, horns, scales, beaks and feathers. The basic unit of hair keratin is the right-handed α-helix which appears to twist together in groups of three (but not helically intertwine as with collagen) in a left-handed coil, termed a 'protofibril'. These protofibrils have been viewed in the transmission electron microscope and are around 2 nm diameter. Nine such protofibrils bundle in a circle around two more to form an eleven-stranded 'cable', termed the 'microfibril', which is approximately 8 nm in diameter. The microfibrils are then embedded in an amorphous glyco-protein matrix of high sulphur content to form an irregular fibrous bundle called the macrofibril, of dimension approximately 200 nm diameter. Thus, if a typical hair fibre of, say, 20 micrometers across is examined, a structure of packed dead cells of
the order of 2 micrometers will be observed. Within these cells lies a residue of macrofibrils oriented parallel to the fibre axis, and each about 200 nm in diameter. Hence, a hair presents an ordered progression of studies: hair fibre, cell, macrofibril, microfibril, protofibril and α-helix. Part of its properties come from the α-helix, but much more come from the way in which it is used.

The α-keratins are very extensible: a wool fibre can be drawn out to twice its original length. When this happens, the α-helices stretch with a breaking of hydrogen bonds between turns of the helix, and an extended β-chain structure is formed. The hydrogen bonds themselves would not be enough to return the fibre to its original state after tension is removed. The helices, however, are cross-linked with disulphide bonds from cystine residues, and these cross-links form both a resistance to stretch to begin with, and a strong restoring force when the stress is removed. The helices, however, are cross-linked with disulphide bonds from cystine residues, and these cross-links form both a resistance to stretch to begin with, and a strong restoring force when the stress is removed. The α-keratins are classed as soft or hard, according to their sulphur content: the low sulphur content keratins of skin and callus are flexible and much more extensible than the high sulphur hard keratins of horns, claws and hooves. Thus the physical behaviour of low keratin arises from the presence of α-helices hydrogen-bonded within the chain but connected to neighbouring helices by disulphide bonds.

Collagen and keratin might be considered the closest in vivo neighbours of elastin. All three are found in close association with each other in various parts of mammalian bodies. Amino-acid analyses, however, preclude the possibility of either being structurally similar to elastin. Proline, for example, of which elastin contains approximately 11%, is generally incompatible with a predominantly α-helical structure, although it does not rule out the possibility of limited regions existing.
Elastin is noted for its high percentage of glycine and alanine (being 32% and 22% respectively) and biological structures with similar high percentages are those of silks with approximately 40% glycine and 30% alanine. Silks are built from extended polypeptide chains stretched parallel to the fibre axis with neighbouring chains intertwining in opposite directions and hydrogen bonded together. Pauling and Corey named this structure the antiparallel pleated sheet: the need to make good hydrogen bonds keeps the chain from being fully extended, and this produces the pleating effect in the sheet. The amino acid composition is such that all the glycine residues will be on one side of the β-sheet and all the alanine on the other. The sheets thus pack together with glycines to glycines and alanines to alanines. The distance between alternate sheets is either 0.35 nm or 0.57 nm.

The result is a fibre which is very strong because the resistance to tension is borne directly by the covalent bonds of the polypeptide chain. It is not appreciably extensible for the chain is already stretched as far as it can go without breaking the hydrogen bonds which hold the sheet together. Nevertheless, since the sheets themselves are held together only by Van der Waal's forces between unbonded side chains, the silk is quite flexible.

There is also another part of silk structure which has marked effects on its mechanical properties. In similar manner to elastin, the amino acid sequences of silk contain a proportion of bulky side-chains such as tyrosine. There is no room in the sheet to incorporate those bulky side-chains, and so, in similar manner to a fringed micelle or fringed fibril structure, the ordered regions alternate with disordered regions. The disordered regions contain in addition to the three primary residues (glycine, alanine, serine) all the large side-chains. The amorphous
regions are responsible for the extensibility of the silk fibre and a given chain can run through several crystalline regions with disorder in between.

Different species of silkworm produce proteins with different proportion of bulky amino acids with the consequent differences in crystalline/amorphous structural ratio. Thus, anaphe moloneyi silk has approximately 80% crystallinity, and demonstrates Hookean elasticity up to a comparatively low breaking-point at 12.5% strain, while B. mori silk has 60% crystallinity and is almost completely elastic up to its breaking-point of 24% strain. Silks with less crystallinity show greater extensibilities but with an increasing proportion of viscous flow demonstrating a low degree of cross-linking in the amorphous regions.

There is notable similarity between the amino acid composition of the silks and that of elastin, yet caution must be applied in making subsequent conclusions about similarities in structure. There is little similarity between X-ray diffraction data obtained from the two materials. Silk presents a characteristic banded pattern, whereas only amorphous rings are obtained from elastin. Nevertheless, where an analogy is useful is in showing that an 'amorphous' structure can co-exist in a biological material with a crystalline one in a similar manner to the semi-crystalline polymer models described in section 8.2.

In elastin Grey et al. have demonstrated that instead of each type of amino acid being evenly distributed along the chain, two different distributions tend to occur – alanine and lysine-rich regions, and glycine, proline and valine-rich regions. They suggested that the former was concerned with cross-linking and the latter with the extensibility of the material. They also pointed out that in the cross-link regions pairs of lysines occurred in sequences such as Ala-Ala-Ala-Ala-Lys-Ala-Ala-Lys-Tyr-Gly-Ala-Ala-... The sequences could be much longer with more lysines and
with longer runs of alanine at the amino end. Such regions would favour an \( \alpha \)-helical conformation with lysines protruding from the same side of the helix. This would facilitate inter-chain condensations between oxidised lysines. Subsequent inter-chain condensations would lead to desmosine and isodesmosine linking two \( \alpha \)-helical chains with very little strain.

From circular dichroism experiments the presence of limited areas of \( \alpha \)-helix structure has been reported in solutions of chemically dissolved elastin\(^8\text{-}22,8\text{-}23\) in amounts varying from 10% to 15%. The amount is reported to increase with the ethanol content of the solvent\(^8\text{-}24\).

Grey et al\(^8\text{-}21\) proposed a structure of the remaining amino acids of large left-handed \( \beta \)-coils and hence produced their 'oiled coil' model as described in Chapter 1. A main part of their argument for the oiled coil structure, however, rests on Weis-Fogh and Andersen's liquid-drop type elastic recoil mechanism which has been shown in the earlier sections of this thesis to be with little experimental backing. The model also lacks substantiation from X-ray diffraction data and it is thus considered much more likely that the remaining structure would be that of the random coil classical rubber type amounting to some 80% to 90% of the bulk material.

Additional evidence for the random coil structure arises from work by Torchide and Piez\(^8\text{-}25\) who studied elastin using the technique of nuclear magnetic resonance. They established that solvated elastin was composed largely of highly mobile chains and this strongly suggested a random coil structure with configurational entropy playing a major role in elastic properties.

The phenomenon of increasing \( \alpha \)-helix content in certain solvents\(^8\text{-}24\) has also been found in soluble bombyx silk. Solutions of this material in dichloroacetic acid and in aqueous lithium bromide have been found\(^8\text{-}26\).
to have the structure of a randomly coiled polymer. However, when the solution in dichloroacetic acid is diluted with ethylene dichloride or with ethylene chlorohydrin, significant formation of \( \alpha \)-helices occurs. The water soluble silks of antherea mylitta and anaphe moloneyi have also shown \( \alpha \)-helical forms when prepared as films\(^{8-27,8-28}\) and it is interesting to compare this with the work of Urry et al\(^{8-29,8-30,8-31}\) on coacervated films of solubilized elastin. From Urry's studies the elastin films were shown to be in a more ordered state than in solution and to contain electron-microscopically observable 'Gotte fibril' structures (see Chapter 7).

The hypothesis of elastin being in fairly delicate balance between a crystalline and amorphous structure (at least in certain areas, if not overall) thus appears a reasonable possibility. Consideration of the topic is not complete, however, without an examination of the X-ray diffraction data which has been obtained from the material. This is discussed in the following section.

### 8.4 X-Ray Diffraction Results from Elastin

For structural investigations the technique of X-ray diffraction is probably one of the most powerful tools available to materials scientist and molecular biologist alike. Nevertheless, results obtained from elastin have been comparatively uninformative.

The early work on elastin by Kolpak\(^{8-32}\) demonstrated a similar pattern to collagen. This, however, was later criticised by Astbury\(^{8-33,8-34}\) as being due to impurities of that material. Astbury's own results\(^{8-33,8-34}\) on unpurified ligamentum nuchae demonstrated an amorphous pattern of randomly oriented collagen fibres superimposed on a faint 0.44 nm diffuse
ring which he attributed to the elastin. When the ligament was treated in a steam bath for a week, the collagen pattern was found to have disappeared. From this work Astbury suggested that elastin might be a member of the collagen group whose thermal transformation temperature was below room temperature.

Ramachandran and Santhanam performed experiments on autoclaved ligamentum nuchae and found three rings, a 0.22 nm, a 0.44 nm and a faint central halo at 0.9 - 1.0 nm. Comparing these results with chemically denatured and thermally shrunk collagen, he arrived at a similar conclusion to Astbury – that elastin was in fact a form of denatured collagen.

Studies on a variety of purified, elastin-rich tissues were performed by Cox and Little and were perhaps the first to produce any really consistent results. Cox and Little used samples of human and animal aorta and ligamentum nuchae purified by acid and alkaline treatment, and obtained diffraction patterns of the material in its dried, and stretched and dried, state. All the specimens were found to show the same two amorphous haloes at 0.46 nm and 0.78 nm respectively. There was no sign of the characteristic collagen halo of 0.286 nm and the pattern remained the same even when stretched 100 - 150%.

A very comprehensive study was performed by Gotte et al who obtained patterns from autoclaved ligamentum nuchae, before and after ultrasonication, treatment in methanol, formdimethylamide, and dimethylsulphoxide, with and without stretching. Results were also obtained from the material in its water swollen state. Dry autoclaved elastin both before and after ultrasonication was found to exhibit a series of diffuse rings at 0.89 nm, 0.44 nm, 0.22 nm and 0.112 nm. A diffuse halo extending from the 0.44 nm ring to a distance of 0.28 nm was observed, possibly due to residual collagen in the material, and the
0.22 nm and 0.112 nm rings were very weak. Swelling in methanol, stretching 100% and drying, showed a strengthening of 0.89 nm and 0.44 nm rings with no significant orientation effects. Swelling in formdimethylamide and dimethylsulphoxide increased the 0.89 nm ring to 1.3 nm, but left the 0.44 nm ring unchanged, and stretching these swollen fibres produced no orientation effects. On drying, the pattern reverted to the previously observed 0.89 nm and 0.44 nm rings. Swelling in water appeared to affect the 0.44 nm ring, shifting it to 0.35 nm and finally, ultrasonicated elastin, extruded as a paste and dried, gave a much sharper definition of the still unoriented 0.44 ring.

The most recent X-ray diffraction results have come from Serafini-Fracassini et al8-37 who obtained both wide and low-angle diffraction patterns from collagenase purified bovine ligamentum nuchae in dried, stretched and unstretched states. In similarity with earlier studies, wide-angle patterns revealed two amorphous rings at 0.45 nm and 0.93 nm respectively. These rings were unaffected by strains of 140% to 165%. Low-angle X-ray diffraction of unstretched material gave patterns in which no significant features were visible. Samples oriented by extension to 140% of their original length produced a diffuse, almost complete diffraction ring corresponding to a spacing of 5 nm, which on further elongation to 164% became almost entirely equatorial and was accompanied by an additional reflection corresponding to a spacing of 4.6 nm. Serafini-Fracassini et al8-37 suggested that the 5.0 nm and 4.6 nm refractions represented inter-filament distances, possibly from two separate types of packing arrangement within the fibrils. In addition it was considered that this packing might be tetragonal at moderate strain, becoming hexagonal at higher strains, an arrangement which would yield inter-fibril distances of 5.0 nm and 4.4 nm respectively.
There thus is a wide range of amorphous rings reported throughout the literature to constitute the complete diffraction pattern of elastin. Two groups of these, however, appear predominant. These consist of the 0.44-0.46 nm rings, decreasing to 0.35 nm when swollen in water\textsuperscript{8-20} and the 0.78-0.93 nm rings, increasing to 1.3 nm when swollen in DMSO or formdimethyamide.

The subsequent step would thus appear to be to attempt to interpret these reflections in terms of helical or other crystalline structures. It is here, however, where caution should be applied. Amorphous synthetic polymers also typically show diffuse diffraction rings; rings which correspond to the more frequent Van der Waal's distances and to the more probable inter-chain separations within the structure\textsuperscript{8-38,8-39}. Nevertheless, Gotte et al\textsuperscript{8-40} have attributed the 0.44 nm ring to the translation distance per repeat along a chain spiral axis. These researchers conclude that elastin consists of an assembly of rope-like structures each with an overall diameter of 3.5-4.0 nm of paired elements each 1.5 nm in width and periodically linked longitudinally at a distance capable of varying 3.5-4.5 nm according to the degree of stretching. How such a rigid molecular structure is able to generate the predominantly entropy elastic recoil mechanism of solvated elastin remains by them unexplained. Hence, although Gotte et al's\textsuperscript{8-40} proposals might well apply to the dried material, it must be treated with caution when considering elastin in its in vivo condition.

Perhaps the most significant of all the results described in this section were the low-angle reflections of Serafini-Fracassini et al\textsuperscript{8-37}. The fact that significant features in the patterns only become visible on the application of considerable strain to the specimen is in clear support of the hypothesis of Chapter 7 where it was suggested that fibrillar
structures observed in elastin at the highest electron-microscope magnifications were a direct result of the preparation process and not typical of the material under general in vivo conditions.

8.5 Final Conclusions on Elastin Structure

It is the author's opinion that the evidence for solvated elastin conforming to a predominantly random coil structure is overwhelming. There is virtually no experiment which has been performed on the bulk material which does not in the final analysis support the random coil theory: results to the contrary all stem from experiments involving severe specimen preparation treatments employing fragmentation, drying or dissolving. In independent research centres in various parts of the world at least three research groups have measured the f/f ratio of the material to be in the region of 0 to 0.1, thus showing clear entropy predominance of its elasticity (see Chapters 2 and 6). Furthermore, the evidence for its random coil structure has been given added confirmation by nuclear magnetic resonance studies. The work of this thesis has shown one of the main pieces of supporting evidence for the globular and oiled coil structures to be unfounded (see Chapter 4) and this, coupled with elastin's twin ring X-ray pattern typical of amorphous polymers, provides a powerful argument for the random coil.

It would nevertheless be pertinent to make a final critical summary of the arguments and counter-arguments for elastin structures other than random coil:

(a) Globular Model: Evidence was claimed to arise from biochemical considerations (ie: tendency of hydrotropic chains to reduce surface area to minimum and form globules), diffusion column experiments, thermo-mechanical

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Considering each of these separately:

(i) Biochemical considerations - although globular structures are well known in the general field of protein structure ordered to allow minimum exposure of their hydrophobic groups, it does not necessarily follow that all predominantly hydrophobic proteins will be globular. The structure of silk fibrils has already been discussed, and their similarity of amino-acid composition and hydrophobicity to elastin pointed out. Their structures, however, are far from globular.

(ii) Diffusion column experiments (see Chapter 1) - these experiments have been criticized by Hoeve and Flory\(^8-41\) who reject Partridge's assumption (see Chapter 1) that retardation of solutes is governed by the size of discrete inter-particle pores. Hoeve and Flory claim that Partridge ignores the theory of retention of solutes within a gel matrix on the basis of their size and solubility, a theory well established in gel phase chromatography using cross-linked polymers as matrices.

(iii) Thermo-mechanical experiments of Weis-Fogh and Andersen - much has already been said regarding these results, so they will not be further discussed.

(iv) Electron microscopy - the results put forward by Partridge in support of the globular theory (see Chapter 1) indicate a structure which could be interpreted in a number of ways without the necessity of invoking a globular model. The globular appearance could, for example, be due to the type of domain structure observed by Geil (ref. 7-46) in his studies on amorphous elastomers. Even if this was not the case, however, Hoeve and Flory\(^8-41\) have pointed out that specimens of polymeric materials prepared in thin films often show similar rippled appearances when examined under the transmission electron microscope.
(b) Oiled-Coil Model: Supporting evidence claimed from (i) liquid drop mechanism; (ii) molecular modelling and circular dichroism studies suggesting evidence of α-helices\(^8-22,6-23\).

As with the globular model the validity of the liquid drop mechanism has already been discussed, so will not be repeated. Circular dichroism studies are necessarily performed on solutions of the material which, owing to its naturally insoluble nature must first be rendered soluble by fairly vigorous action of chemical reagents such as formic acid. Because of this, the extrapolation of results obtained from these solutions to the bulk material is not necessarily a justifiable procedure. Gotte et al\(^8-22\) themselves acknowledge this in their original paper on the subject, stating 'This finding does not necessarily mean that the same conformation (α-helix) must also be present in the native insoluble protein because of the chemical degradation used for its solubilization'. A similar argument applies to circular dichroism structures on the precipitated coacervate\(^6-23\). The postulation for the other portion of the structure, the β-turns, is based largely on molecular modelling. Again, however, Partridge's diffusion column experiment is cited with the acceptance of his interpretation of results, and consequently the same criticism must apply as stated in the previous section.

(c) Fibrillar Model: Support claimed from electron microscopy (see Chapter 7) low-angle X-ray analysis\(^8-37\) and α-helix evidence from circular dichroism. Each of these three aspects have already been discussed so will not be further enlarged upon. The possibility of considerable crystallization on drying or with a combination of drying and staining appears to be a likely one and it is interesting to note that the oiled coil theory actually includes a mechanism for such a process, leading to a rigid cross-β structure.
It thus follows that there is little substantial evidence for any but a predominantly random coil structure for solvated elastin in its physiological condition. Precisely how the electron microscopically and low-angle X-ray observed fibrillar structures occur must remain a topic for further research.

One last argument which can be put forward in favour of the random coil structure should also be mentioned, although it is perhaps of slightly more philosophical than scientific nature. This concerns nature's need for elastin in the body. Elastin is always found in conjunction with strong and tough protein collagen and it is this material which provides the strength needed for the composite. Elastin itself has no need of strength; its in vivo specification is a material to provide pure elasticity. Collagen provides the strength, elastin the elasticity, and it seems impossible to 'design' a more efficient ultrastructure to produce medium to long-range (10% - 100%) elasticity than random coil structures whose densities are low and require a no more intricate formation mechanism than a simple random cross-linking process.

The final section of this chapter will be devoted to what in the writer's view is the most important aspect of a thesis such as this: a consideration of implications of any results generated by the work.

8.6 Application of Results to Medical Research

Undoubtedly the most important application of elastin research in the field of medicine today is in attempting to elucidate the causes of arterial disease. The field is a vast one, and to review it is not only beyond the scope of the thesis, but would occupy many volumes. Two aspects, however, have been shown to be particularly associated with elastin - the
formation of atherosclerotic plaques and the calcification of those plaques and of the aortic wall itself.

The formation of atherosclerotic plaques appears to result from the deposition of lipids due to hydrophobic interaction, whereas calcium binding, the initial step for calcification, occurs at actual sites, i.e. at the peptide carbonyl oxygens, within the elastic fibres; both processes lead to general arterial wall degeneration. The individual steps involved with these processes, however, are complex and for excellent general reviews of these subjects reference should be made to Ref. 8-8.

One fact which emerges from the literature is that, in general, research into chemical and biochemical causes of these degenerative mechanisms has far outstripped research into physical initiating factors. It is only comparatively recently that the importance of physical factors is now beginning to be realized.

As a first step in examining possible physical influences on the arterial wall in the process of disease, it is necessary to have a clear understanding of how the material of the wall functions under normal, healthy in vivo conditions. It is here that speculation can be made from the work of this thesis:

It is a general assumption in the literature of mechanical studies on the deformation of elastin within the arterial wall to assume that the material behaves like a dry, incompressible rubber with a volumetric strain approximately zero$^{3-4}$. The work of Chapters 4, 5 and 6 of this thesis, however, has shown this not to be the case. From these studies it follows that elastin in the vasculature operates under conditions of time, temperature and solvent uptake which place it in the viscoelastic transition zone. The equivalent time in the step-function experiments of
Chapter 6 can be approximated by the formula $t = (2\pi q)^{-1}$ where $q$ is the in vivo oscillation frequency. If $q = 1 \text{ hz}$, then $t = 0.16 \text{ sec}$, which can be seen from Chapter 6 to place the material considerably further into a viscoelastic state than in the shortest times (2 sec) used in the experiments. The most significant physiological implications of this are mechanical damping of the system and the response of the material to the hydrostatic component of the general stress tensor. As has been discussed, there is a liquid uptake of the water swollen specimen with length:

$$(\partial V_s/\partial l)_{P,T,eq} > 0,$$

in which eq denotes the specimen is in equilibrium with a reservoir of diluent. When the stress is applied, the specimen absorbs liquid which is exuded when released, and if this true for diluted elastin, then a pulsating stress in an element of elastin will generate a molecular flux along a concentration gradient which may well be considerably larger than the flux due to normal diffusion processes. What is more, the efficiency of this stress assisted 'diffusion pump' will be facilitated by the laminated texture of the arterial wall, and it is quite possible that nutrition of the inner regions of the wall depends upon this pump mechanism. Any contribution from other tissues present is likely to be small$^{8-45}$ and also of visco-elastic nature$^{8-46}$.

It is hence conceivable that a critical balance is established within the wall of the vessel, with the diffusion pump plus natural diffusion gradient effects exactly supplying the nutritional needs of the cells deep within the wall, and it follows that any factor which upsets this equilibrium will necessarily cause degeneration of the wall. Such a factor which could upset the system is the binding of lipids to the elastin on the intimal layers of the wall. This would generally create an increase in hydrophobicity of the region and act as a barrier to fluids diffusing through the wall.
Another known influence on arterial wall degeneration is the condition of hypertension. Recent studies by Sharma and Hollis indicate a considerable stiffness increase in the aortic walls of rabbits under induced hypertensive conditions which they attribute to an overall increased water content of the wall. An increased stiffness is likely to reduce the diffusion pump mechanism by reducing the volume change of the material per cycle. Hence, although the overall liquid composition of the wall may increase, the diffusive flux will decrease, leading to wall degeneration.

Although such mechanisms must remain for the moment in hypothetical form, they nevertheless serve to illustrate how physical parameters may be important for the healthy functioning of the arterial wall. It is hard to believe that elastin's 'built in' viscoelasticity is not there for a purpose!

8.7 Conclusion

As each experimented chapter has stated its own specific conclusions, these will not be reiterated. The main conclusion of the complete thesis is, however, that contrary to numerous other proposals, the protein elastin under typical in vivo conditions consists of a randomly coiled molecular structure typical of amorphous rubbers. Nevertheless, its mechanical properties in this condition place it in the viscoelastic transition zone in a region just below the positive slope linear region relating to conventional rubbers. In spite of this, its elastic response is predominantly entropy elastic.
8.8 Suggestions for Further Work

As can be perceived by turning through the pages of this thesis, the general philosophy of the work has been to use a wide range of available techniques to investigate elastin structure. The advantage of this method is that a general scan of a variety of methods is carried out, and any which appear particularly fruitful can be pin-pointed for further work. This thesis might be considered as having carried out the 'scan' and hence useful further research would best be aimed at more detailed work on any one of the techniques employed.

It is the author's opinion that probably the most fruitful areas would be in further dynamic or step-function experiments of the type described in Chapters 5 and 6 to examine parameters such as tissue age, source, purification and condition (healthy, diseased, calcified, etc.). The bulk of the mechanical mechanical relaxation spectrum of both dry and solvated elastin and unpurified elastic tissue remains to be investigated.

Electron microscopy and X-ray analysis have been well investigated and it appears that it is unlikely that these techniques alone will produce any major advances in current elastin structural knowledge. An investigation of the bonding relationship of elastin to its neighbouring tissues using these and related techniques, however, might prove fruitful.

Finally, mechanical 'in vivo' and diffusion experiments on whole aortas, similarly in vivo conditions, would provide valuable information on the proposed diffusion pump mechanism.
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APPENDIX 1

THE THEORY OF RUBBER ELASTICITY

Introduction

The phenomenon of rubber elasticity is unique in nature. A cross-linked rubber strip, extended to several times its original length, will return when released to its original length exhibiting little or no permanent deformation as a result of the extension. In marked contrast to this is the behaviour of crystalline solids and glasses which cannot normally be extended to more than a very small fraction of their original length without undergoing fracture. On the other extreme lie the ductile metals which can undergo large deformations without fracture, but do not return to their original length on removal of the deforming stress.

Over the years, rubber elasticity has been examined using three different approaches: thermodynamic, statistical and phenomenological. The classical thermodynamic approach is concerned only with the macroscopic behaviour of the material under investigation and has nothing to do with its molecular structure, this being left to the statistical approach. The phenomenological treatment provides a fuller description of the elasticity at large deformations.

Thermodynamic Treatment

From the first and second laws, for reversible processes:
\[ dU = TdS - dW \]  
(1)

\( dU \) = internal energy

\( dS \) = entropy

\( T \) = temperature

\( dW \) = All the work performed by the system

(ie: electrical, mechanical, pressure, volume, chemical, etc.)
For an ideal gas, \( dW \) simply becomes pressure-volume work \( PdV \).

In the case of a rubber, however, deformed by an amount \( dl \) in equilibrium tension exerting a restoring force \( f \), the mechanical work done on the system to accomplish the deformation, namely \( fdl \), must also be included in \( dW \). Thus, for a rubber strained uniaxially in tension under equilibrium conditions:

\[
dW = PdV - fdl
\]  

(2)

In a simple tensile test, \( P \) would be the atmospheric pressure and \( dV \) the volume dilation accompanying the elongation of the rubber. Generally, \( PdV \) is much smaller than \( fdl \) and may sometimes be neglected in comparison to \( fdl \). However, for completeness, the following treatment will include pressure-volume work. With these considerations, equation (1) thus becomes:

\[
dU = TdS - PdV + fdl
\]  

(3)

Maintaining constant pressure, i.e., atmospheric, it is natural to use the thermodynamic entity \( H \), the enthalpy defined as:

\[
H = U + PV
\]  

(4)

Differentiating (4) gives:

\[
dH = dU + PdV
\]  

(5)

And substituting for \( dU \) from (3), we obtain:

\[
dH = TdS + fdl
\]  

(6)

or \( fdl = dH - TdS \)

Thus, the restoring force exerted by the rubber when it has undergone a deformation by the amount \( dl \) at constant temperature and pressure is:

\[
f = \left( \frac{\partial H}{\partial l} \right)_{T,P} - T \left( \frac{\partial S}{\partial l} \right)_{T,P}
\]  

(7)

Equation (7) shows that the restoring force originates in the enthalpy and entropy changes which occur in the elastomer as a result of its deformation.
If stress vs temperature curves are plotted for a piece of uniaxially strained rubber under equilibrium conditions, at different elongations, the slope of the curves is found to change from negative values at low degrees of elongation to positive values at high elongations. This effect is known as the thermoelastic inversion phenomenon. In order to see why this should occur, it is necessary to obtain a thermodynamic expression for the slope of the curve, that is an expression for \( \frac{\partial f}{\partial T} \)\_P,\_1.

Thus:

The Gibbs free energy is defined by:

\[
\mathrm{d}E = -S \mathrm{d}T + V \mathrm{d}P + f \mathrm{d}l
\]  

(8)

And taking one of Maxwell's relations which states:

\[
\left( \frac{\partial S}{\partial l} \right)_{T,P} = - \left( \frac{\partial f}{\partial T} \right)_{T,1}
\]  

(9)

and substituting for \( \left( \frac{\partial S}{\partial l} \right)_{T,P} \) in (7) gives:

\[
f = \left( \frac{\partial H}{\partial l} \right)_{T,P} + T \left( \frac{\partial f}{\partial T} \right)_{P,1}
\]  

(10)

which can be re-arranged to give the desired result:

\[
\left( \frac{\partial f}{\partial T} \right)_{P,1} = \frac{f - \left( \frac{\partial H}{\partial l} \right)_{T,P}}{T}
\]  

(11)

Thus, in order for the slope of the \( f \) vs \( T \) curves to be negative, it must be that:

\[
\left( \frac{\partial H}{\partial l} \right)_{T,P} > f
\]

while in order for the slope to be positive it must be that:

\[
f > \left( \frac{\partial H}{\partial l} \right)_{T,P}
\]
Experimental results show that the former inequality holds at low elongations, while the latter inequality holds at high elongations.

At sufficiently high elongations, it is possible to neglect \( \frac{\partial H}{\partial l} \) in comparison with \( f \), and as a result, under these conditions:

\[
f = T \left( \frac{\partial f}{\partial T} \right)_{P, l} = -T \left( \frac{\partial S}{\partial l} \right)_{T, P}
\]

ie: \( f \) becomes directly proportional to the absolute temperature or the elastic response of the rubber is entirely governed by the decrease in entropy which it undergoes upon extension.

Although equation (12) adequately describes the behaviour of an elastomer at high extensions (> 10% for natural rubber), it is nevertheless true that the coefficient \( \frac{\partial H}{\partial l} \) has a finite value which cannot be neglected in an exact treatment of rubber elasticity. (It should be noted that equation (12) is inadequate to describe the behaviour of most elastomers at very high extensions. This is because many elastomers undergo crystallization at sufficiently high extensions and, when this occurs, the term \( \frac{\partial H}{\partial l} \) once again becomes important and may actually outweigh the term \( -T \left( \frac{\partial S}{\partial l} \right) \).) In order to explore the origins of the coefficient \( \frac{\partial H}{\partial l} \), it is necessary to return to equation (5).

Differentiation of equation (5) at constant temperature yields:

\[
\left( \frac{\partial H}{\partial l} \right)_{T, P} = \left( \frac{\partial U}{\partial l} \right)_{T, P} + P \left( \frac{\partial V}{\partial l} \right)_{T, P}
\]

It is obviously desirable to separate effects arising from volume changes from effects arising from deformations in the material. This can be done as follows:

Expressing \( U \) as a function of length and volume:

\[
dU = \left( \frac{\partial U}{\partial V} \right)_{l, T} dV + \left( \frac{\partial U}{\partial l} \right)_{V, T} dl
\]
And differentiating, gives:

\[
\left( \frac{\partial U}{\partial V} \right)_{T,P} = \left( \frac{\partial U}{\partial V} \right)_{1,T} + \left( \frac{\partial U}{\partial V} \right)_{V,T} \tag{15}
\]

which, upon substitution into (13) with re-arrangement gives:

\[
\left( \frac{\partial S}{\partial V} \right)_{T,V} = \left( \frac{\partial S}{\partial V} \right)_{1,V} + P \left( \frac{\partial U}{\partial V} \right)_{T,P} \tag{16}
\]

Equation (16) shows that the coefficient \(\frac{\partial S}{\partial V}\) consists of a term arising from internal energy changes occurring during a change of length at constant volume and a volume dependent term. Although the thermodynamic development is not concerned with the molecular nature of the rubber, the coefficient \(\frac{\partial U}{\partial V}\) can, in fact, be related to intramolecular energy effects. This is so because of the constant volume condition of a rubber. Real rubbers usually have non-zero values for this coefficient.

The coefficient \(\frac{\partial S}{\partial V}\) may be transformed into experimentally accessible quantities as follows. The Helmholtz free energy \(dF\) is given by:

\[
dF = -SdT - PdV + fdl \tag{17}
\]

From this it follows that:

\[
\left( \frac{\partial S}{\partial V} \right)_{T,1} = \left( \frac{\partial P}{\partial T} \right)_{V,1} \tag{18}
\]

Returning to (3) and differentiating with respect to \(V\) at constant \(T\) and \(1\)

\[
\left( \frac{\partial U}{\partial V} \right)_{T,1} = T \left( \frac{\partial S}{\partial V} \right)_{T,1} - P \tag{19}
\]

or, making use of (18) and (19)

\[
\left( \frac{\partial U}{\partial V} \right)_{T,1} = T \left( \frac{\partial P}{\partial T} \right)_{V,1} - P \tag{20}
\]
Then, by partial differentiation:
\[
\left(\frac{\partial P}{\partial T}\right)_{V,1} = -\left(\frac{\partial P}{\partial V}\right)_{T,1} \left(\frac{\partial V}{\partial T}\right)_{P,1}
\]  
(21)

Now, the coefficient of volume expansion is defined by:
\[
\beta_V = \frac{1}{V} \left(\frac{\partial V}{\partial T}\right)_{P,1}
\]  
(22)

and the coefficient of isothermal compressibility by:
\[
\beta_P = \frac{1}{V} \left(\frac{\partial V}{\partial P}\right)_{T,1}
\]  
(23)

(Note that, as defined above, both \(\beta_V\) and \(\beta_P\) are functions of length inasmuch as the volume of the elastomer undergoes changes on extension.)

Using these definitions we thus have:
\[
\left(\frac{\partial P}{\partial T}\right)_{V,1} = \left(\frac{\partial V}{\partial T}\right)_{P,1}
\]  
(24)

and
\[
\left(\frac{\partial U}{\partial V}\right)_{T,1} = \frac{T}{\beta_P} - P
\]  
(25)

Inserting (25) into (16)
\[
\left(\frac{\partial H}{\partial L}\right)_{T,P} = \left(\frac{\partial U}{\partial L}\right)_{T,V} + T \frac{\partial V}{\partial P} \left(\frac{\partial V}{\partial L}\right)_{T,P}
\]  
(26)

The quantity \(\frac{\partial H}{\partial L}\)_{T,P} is directly accessible from experimentally obtained \(f\) versus \(T\) curves as the intercept at \(T = 0\) of the tangent to the experimental curve at any desired temperature (see (10)). Its measurement involves no difficulty for any elastomer provided equilibrium constant pressure stress-temperature data are available for that elastomer. In principle, the second term on the right hand side of (26) is also directly measurable, but precise values turn out to be very difficult in practice. Measurements of \(\beta_V\) and \(\beta_P\) can be carried out with relative ease,
but the coefficient $\left(\frac{\partial V}{\partial L}\right)_{T,P}$ is very small for most rubbers and usually depends on extension. Thus great experimental skill is required in order to obtain a measurement of any accuracy\(^1\),\(^2\).

By combining equation (26) with (10) the energetic component of the elastic force $f_e$ becomes:

$$f_e = \left(\frac{\partial U}{\partial l}\right)_{T,V} = f - T \left(\frac{\partial f}{\partial T}\right)_{P,L} - T \left(\frac{\partial V}{\partial l}\right)_{T,P}$$

Thus from (27) it should be possible to compute the relative importance of contributions from energy and entropy to rubber elasticity. Once again, however, the unavailability of accurate values of the partial derivative $\frac{\partial V}{\partial L}$ renders this separation difficult at best.

In general, direct measurements\(^3\) have not been fruitful and various approximate methods have been devised.

Statistical Treatment

The molecular model for the ideal gas is a collection of point masses in ceaseless random thermal motion. The motion of each of the point masses is completely unrelated to the motion of any other. The counterpart to this in the case of the ideal rubber is a collection of volumeless, long, flexible chains which are constantly undergoing conformation re-arrangements due to thermal motion but with all conformations equally accessible and without energy differences between them (that is, all conformations are isoenergetic). Upon stretching, some chain conformations become inaccessible, leading to a decrease in entropy and hence to the restoring force exerted by the rubber.

In order for a rubber to exhibit an equilibrium elastic stress, it is necessary for the collection of linear polymer chains to be tied together into an infinite network. Otherwise Brownian motions of the macromolecules
will cause them to move past each other, thus exhibiting flow. Cross-linking reactions are many and varied in different elastomers, but it is sufficient to simply consider a cross-link to be a permanent tie-point between two chains.

To compare an ideal rubber with the ideal gas on a more quantitative basis:

from equation (3)

\[
f = \frac{\partial U}{\partial l} \left( T, V \right) - T \frac{\partial S}{\partial l} \left( T, V \right)
\]  

(28)

which resembles the gas relation:

\[
-P = \frac{\partial U}{\partial l} T - T \frac{\partial S}{\partial V} T
\]  

(29)

For ideal gases, the internal energy is independent of volume, \((\partial U/\partial V)_T = 0\) and the entropy has two components. One of these is associated with the heat capacity of the gas, but independent of volume, and the other is related to the configurational entropy of the system and thus a function of volume. By analogy, the ideal rubber may be looked at in the same way. Its internal energy is independent of elongation as required by the model and thus \((\partial U/\partial l)_T, V = 0\) and the stress can be attributed to configurational entropy alone.

In the formulation of the statistical theory of rubber elasticity, the following simplifying assumptions are made:

1. The internal energy of the system is independent of the conformations of the individual chains.

2. An individual network chain is freely jointed and volume-less (ie: it obeys Gaussian statistics).
3. The total number of conformations of an isotropic network of these Gaussian chains is the product of the number of conformations of the individual network chains.

4. Cross-link junctions in the network are fixed at their mean positions. Upon deformation, these junctions transform affinely, i.e. in the same ratio as the macroscopic deformation ratio of the rubber sample.

From (27):
\[ f = \left( \frac{\partial F}{\partial \lambda} \right)_{T,V} \]  

but by definition:
\[ F = U - TS \]  

Because of assumption 1, however, it is not necessary to find an explicit expression for \( U \) and hence it is sufficient to use the entropy expression. The Boltzmann relation gives:
\[ S = k \ln \Omega \]  

where \( \Omega \) is the total number of conformations available to the rubber network. According to assumption 2, the number of conformations available to the \( i \)th individual chain is given by the Gaussian distribution function:
\[ a(x_i, y_i, z_i) = \left( \frac{b}{\sqrt{\pi} d^2} \right)^3 \exp \left[ -b^2 (x_i^2 + y_i^2 + z_i^2) \right] \]  

Equation (33) refers to a chain having an end to end vectorial distance \( r_e \) with one end at co-ordinates \( (x_i, y_i, z_i) \) in the unstrained state, the other end being at the origin of the cartesian co-ordinate system. Following assumption 3, the total number of confirmations available to a network of \( N \) such chains is:
\[ \Omega = \prod_{i=1}^{N} \omega (r_e) \]
and the conformational entropy of the undeformed network is just:

\[ S = 3k \ln \left( \frac{b}{n^{1/2}} \right) - k \sum_{i=1}^{N} b^2(x_i^2 + y_i^2 + z_i^2) \]  \hspace{1cm} (35)

or

\[ F = F_0 + kT \sum_{i=1}^{N} b^2(x_i^2 + y_i^2 + z_i^2) \]  \hspace{1cm} (36)

where \( F_0 \) is that proportion of Helmholtz free energy which is not related to conformational entropy changes.

In the strained state, the chain is deformed to \( r_i' \) with the chain end now at co-ordinates \((x_i', y_i', z_i')\). To relate the microscopic strain of the chains to the macroscopic strain of the rubber sample, the deformation is assumed to be affine (assumption 4).

Considering a unit cube of an isotropic rubber sample

In the general case of a pure homogeneous strain, the cube is transformed into a rectangular parallelepiped
The dimensions of the parallelepiped are $\lambda_1$, $\lambda_2$, $\lambda_3$ in the three principal axes, where $\lambda$'s are known as the principal extension ratios.

Choosing the co-ordinate axes for the chain to coincide with the principal axes of strain for the sample, then:

$$x_i' = \lambda_1 x_i, \quad y_i' = \lambda_2 y_i, \quad z_i' = \lambda_3 z_i$$  \hspace{1cm} (37)

And thus, the Helmholtz free energy of the deformed network can be written as:

$$F = F_0 + kT \sum_{i=1}^{N} b^2 (\lambda_1^2 x_i^2 + \lambda_2^2 y_i^2 + \lambda_3^2 z_i^2)$$  \hspace{1cm} (38)

Hence the difference between (26) and (38) represents the total change in free energy due to the deformation, ie:

$$\Delta F = kT \left[ \sum_{i=1}^{N} (\lambda_1^2 - 1) b^2 x_i^2 + \sum_{i=1}^{N} (\lambda_2^2 - 1) b^2 y_i^2 + \sum_{i=1}^{N} (\lambda_3^2 - 1) b^2 z_i^2 \right]$$  \hspace{1cm} (39)

Now by definition:

$$r_c^2 = x_i^2 + y_i^2 + z_i^2$$  \hspace{1cm} (40)

And for a random isotropic network, all directions are equally probable so:

$$x_i^2 = y_i^2 = z_i^2 = \frac{1}{3} r_c^2$$  \hspace{1cm} (41)

which causes (39) to become:

$$\Delta F = \frac{1}{3} kT \sum_{i=1}^{N} b^2 r_c^2 (\lambda_1^2 + \lambda_2^2 + \lambda_3^2 - 3)$$

$$= \frac{1}{3} kNTb^2 r_c^2 (\lambda_1^2 + \lambda_2^2 + \lambda_3^2 - 3)$$  \hspace{1cm} (42)

where $\overline{b^2 r_c^2} = \frac{1}{N} \sum_{i=1}^{N} b^2 r_c^2/N$

from the definition $b^2 = \frac{3}{2} \overline{r_c^2}$  \hspace{1cm} (43)
If the network chains in the unstrained state have the same distribution of conformations as an ensemble of free chains, then \( \overline{b^2r_c^2} = \frac{2}{3} \).

However, in real networks, this condition may not be met. For instance, some of the chains may already be partially strained during the cross-linking process. The details of the cross-linking process, e.g., whether it is carried out in solution or in bulk, may also affect the state of the network.

It is, therefore, more general to write:

\[
\overline{b^2r_c^2} = \frac{b^2}{N} \sum_{i=1}^{N} r_i^2 / N = \frac{3\langle r^2 \rangle_i / \langle r^2 \rangle_0}{N} \quad (44)
\]

where \( b^2 \) is averaged over all the free chains

\[ b^2 = \frac{3}{2} <r^2>_0 \]

Here \( <r^2>_0 = \sum r_i^2 / N \) refers to the mean square end-to-end distance of the isolated chain and \( <r^2>_i \) to the chain in its cross-linked condition. Substitution of (44) into (42) gives:

\[
\Delta F = k N \frac{1}{2} \left( \frac{\langle r^2 \rangle_i}{\langle r^2 \rangle_0} \right) (\lambda_1 + \lambda_2 + \lambda_3 - 3) \quad (45)
\]

The parameter \( \langle r^2 \rangle_i / \langle r^2 \rangle_0 \) sometimes referred to as the 'front factor', can be regarded as the average deviation of the network chains from the dimensions they would assume if they were isolated and free from all constraints. For an ideal rubber, the front factor = 1.

Now, assuming a unit cube of volume \( V_0 \) and length \( l_0 \); after a uniaxial extension (\( \lambda_1 = 1/l_0 \)) is applied, the length in direction of stretch is 1, and the volume dilates to \( V \). Although the strain induced volume dilation for unfilled rubbers is very small, of the order of magnitude \( 10^{-11} \), the deformation process is nevertheless not a volume preserving one as required by (30). The method that is generally employed...
to circumvent this difficulty is to redefine the reference state. A hypothetical hydrostatic pressure is imagined to have been applied to the sample so that its volume in the unstretched state is also \( V \). The initial length is then no longer \( l_0 \), but is:

\[
l' = l_0 \left( \frac{V}{V_0} \right)^{\frac{1}{3}}
\]

(46)

and the extension ratio in the direction of uniaxial stretch becomes:

\[
1^* = 1/l'
\]

(47)

The average chain dimensions become

\[
\bar{r}^2 = \left< r^2 \right> = \left( \frac{V}{V_0} \right)^{\frac{1}{3}}
\]

Now the condition of incompressibility requires that:

\[
\alpha_1^* \alpha_2^* \alpha_3^* = 1
\]

(48)

while previously

\[
\lambda_1 \lambda_2 \lambda_3 = V/V_0
\]

(49)

Since the network is isotropic, the contractions along the two lateral axes are equal, and:

\[
\lambda_2 = \alpha_1^* (V/V_0)^{\frac{1}{3}}
\]

\[
\lambda_2 = \lambda_3 = \alpha_1^* (V/V_0)^{\frac{1}{3}}
\]

(50)

The uniaxial extension of the unit cube is illustrated below:
Inserting (50) into (45), the change in Helmholtz free energy on deformation becomes:

\[ \Delta F = \frac{T}{2} Nk \frac{\langle r^2 \rangle}{\langle r^2 \rangle_0} (\alpha^2 + 2/\alpha^2 - 3) \] (51)

for simple uniaxial extension; since

\[ \left( \frac{\partial F}{\partial l} \right)_{T,V} = \left( \frac{\partial F}{\partial \alpha^2} \right)_{T,V} \left( \frac{\partial \alpha^2}{\partial l} \right)_{T,V} \] (52a)

\[ f = \frac{T}{l} \left( \frac{\partial F}{\partial \alpha^2} \right)_{T,V} \] (52b)

So performing the indicated differentiation on (51), the equation of state for rubber elasticity is obtained:

\[ f = \frac{NkT}{l} \frac{\langle r^2 \rangle}{\langle r^2 \rangle_0} \left[ \alpha^2 - \frac{1}{\alpha^2} \right] \left( \frac{V}{V_0} \right)^{2h} \] (53)

Equation (53) gives the total elastic restoring force exerted by the sample. However, for many purposes, it is more convenient to deal with expressions relating the stress to the deformation rather than the total force as in equation (53). Thus, stress is defined

\[ \sigma = \frac{f}{A_0} \]

where \( A_0 \) is the area of the undeformed sample.

Furthermore, \( N_0 \) is defined:

\[ N_0 = \frac{N}{V_0} \]

as the number of network chains per unit volume of the undeformed sample, where \( V_0 = 1_0 A \).

Also, \( N_0 \) is expressed in terms of the number of moles of network chains.
Thus with the aid of (47), (53) can be re-written:

\[ \sigma = N_0RT \frac{<r^2>_i}{<r^2>_0} (\lambda - V/V_0\lambda^2) \]  \hfill (54)

where \( R \) is the ideal gas constant

\( R = N_{av}k \); \( N_{av} \) being the Avagadro's No

The ratio \( V/V_0 \) 1 so thus it is often sufficient to write (54) as:

\[ \sigma = N_0RT \frac{<r^2>_i}{<r^2>_0} (\lambda - 1/\lambda^2) \]  \hfill (55)

The difference between (54) and (55) is numerically trivial, although conceptually important.

The extension ratio may be written:

\[ \lambda = 1 + \varepsilon \]  \hfill (56)

where \( \varepsilon \) is the tensile strain, \( \Delta l/\Delta l_0 \). By the binomial expansion:

\[ \lambda^{-2} = (1 + \varepsilon)^{-2} = 1 - 2\varepsilon + \ldots \ldots \]  \hfill (57)

For very small strains, higher order terms can be neglected and (53) may be recast as:

\[ E = \frac{\sigma}{\varepsilon} = 3N_0RT \frac{<r^2>_i}{<r^2>_0} \]  \hfill (58)

where \( E \) is the time-dependent tensile modulus.

Since, for cross-linked rubbers, the tensile modulus is three times the shear modulus \( G \), then:

\[ G = N_0RT \frac{<r^2>_i}{<r^2>_0} \]  \hfill (59)

And thus, the equation of state may be re-cast as:

\[ f = G \lambda (\lambda - 1/\lambda^2) \]  \hfill (60a)

or

\[ \sigma = G (\lambda - 1/\lambda^2) \]  \hfill (60b)

where \( G \) is determined by the initial slope in the stress-strain curve and defined by equation (59)
Energy Contribution

In the statistical treatment it was assumed that the elastic force is entirely attributable to the conformational entropy of deformation, energy effects being neglected. In real rubber, however, energy effects cannot be entirely absent and deviations from the ideal rubber model may be expected to occur.

The extent to which the neglect of the energy effects is justified is examined below.

Re-writing equation (28):
\[ f = f_e + f_g \]  \hspace{1cm} (61)
with
\[ f_e = \left( \frac{\partial U}{\partial l} \right)_{T,V} \]  \hspace{1cm} (62)
\[ f_g = -T \left( \frac{\partial S}{\partial l} \right)_{T,V} \]  \hspace{1cm} (63)

Since \( f \) can be experimentally determined and \( f_g \) obtained from the thermodynamic identity (equation (9)):
\[ \left( \frac{\partial S}{\partial l} \right)_{T,V} = - \left( \frac{\partial f}{\partial T} \right)_{V,1} \]  \hspace{1cm} (64)

\( f_e \) can be found by:
\[ f_e = f - T \left( \frac{\partial f}{\partial T} \right)_{V,T} \]  \hspace{1cm} (65)

Unfortunately, the condition of constant volume is very difficult to achieve experimentally, and similarly the alternative expressions tend to contain terms which are not accurately measurable.

However, advantage can be taken of the expression from statistical theory:
\[ f = G A \left( \lambda - V/V_o \lambda^2 \right) \]  \hspace{1cm} (66)

where \( G \) is defined by equation (59).
The shear modulus $G$ is a material constant, which is invariant whether the experimental condition is constant volume or constant pressure. Equation (66) can then be differentiated with respect to $T$ keeping $V$ and $L$ constant. (Note, however, $V_0$ and $L_0$ are not constant.) The result is:

$$\left( \frac{\partial f_e}{\partial T} \right)_{V,L} = \frac{fG}{GdT} + \frac{\beta_v f}{3}$$

(67)

And combining (67) and (65):

$$f_e = f - \frac{fG}{dlnT} - \beta_v T f/3$$

(68)

The advantage of equation (68) is that experimental errors in stress-strain data at various temperatures are averaged out in plotting $f$ against $(\lambda - \lambda^{-2})$, the slope of which is the shear modulus.

Knowing shear moduli as a function of temperature, values of $f_e$ can be readily calculated.

For the sake of comparison, however, it is more useful to use the relative energy contribution $f_e/f$. Thus, equation (68) can be re-written as:

$$f_e/f = 1 - \frac{dlnG}{dlnT} - \beta_v \frac{T}{3}$$

(69)

Values of $f_e/f$ calculated from (69) are independent of $\lambda$ so long as they are obtained within the region of strain for which the Gaussian theory is valid.

Now one of the assumptions (assumption 3) for the statistical theory was that the entropy of the network was the sum of the entropies of individual chains of the network. In order for this requirement to be valid, it is required that chains in the network behave as if they were in free space and unaffected by the presence of other chains. This stipulation can only be satisfied if interchain interactions are absent. Thus the energy effects present in rubber elasticity must only come from
interchain interactions, such as the energy barriers hindering rotations along the polymer chain. It is reasonable to expect that real polymer chains are not isoenergetic, i.e., energies of the individual chains are not constant as a function of their conformations. It follows that changes in the supply of thermal energy (changes in temperature) would produce changes in the mean chain dimensions as well. An expression for the temperature coefficient of the unperturbed dimension of the polymer chain can be arrived at by differentiating (59) and remembering that \( N_0 = N/V_0 \) and \( T^2_0 \) is proportional to \( V_0^{2\beta} \).

\[
\frac{d\ln\langle r^2 \rangle_0}{d\ln T} = 1 - \frac{d\ln C}{d\ln T} - \frac{\beta V T}{3}
\]  

(70)

Inserting (70) into (69):

\[
\frac{d\ln\langle r^2 \rangle_0}{d\ln T} = \frac{f_e}{f}
\]  

(71)

Thus energy effects in rubber elasticity arise from the interchain interaction energies of the network chains.

Phenomenological Treatment

The statistical theory was arrived at through considerations of the underlying molecular dynamics. The equation of state is obtained directly from the Helmholtz free energy of deformation (or simply conformational entropy of deformation, since energy effects were assumed to be absent) which can be re-cast using (45) and (59) as:

\[
F = -TS = \frac{1}{2} G (\lambda_1^2 + \lambda_2^2 + \lambda_3^2 - 3)
\]  

(72)

The phenomenological theory concerns itself only with the observed behaviour of rubbers. It is not based on considerations of the molecular structure of the polymer. The central problem here is to find an expression
for the elastic energy stored in the system, analogous to the free energy expression in the statistical theory, i.e.: equation (72). If the deformation of a unit cube is again considered, in order to arrive at the state of strain, a certain amount of work must be done which is stored in the body as strain energy:

$$W = W(lij) = \int_{\lambda_1=1}^{\lambda_1} \int_{\lambda_2=1}^{\lambda_2} \int_{\lambda_3=1}^{\lambda_3} \left( \sigma_1 d\lambda_1 + \sigma_2 d\lambda_2 + \sigma_3 d\lambda_3 \right)$$

(73)

where the λ's are again the principal extension ratios.

The energy is a unique function of the state of strain, and if the amount of it is known as a function of strain, the elastic properties of the material can then be completely defined.

Although the strain energy function expressed in terms of the principal extension ratios is chosen without any regard for the molecular mechanism, it must satisfy certain logical constraints in the case of isotropic solids. These considerations lead to the expression of W in terms of the so-called strain invariants.

$$W = W(I_i), \quad i = 1, 2, 3$$

(74)

where:

$$I_1 = \lambda_1^2 + \lambda_2^2 + \lambda_3^2$$

$$I_2 = \lambda_1^2 \lambda_2 + \lambda_2^2 \lambda_3 + \lambda_3^2 \lambda_1$$

$$I_3 = \lambda_1^2 \lambda_2^2 \lambda_3^3$$

(75)

The third invariant is obviously:

$$I_3 = \left(\frac{V_0}{V}\right)^2$$

(76)

which = 1 for an incompressible material.
The most general form of the strain energy function for an isotropic material is the power series:

\[
W = \sum_{i,j,k=0}^{\infty} C_{i,j,k} (I_1 - 3)^i (I_2 - 3)^j (I_3 - 1)^k
\]  

(77)

Quantities in parenthesis are so chosen that the strain energy vanishes at zero strain. Since the set of terms in equation (77) cannot be determined a priori, it is necessary to examine the lowest members of the series.

For \(i = 1, j = 0, k = 0\):

\[
W = C_{100} (I_1 - 3)
\]  

(78)

which is functionally identical to the Gaussian free energy of deformation (72).

The stress-strain relation can then be obtained from equation (73) by differentiation.

For the special case of uniaxial extension, using (5) and setting \(C_{100} = C_1\), and observing the incompressibility condition, it follows that:

\[
\sigma = \frac{\partial W}{\partial \alpha^k} = 2C_1 (\alpha^k - 1/\alpha^2)
\]  

(79)

which is known as the neo-Hookean equation. If \(C_1 = G/2\), equation (79) is just the statistical expression (60).

Suppose an additional term is retained with \(i = 0, j = 1, k = 0\), then:

\[
W = C_{100} (I_1 - 3) + C_{010} (I_2 - 3)
\]  

(80)

For uniaxial extension: \((\alpha_x = \alpha, \alpha_y = \alpha_z = 1/\alpha^2)\)

\[
\sigma = 2C_1(\alpha^k - 1/\alpha^2) + 2C_2 (1 - 1/\alpha^3)
\]  

(81)

where again, for simplicity, \(C_1\) and \(C_2\) have been set \(C_1 = C_{100}, C_2 = C_{010}\).
Equation (81) is known as the Mooney-Rivlin Equation which can alternatively be expressed as:

\[ \sigma = 2(C_1 + C_2/\lambda)(\lambda - 1/\lambda^2) \]  

(82)

since \( \lambda = \alpha^* \) in the incompressible case.

**Effect of Swelling**

Linear polymers are capable of dissolving in appropriate solvents to form solutions, but if cross-links are introduced then the material is unable to dissolve and instead the solvent is absorbed into the polymer network giving rise to the phenomenon of swelling. As solvents fill the network, chains are extended. The resultant retractive force operates in opposition to the swelling force and there is a maximum degree of swelling at which these two forces are at equilibrium.

If rubber is swollen to below the equilibrium swelling, so that no de-swelling will occur upon deformation, the statistical expression for the shear modulus (59) can be modified defining \( v \) as the ratio of the unswollen volume to the swollen volume. The number of network chains per unit volume then becomes \( vN_0 \) and the mean square end-to-end distance of the network chain is now \( \langle r^2 \rangle^i / v^2 \).

Equation (59) is then:

\[ G_s = N_0 RT v^{1/3} \frac{\langle r^2 \rangle^i}{\langle r^2 \rangle_0} \]  

(86)

For swollen rubbers, the equation of state is now:

\[ \sigma_s = N_0 RT v^{-1/3} \frac{\langle r^2 \rangle^i}{\langle r^2 \rangle_0} (\lambda_s - 1/\lambda_s^2) \]  

(87)

where subscripts \( s \) refer to the swollen sample. If stress is expressed in terms of per unit cross-sectional area of unswollen sample, then since:

\[ A_d = A_s v^{1/3} \]  

where subscripts \( d \) refer to the dry (unswollen) sample, then:
An expression based on the Mooney–Rivlin strain energy function for swollen rubbers can also be derived. A dry rubber sample will undergo two types of deformation: one due to swelling and the other due to extension. The strain energy function per unit volume of swollen rubber is related to that of the dry sample by:

$$\overline{w}_s = \overline{w}_d + v \left[ C_1 (I_1 - 3) + C_2 (I_2 - 3) \right]$$ (89)

where subscripts s and d refer to swollen and dry samples respectively.

In equation (89) the strain invariants $I_1$ and $I_2$ are defined by $\lambda d$'s, i.e. strains suffered by the dry rubber (both swelling and extension). The deformation due to isotropic swelling is just $v^{-1/3}$ for all three principal axes, thus the $\lambda d$'s are related to the $\lambda s$'s (deformation of swollen rubber by extension) by:

$$\lambda_{1d} = \lambda_{1s} v^{-1/3}, \quad \lambda_{2d} = \lambda_{2s} v^{-1/3}, \quad \lambda_{3d} = \lambda_{3s} v^{-1/3}$$ (90)

For the case of simple uniaxial extension, assuming incompressibility for the sake of convenience, using equations (49) and (50), the following is obtained:

$$\overline{w}_s = C_1 v^{1/3} (\lambda_s + 2/\lambda_s - 3) + C_2 v^{2/3} (2\lambda_s + 1/\lambda_s^2 - 3)$$ (91)

The stress–strain relation can thus be obtained directly by differentiating with respect to $\lambda_s$ (73):

$$\sigma_s = 2 C_1 v^{1/3} (\lambda_s - 1/\lambda_s^2) + 2 C_2 v^{2/3} (1 - 1/\lambda_s^3)$$ (92)

or, put in terms of unit cross-sectional area of unswollen sample:

$$\sigma_d = 2v^{-1/3} (C_1 + C_2 v^{1/3}/\lambda_s) (\lambda_s - 1/\lambda_s)$$ (93)
The $C_1$ term of the Mooney-Rivlin equation is often identified with the shear modulus of the statistical equation; they both depend on $v$ in the same manner (compare (92) with (89) or (93) with (88)).

References