PHYSICOCHEMICAL STUDIES OF COLLAGEN AND COLLAGEN-MUCOPOLYSACCHARIDE COMPOSITE MATERIALS
(Model Materials for Skin)

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ABSTRACT

PHYSICOCHEMICAL STUDIES OF COLLAGEN AND

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Submitted to the Department of Mechanical Engineering on February 8, 1974 in partial fulfillment of the requirements for the Degree of Doctor of Science.

The main objective of this thesis is the design of materials which may be suitable for the replacement of skin. The choice of the materials, collagen and mucopolysaccharides (MPS), had been influenced largely by the fact that collagen is the major structural protein in skin and that MPS forms the major component of the "ground substance" that are generally found associated with collagen fibers.

Two forms of collagen-MPS composite materials were prepared. The first form consists of a collagenous core coated with MPS on the surface while the second form consists of collagen fibers dispersed randomly within a MPS matrix.

A number of methods were developed for the characterization of the collagen and collagen-MPS composite materials. These include the measurement of (a) helical content of collagen (using x-ray diffraction, infrared spectroscopy and optical rotation studies); (b) extent of cross-linking of collagen (by the application of the theory of rubber elasticity on the denatured collagen); (c) morphology and ulstrastructure of the materials (by use of optical and scanning electron microscopes); (d) susceptibility of the collagen to *in vitro* enzymatic degradation (by a mechanochemical method); (e) collagen and mucopolysaccharide contents (by hydroxyproline and hexosamine analyses, respectively); (f) tensile and compressive properties; and (g) swelling characteristics.

From these studies, it became apparent that the presence of a limited amount of MPS (<11 wt-%) can strengthen mechanically the collagen fibers as well as render them more resistant to *in vitro* enzymatic degradation. However, the presence of an excess amount of MPS (>11 wt-%) has a deleterious effect on the mechanical strength of the collagen fibers while rendering more susceptible to *in vitro* enzymatic degradation. A model was presented to explain this phenomenon.

The collagen and collagen-MPS composite materials were implanted in experimental animals (guinea pigs) to assess their usefulness as materials for skin replacement. The materials were characterized prior to and after implantation and from the change in their properties, such informations as to the extent of their *in vivo* degradation, the possibility of tissue attachment to the materials and the nature of materials attached to them were obtained. A direct correlation between *in vivo* and *in vitro* enzymatic degradation was observed. Accordingly, the collagen-MPS composite materials were found to be more resistant to *in vivo* degradation than the pure collagen.

Furthermore, the attachment of surrounding tissues to the collagen-MPS composite materials were found to be much stronger than those found for pure collagen. Histological studies of the implantation sites revealed that the collagen-MPS composite materials induced a lesser inflammatory response than the pure collagen.

These observations, coupled with the observation that the MPS can strengthen the collagen fibers, suggest that the collagen-MPS materials are far superior as materials for skin replacement than the pure collagen. However, this conclusion has been based on studies involving only subcutaneous implantation of the materials in experimental animals. It must be confirmed by direct clinical application.

Thesis Supervisor: Ioannis V. Yannas

Title: Associate Professor of Mechanical Engineering

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February, 1974

TABLE OF CONTENTS

TABLE OF CONTENTS (CONTINUED)

TABLE OF CONTENTS (CONTINUED)

TABLE OF CONTENTS (CONTINUED)

TABLE OF CONTENTS (CONTINUED)

LIST OF FIGURES

LIST OF FIGURES (CONTINUED)

LIST OF FIGURES (CONTINUED)

Page

LIST OF FIGURES (CONTINUED)

LIST OF TABLES

LIST OF TABLES (CONTINUED)

Chapter 1

INTRODUCTION

One of the long term objectives of the research conducted in our laboratory is to design materials which will be suitable for the temporary substitution or replacement of human skin damaged by burns or mechanical injury. The need for better treatment of patients with severe burns is great. A report from the National Fire Protection indicated that approximately 1,800,000 persons sustain burns yearly and occupy 11,000 hospital beds per day (Tyron, 1962). The annual death toll is estimated to be 12,000. According to Pruitt (1970), the three most important advances needed to reduce mortality are: (1) the development of materials suitable for the replacement of burnt skin; (2) advances in altering the immune response; and (3) the development of topical agents to control infections leading to septicemia and death.

While some progress has been made in understanding the immune response (Merill, 1964; Cochrane, 1966; Reisfield and Kahn, 1969; Mudd, 1970) and the effect of infection on wound repair (Burke, 1966, 1971; Burke et al., 1969; Mudd, 1970), little progress has been made in the design of suitable materials to replace skin. The problem involved appears to be a lack of established rules and guidelines on characteristics of materials which will render them suitable for skin replacement. We believe that a complete characterization of any material prior to and after its implantation in experimental animals, coupled with clinical studies of these animals, is essential for the establishment of such rules and guide-

lines. The main objectives of this thesis are the design of materials which may be suitable for the replacement of skin and the development of methods which will be suitable for their characterization prior to and after implantation in experimental animals.

Scope of the Thesis

In order to understand the problems involved in designing materials for skin replacement, it is important to have a knowledge of the structure and functions of skin. Chapter 2 surveys this subject, with special emphasis on the connective tissue matrix, which forms the bulk of the dermal and subcutaneous layers of the skin. In order to understand the type of cells that implanted materials may encounter, a survey of the connective tissue cells is made. Furthermore, a survey of the mechanism by which the connective tissue matrix is degraded provides the necessary information for understanding the degradative environment surrounding an implanted material. The changes that occur during wound healing of the skin is also reviewed so that materials can be designed to cope with these changes. Finally, the rationale for choosing collagen and composite materials based on collagen and mucopolysaccharides as candidates for skin replacement is presented. Most of the available books and reviews on the subjects covered in Chapter 2 tend to be either too detailed or too narrow in scope. It is hoped that the survey in Chapter 2 will provide sufficient information to a person who is interested in this area of investigation but who does not wish to spend months ploughing through the vast amount of information available in the literature.

Chapter 3 covers a variety of methods developed in our laboratory for the physicochemical studies of collagen in the solid phase. Most of the previous physicochemical studies on collagen have been done on dilute solutions of the collagen molecule, with much less attention being paid on collagen in the solid phase. As the collagen to be used for skin replacement must necessarily be in the solid phase, methods must be developed to study the molecules in this phase. The properties that are of primary interest in this Chapter are the helical content, the extent of crosslinking, the state of aggregation and the enzymatic degradation of the collagen in the solid phase. The helical content is studied using wide-angle x-ray diffraction, infra-red spectroscopy and optical rotation studies. The extent of crosslinking is studied using a method which relies on the fact that collagen, when denatured, behaves like a rubber and hence its mechanical behaviour is amenable to analysis by the theory of rubber elasticity (Wiederhorn and Reardon, 1952). The state of aggregation of collagen is studied by the use of scanning electron microscopy.

The presence of a collagen-degrading enzyme (collagenase) in human skin has recently been demonstrated (Eisen et al., 1971). The enzymatic degradation of collagen induces a loss in the mechanical strength of the material (Salthouse et al., 1969)and seriously impairs its usefulness as a material for skin replacement. The last section of Chapter 3 describes a method, developed in our laboratory, for the study of the enzymatic degradation of insoluble collagen. The method is similar to the one used by Tobolsky et al. (1944) for the study of oxidative degradation of rubbers. Using this method, the effect on the collagen degradation of temperature, pH, concentration and presence of inhibitors in the col-

lagenase solution is investigated. The effect of strain on the collagen, of its helical content and extent of crosslinking is also studied.

The interactions between collagen and mucopolysaccharides (MPS) have been extensively studied for the last decade. While the nature of the interactions is relatively well understood, the physicochemical characterization of composite materials of the two macromolecules remains substantially unexploited. Chapter 4 describes a few physical and chemical analyses of these composite materials. The susceptability of these materials to collagenase degradation is also studied in order to investigate the effect of the MPS on the degradation.

Laboratory (*in vitro*)studies of the materials to be assessed for skin replacements are irrelevant unless a correlation exists between these studies and the *in vivo* studies derived from implantation of the same materials in experimental animals (guinea pigs). Chapter 5 describes methods to obtain such a correlation. Collagen and collagen-MPS composite materials with varying degrees of resistance to collagenase degradation (as measured by the *in vitro* experiments of Chapters 3 and 4) are implanted in the animals for a fixed period of time and the extent of degradation *in vivo* (as measured by the fractional weight loss of the implanted materials) is correlated with the extent of degradation *in vitro*. Changes in the physicochemical properties of the implanted materials (at 4, 10 and 20 days post implantation) are also studied and correlated with the histological studies of the animal tissues at and around the site of implantation. Based on these studies, assessment of each material for suitability as skin replacement is made.

Chapter 2

THE SKIN

2.1. Structure and Function of the Skin

A knowledge of the structure and function of the skin is essential for the design of any material intended for the replacement of the skin. Since a number of books on the subject are available (Rothman, 1954; Montagna, 1962; Elden, 1971), only a brief description of the structure and major functions of the skin will be presented in this section.

2.1.1. Measurements. The skin covers the entire human body and has a surface area of about 0.25 square meters in the newborn, 1.85 square meters in the average-sized man and 1.6 square meters in the average-sized woman. The thickness of the entire skin (exclusive of the subcutaneous fat) varies from 0.5mm in the eyelid to 3-6mm on the palm or sole. The weight of the skin is about 16 percent of the body weight.

2.1.2. General Anatomy. Figure 2.1 shows a schematic representation of the general anatomy of the mammalian skin . At the surface is a stratified layer of epithelial cells called the epidermis, and underneath this is a connective tissue called the dermis (or corium). Between the epidermis and the dermis is a thin layer of loose connective tissue called the basement membrane. Underneath the dermis is the fatty layer (or panniculus adiposus) and below this layer is a discontinuous sheet of skeletal muscles called panniculus carnosus. A bed of loose (areolar) connective tissue called the subcutaneous tissue (or tela subcutanea) binds the skin

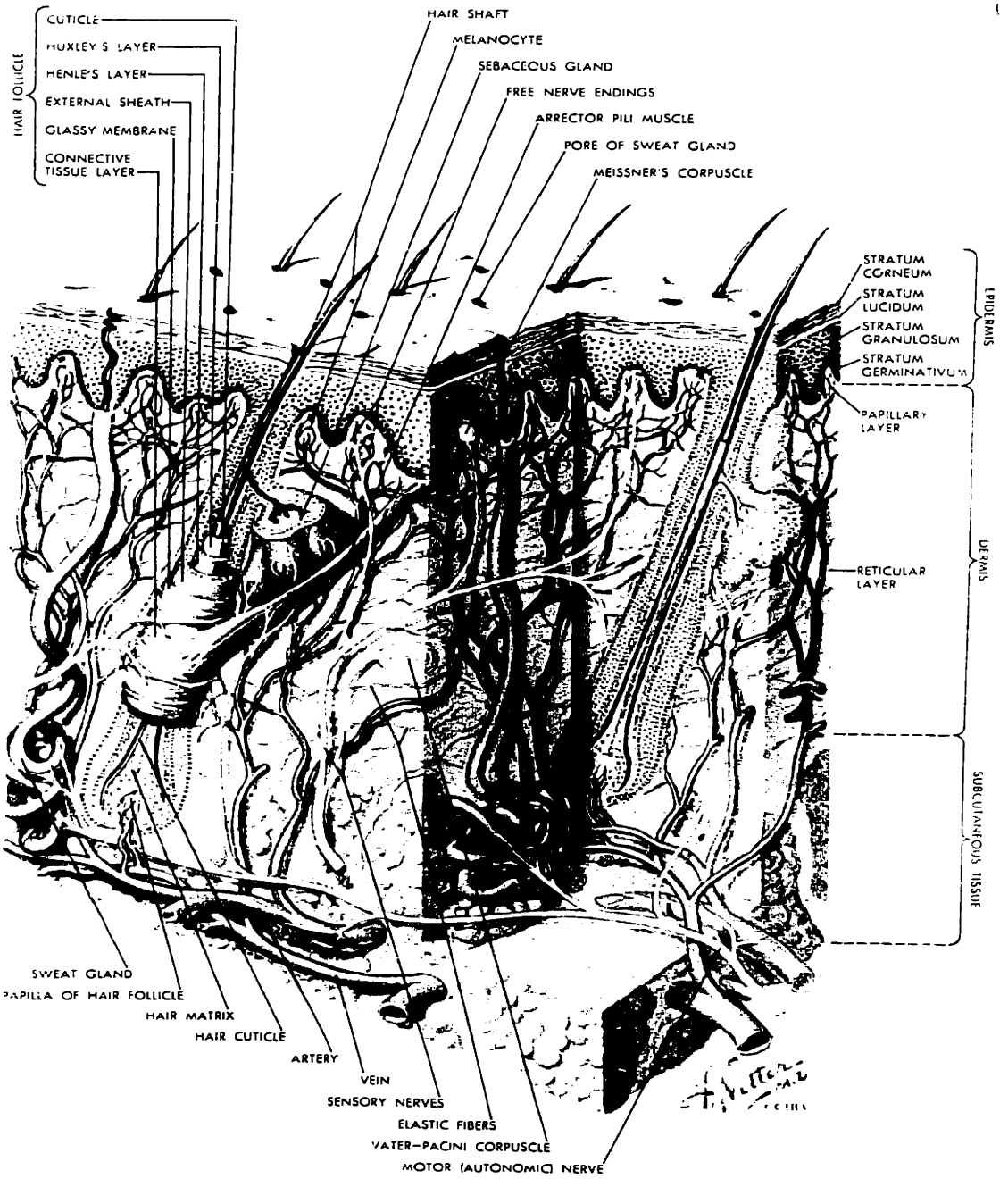


Fig. 2.1. General anatomy of the mammalian skin. From Netter (1973).

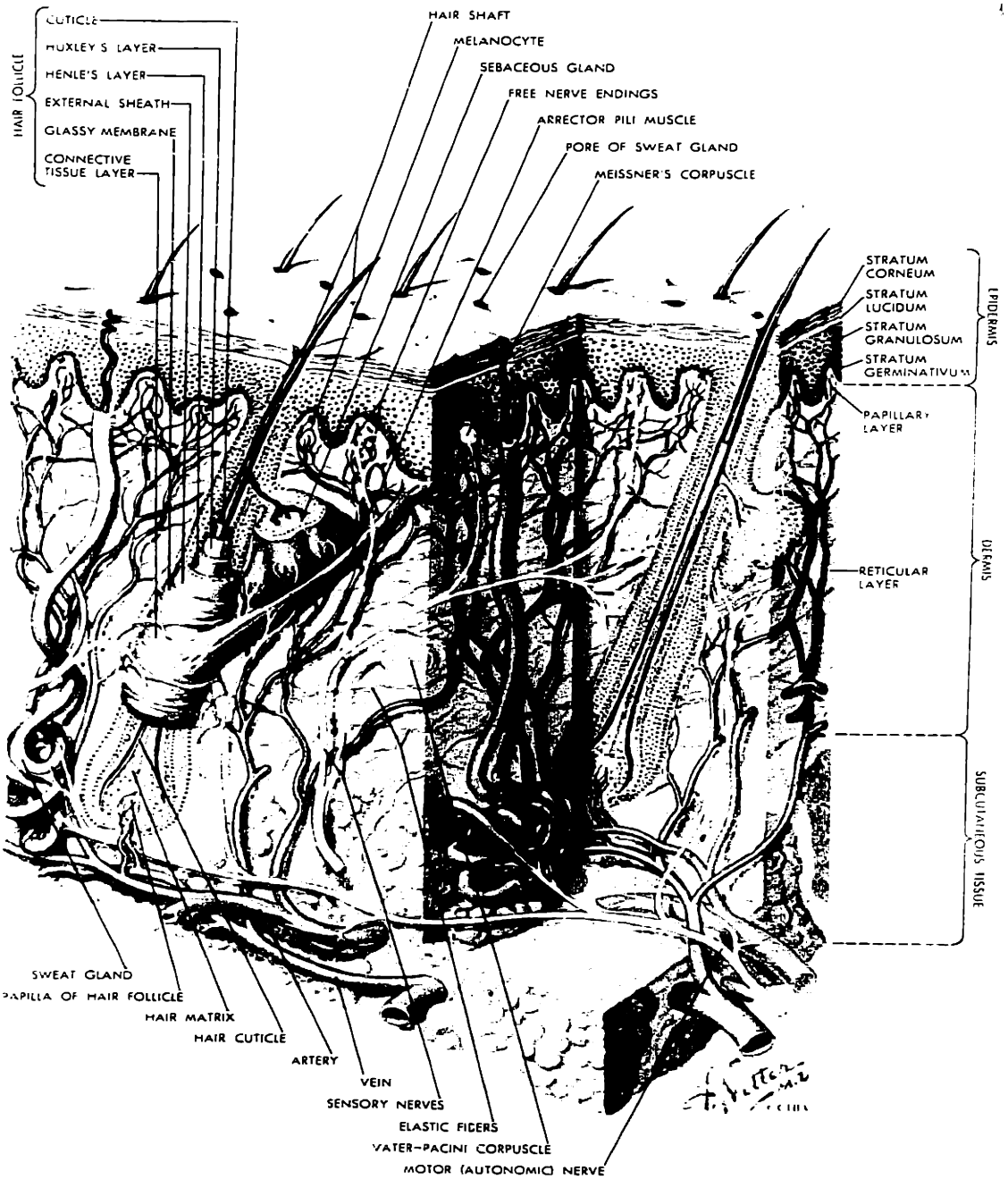


Fig. 2.1. General anatomy of the mammalian skin. From Netter (1973).

to the superficial muscles and to other tissues.

Appendages such as hairs, nails and cutaneous glands grow directly from the epidermis which extends into the dermis and the subcutaneous tissue along the hair follicles and sweat ducts. Dispersed within the dermal and subcutaneous layers are the blood and lymphatic vessels. Nerve fibers, terminating in a variety of nerve endings and organs branch throughout the epidermis, dermis and subcutaneous tissue. A variety of connective tissue cells are sparsely distributed within the dermal and subcutaneous layers.

2.1.3. The Epidermis. The epidermis is made up of stratified layers of epithelial cells, the cells being distinguished mainly by their shapes when strained and viewed under the microscope. The outermost layer is a flat, hornified layer of dead cells called stratum corneum. A thin, homogenously dense layer of cells called stratum lucidum dies immediately below the stratum corneum. Underneath the stratum lucidum lies a number of layers of granular cells called stratum granulosum. Below the granular layer is the stratum germinativum (or Malpighian layer) which is irregularly ridged at its lower surface where it is in contact with the dermis. The stratum germinativum can be divided into two layers: the lower basal layers, called stratum basale, which is made up of a one-cell layer of columnar cells and the upper spinous layer, the stratum spinosum which is made up of cells that are polyhedral in shape and are characterized by the presence of uniformly spaced intercellular bridges (or spines) which separate them. Each intercellular bridge is thickened near its centre by a granule called desmosome. The intercellular bridges together with the desmosomes serve to

hold the cells together and provide them with strength and elasticity.

The intercellular bridges are made up of bundles of filaments called tonofilaments which are believed to be the precursors of keratin (Selby, 1955), the fibrous protein which is found throughout the epidermis, hair and nails of human beings. By a rather complicated "keratinization process" (Rothman, 1954, p. 366), the tonofilaments can be converted to the "soft keratin" of the epidermis or the "hard keratin" of the hair and nails. The difference between these two forms of keratins appear to be the extent of crosslinking, as evidenced by the low sulfur content (1-3%) of the "soft keratin" and high sulfur content (3-5%) of the "hard keratin" (Hawk et al., 1947).

Besides being involved in the important process of keratinization, the epidermal cells perform some other very important functions. The whole of the epidermis appears to be tailored in every detail to protect the body against penetration of a variety of agents from the environment. The surface of the skin is generally coated with a complex layer of lipids and organic salts, liberated by the keratinizing cells and secreted by the sebaceous and sweat glands. This layer, which has a pH of 4.5 to 6.0 is called the "acid mantile" and is said to have antifungal and antibacterial properties (Blank, 1959). It is the first barrier against penetration of potential invading agents. If agents should get past it, they must get through the stratum corneum where the interstices between the cells become progressively smaller in deeper parts, thus serving as both physical and chemical traps.

The stratum lucidum, below the stratum corneum, provides the major resistance to penetration of external agents. The essential barrier is considered to be a complex made up of lipids, proteins and water,

forming a hydrophobic liquid-protein complex (Onken and Moyer, 1963). It also plays an important role in regulating the transfer of water through the skin (Monash and Blank, 1958). The removal of this layer increases the rate of transpiration of water through the skin from 2.9 to 229 gm/sq. meter/hour (Marzulli, 1962). Even with the removal of this layer, the rest of the epidermis offers a significant barrier to penetrating agents (Feldman and Marbach, 1965).

Located just below the basal layer, at the dermo-epidermal junction, are the very important pigment-forming cells, the melanocytes. These melanocytes are activated by the presence of radiation (Bloch, 1917) and the release of the enzyme, tyrosinase (Raper, 1926). The pigments, called melanin, serve to protect the body from an excessive intake of injurious radiation.

2.1.4. The Dermis. This layer is a connective tissue matrix which supports within it the blood and lymphatic vessels, the nerve fibers, the sebaceous and sweat glands, the hair follicles and a variety of connective tissue cells. The connective tissue matrix is made up of a mesh of fibers surrounded by a viscous, water-swollen ground substance. The fibers are made up of the proteins, collagen, elastin and reticulin while the ground substances are made up of muco-polysaccharides (MPS), protein-polysaccharides (PPS) and glycoproteins (GPS). The structure and functions of these six components of the connective tissue matrix will be discussed in detail in Section 2.2.

Histologically, the dermis can be divided into two layers: a superficial papillary layer and a deep reticular layer (Fig. 2.1). In the

papillary layer, the collagenous, elastic and reticular fibers are thin and loosely arranged. In the reticular layer, they are thicker and more densely packed: they run in various directions but are more or less parallel to the surface of the skin. Around the blood vessels and nerves, the fibers are more widely spaced than they are elsewhere.

The main functions of the connective tissue fibers and the associated ground substances appear to be that of providing a mechanical support for the various components of the dermis. (See Section 2.2 for other functions of the connective tissue). The blood and lymphatic vessels in the tissue provide nutrition to the cells of the epidermis as well as to the connective tissue cells. They are also involved in the very important process of thermoregulation of the body. At high atmospheric temperatures or with elevated heat production of the body, the blood vessels are dilated in order to allow an increased flow of blood through them and hence a greater loss of heat from the body through the surface of the skin. At low temperatures, the vessels contract and thus reduce the loss of heat through the skin surface.

The glands embedded in the connective tissue perform some protective as well as excretory functions. The sebaceous glands secrete, through their excretory ducts (which drain into the hair follicles), a mixture of lipids and organic salts (the sebum) which are the products of epithelial cell breakdown. This oily secretion provides lubrication for the hair and skin as well as forming a chemical barrier (the "acid mantle") to the penetration of microorganisms and other agents into the skin. The sweat glands, which have excretory ducts that open into the skin surface, secrete salt, urea and other waste products. They, therefore, act as

accessory excretory organs, aiding the kidney in the removal of waste products from the body. They also play an important role in the regulation of the body temperature. The evaporation of sweat from the skin surface has a cooling effect.

The complex network of nerve fibers, with nerve endings branching into all parts of the skin, makes the skin the principal receptor of tactile, thermal and painful sensations. Besides the free nerve endings, several nerve organs such as the Vater-Pacini Corpuscles and Meissner Corpuscles shown in Fig. 2.1 can be distinguished. Attempts to relate a specific end organ to a specific sense have generally not been successful although the sensation of pressure has been related to the Vater-Pacini Corpuscles (Gray and Sato, 1955).

The connective tissue cells are sparsely distributed among the tissue, more of them being present in the papillary layer than in the reticular layer of the dermis. The fibroblast are the most numerous cells found in normal skin. Next in abundance are the mast cells. The macrophages (or histocytes) are cells which display phagocytic activity (i.e. the ability to engulf large particles of foreign objects or cells). In fact, they can hardly be recognized in the dermis unless they are in the process of phagocytosis. A variable number of white blood corpuscles (leucocytes) can be found in the connective tissue. Two types of pigment cells (chromatophores) can be found in the tissue: cells which synthesize the pigment (the melanocytes) and cells which phagocytose it (the melanophores). The overall functions of the connective tissue cells appear to be that of synthesizing and organizing "new" connective tissue and of the breakdown and removal of "old" connective tissue as well as foreign

agents that are harmful to the tissue. The connective tissue cells will be discussed in greater detail in Section 2.3.

2.1.5. Functions of the Skin. We are now in the position to summarize the various functions of the skin:

(1) The skin provides an external covering for the body, protecting it from a number of mechanical injuries.

(2) The superficial layer of the skin provides protection for the body against penetration of bacteria and other microorganisms.

(3) The skin regulates the transport of water in and out of the body.

(4) The pigments in the skin protect the body from an excessive intake of injurious radiation.

(5) The skin, through its glands acts as an accessory to the kidney in the elimination of waste products from the body.

(6) The skin assists in the regulation of the body temperature.

(7) The skin is the most extensive receptor of tactile, thermal and painful sensations.

(8) The skin is a storehouse for glycogen, cholesterol and water. The action of ultraviolet radiation on ergosterol in the surface of the skin produces vitamin D, a material which plays an important role in calcium and phosphorous metabolism.

It seems clear that an attempt to design an artificial skin which will perform all these functions is bound to be over-ambitious. As the cells are obviously too complicated to imitate, the most logical approach appears to be the design of a material which has properties close

to that of the connective tissue matrix. If such a material can be prepared , it may serve as the seat for cells, blood vessels or even the cutaneous appendages. It is with this goal in mind that we will examine in greater detail the properties of the connective tissue matrix (Section 2.2) and the connective tissue cells (Section 2.3).

2.2. The Connective Tissue Matrix

Research in the area of connective tissue has been exceptionally vigorous in the past two decades. It has culminated in a great number of reviews, monographs and treatises (Tunbridge, 1957; Hall, 1963, 1964, 1965; Fitton Jackson et al., 1965; Fessler, 1968; Farber, 1964; Balazs, 1970; Perez-Tamayo and Rojkind, 1973). The terms "intercellular matrix" and "extracellular matrix" are often used alternatively to designate the connective tissue matrix.

The study of chemistry and biology of collagen has been most intensive and a number of excellent reviews and treatises have been written on the subject (Ramachandran, 1967; Gould, 1968; Harrington and von Hippel, 1961; Seifter and Gallop, 1966; Ramanathan, 1962; Traub and Piez, 1971; Yannas, 1972; Bornstein, 1969). Research on the MPS and PPS have also been very vigorous in the past decade (Muir, 1964; Egami and Oshima, 1962; Quintarelli, 1968; Balazs and Jeanloz, 1965; Brimacombe and Weber, 1964; Dogson and Lloyd, 1968). There has been an increasing interest in the study of elastin (Ayer, 1964; Partridge, 1962; Seifter and Gallop, 1966), but only a limited interest in the study of reticulin (Windrum et al., 1955; Robb-Smith, 1958) and GPS (Quintarelli and Dellovo, 1970; Anderson and Jackson, 1972; Robert et al., 1970 a,b).

In this section, a review of the main chemical and structural characteristics of these materials will be made. Several reviews on the biosynthetic pathways on collagen (Gould, 1968; Priest, 1973), MPS, PPS and GPS (Balazs and Jeanloz, 1965, vol II B; Muir, 1964; Dodgson and Lloyd, 1968) have been made and will not be discussed here.

2.2.1. Collagen. Collagen is the major protein constituent of the tissue matrix. It accounts for one third of the human protein and is found primarily in skin, tendon, cornea, basement membrane, bone and cartilage. In skin, collagen forms about 70 to 80% of the dry weight and up to 30% of the wet weight of the dermis (Harkness, 1961). Its main function is structural and it fulfills this by means of a distinctive molecular conformation arising from special regularities in its amino acid sequence. Through specific aggregation and crosslinking, these molecules form fibrils of unusual strength and stability.

The collagen molecule is secreted by fibroblasts as a precursor "procollagen" which is rapidly converted by a proteolytic mechanism to the tropocollagen molecule (Layman et al., 1971; Bellamy and Borstein, 1971). The tropocollagen monomers have been extensively studied; they have a molecular weight of approximately 300,000 (Lewis and Piez, 1964) and are approximately 3000 A° in length and 15 A° in width. Fig. 2.2 summarizes elegantly the primary, secondary and tertiary structures of the tropocollagen (TC) molecule. The molecule is made up of three polypeptide chains each of 100,000 molecular weight. These chains are called α-chains and they are characterized by the repeating Gly-X-Y triplet which produces the characteristic collagen helix. The

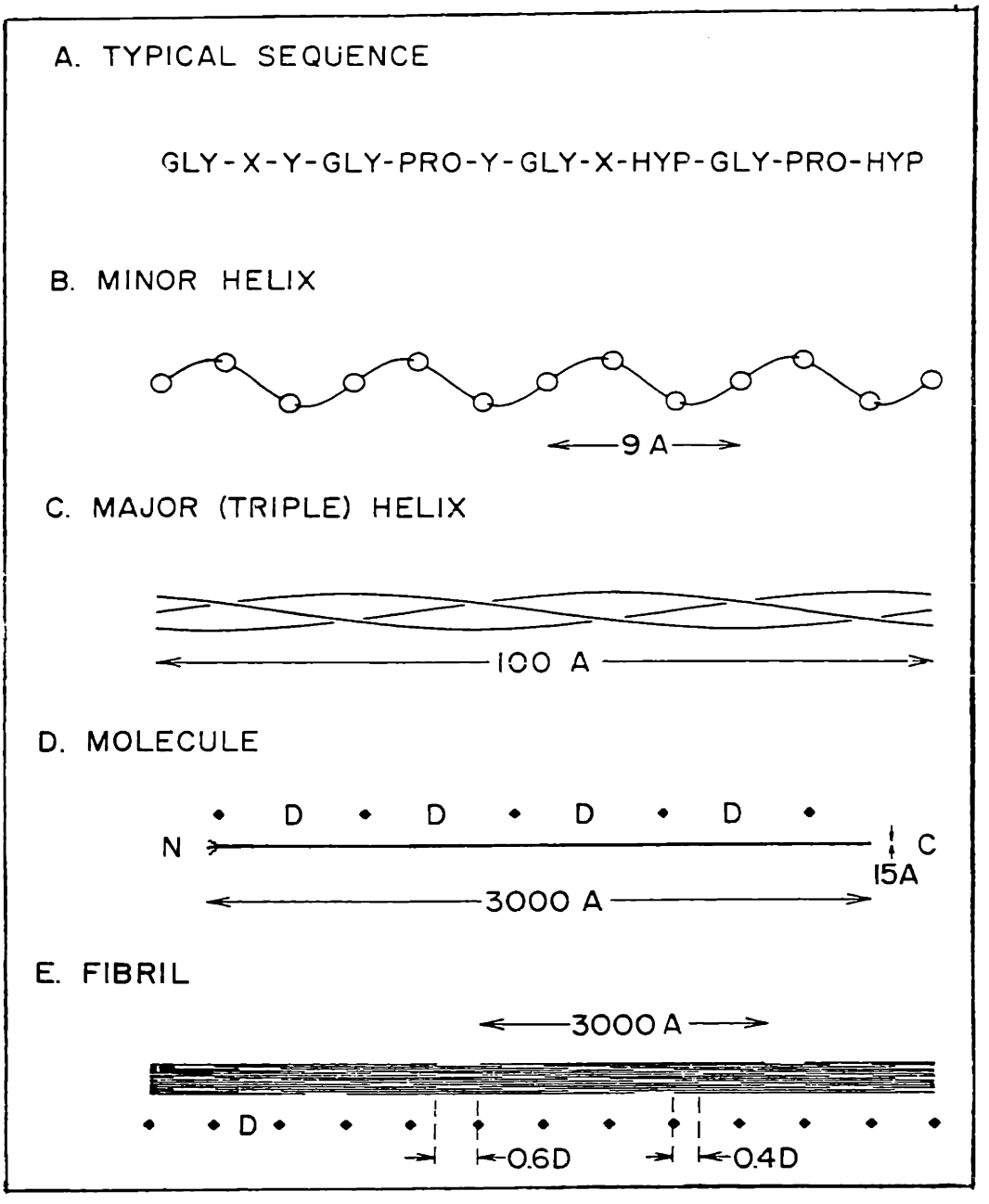


Fig. 2.2. Diagrammatic representation of several levels of order in collagen: A, primary structure; B, secondary structure; C and D, tertiary structure; E, quaternary structure. From Piez (1969).

α- chains each form a left handed helix (with a pitch of about 9 A°) while the three α-chains that form the tropocollagen molecule are twisted with respect to each other to form a very gradual right-handed superhelix (with a pitch of about 100 A°).

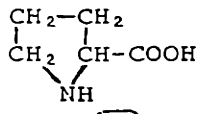
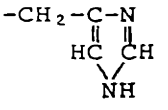
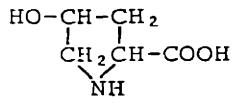
The amino acid composition of collagent is shown in Table 2.1. Glycine accounts for one-third of the total amino acid residue while proline together with the somewhat unique hydroxyproline accounts for another 22% of the composition. Collagen also contains 12% of the acidic amino acids (glutamic and aspartic acids), 11% of alanine, 9% of the basic amino acids (arginine, lysine, hydroxylysine and histidine), 5% of the lipophilic amino acids (leucine, isoleucine, valine and phenylalanine) and 5% of the hydroxyl amino acids (serine, threonine and tyrosine). There are significant differences in the amino acid compositions of the constituent α-chains of collagen. Most collagens are made up of two α1 and one α2 chains (Piez, 1967). The α2 chains differ from α1 in being more basic (because of a higher histidine content) and having larger amounts of lipophilic amino acids and lower amounts of imino acids, proline and hydroxyproline (Piez, 1967). A few exceptions to this somewhat standard chain composition have been found (Piez, 1964; Miller, 1971).

The α-chains may be crosslinked to give β components (2 α-chains) and γ components (3 α-chains). The intramolecular crosslink is formed by oxidative deamination of lysine by lysyl oxidase to form α-aminoadipic-δ-semialdehyde (Pinell and Martin, 1968). Two of these lysine aldehydes then undergo a spontaneous aldol condensation reaction to form the crosslink (Bornstein and Piez, 1966; Schiffman and Martin, 1969). This intramolecular crosslink occurs at the lysyl residues on the ninth position

TABLE 2.1

AMINO ACID COMPOSITION OF COLLAGEN FROM

HUMAN TENDON (From EASTOE, 1967)

Trivial name	R in repeating sequence -NHCHRCO-; or imino acid formula	Number of amino acid residues per 1000 total residues
Alanine	$-CH_3$	110.7
Glycine	$-H$	324
Valine	$-CH(CH_3)_2$	25.4
Leucine	$-CH_2-CH(CH_3)_2$	26.0
Isoleucine	$-CH(CH_3)-CH_2-CH_3$	11.1
Proline		126.4
Phenylalanine	$-CH_2-C_6H_5$	14.2
Tyrosine	$-CH_2-C_6H_4-OH$	3.6
Serine	$-CH_2-OH$	36.9
Threonine	$-CH(CH_3)-OH$	18.5
Methionine	$-CH_2-CH_2-SCH_3$	5.7
Arginine	$-CH_2-CH_2-CH_2-NH-C(=NH)-NH_2$	49.0
Histidine		5.4
Lysine	$-CH_2-CH_2-CH_2-CH_2-NH_2$	21.6
Aspartic Acid	$-CH_2-COOH$	48.4
Glutamic acid	$-CH_2-CH_2-COOH$	72.3
Hydroxyproline		92.1
Hydroxylysine	$-CH_2-CH_2-CH(OH)-CH_2-NH_2$	8.9

from the A- or N-terminal end (Fig. 2.2D) of the molecule (Kang et al., 1966). This unique non-helical area, commonly called the "telopeptide", comprises the first 16 N-terminal amino acid residues and is probably susceptible to attack by proteolytic enzymes other than collagenase (see Section 2.4.1).

Individual collagen molecules, after being secreted by the fibroblast, aggregate into fibrils which when stained and viewed with the electron microscope, show a characteristic cross striation pattern with an axial periodicity of 640 A° (Fig. 2.3). Some of these native collagen fibrils can be dissolved with suitable solvents to form solutions of tropocollagen (Piez, 1967). By adding appropriate agents to change the charge distribution within the tropocollagen molecule (Kuhn et al., 1970), it is possible to reconstitute the collagen from solution to form not only native type fibrils but also two types of aggregates with periodicities of nearly 3000 A°. These are the fibrils of the "fibrous long spacing" (FLS) form (Highberger et al., 1950, 1951) and the tactoids of the"segment long spacing" (SLS) form (Schmitt et al., 1953). The observation in SLS aggregates of molecular lengths roughly four times the native fibril periodicity led Schmitt et al. (1955) to postulate the "quarter-stagger" arrangement of collagen fibrils. This comprises an assembly of parallel molecules regularly displaced by a quarter of their length with respect to their nearest neighbors. Hodge and Schmitt (1960) provided strong support for this theory by demonstrating that an optical synthesis of appropriately displaced SLS patterns showed good agreement with the observed fibril pattern. However, careful comparison of the two patterns indicated that the molecular weight was not 4 but 4.4 times of the native

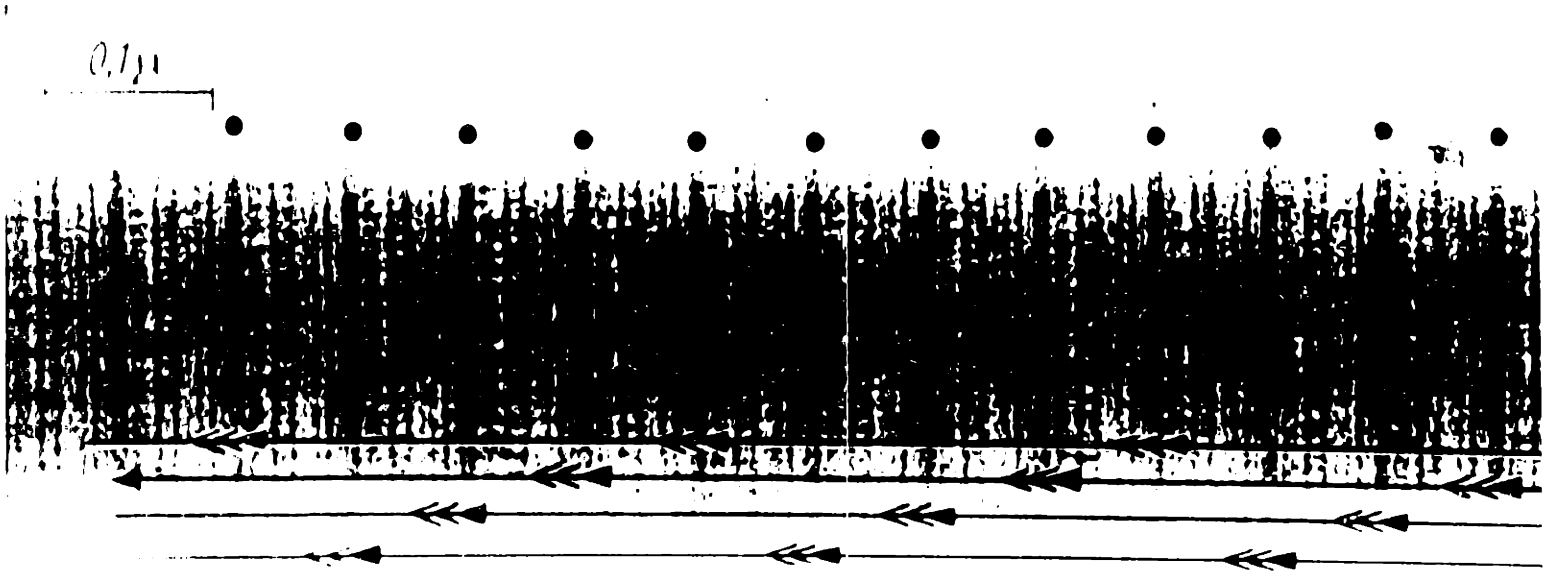


Fig. 2.3. Electron micrograph of a native collagen fibril, showing the typical periodicity of ca. 640A°. Stained with phosphotungstic acid, pH. 4.2. From Hodge and Schmitt (1960).

fibril period (D). The quarter stagger arrangement was consequently modified (Hodge and Petruska, 1963; Petruska and Hodge, 1964) to incorporate holes which are 0.6 D between successive molecules in the same row and an overlap of 0.4 D between molecules in adjacent rows (Fig. 2.2E).

This model for the molecular assembly of collagen in the fibril cannot, however, be extended from two to three dimensions in a way that maintains the same staggered relationship between all nearest-neighbour molecules (Ross and Benditt, 1961; McGavin, 1964; Smith, 1965). To overcome this difficulty, several schemes for the three dimensional arrangement of collagen molecules have been postulated. Grant et al., (1965, 1967) suggested a random stagger of one, two, three, or four times D between collagen molecules, which have five more or less equal bonding zones of length about 0.4 D separated by four nonbonding zones about 0.6 D long. This model has been criticized for not incorporating a systematic set of holes in the fibril (Veis et al., 1967) nor taking account of the unique amino acid sequence of the collagen molecule (Smith, 1968). Veis et al. (1967) have proposed a scheme whereby fibrils are built up of microfibrils which are in turn composed of fundamental packing units of four collagen molecules in staggered array. Successive tetrameric units, in the same azimuthal orientation are lined up with a gap of 0.6 D between molecules, so that the microfibrils have regularly spaced holes and overlaps (Fig. 2.4). However, the individual molecules in this scheme are not quite evenly spaced since they are separated by three successive intervals of 1 D and then one of 2 D. Another model for a microfibril has been recently proposed by Smith (1968). In this model, the modified quarter-stagger arrangement of HOdge and Petruska (1963) is maintained throughout the

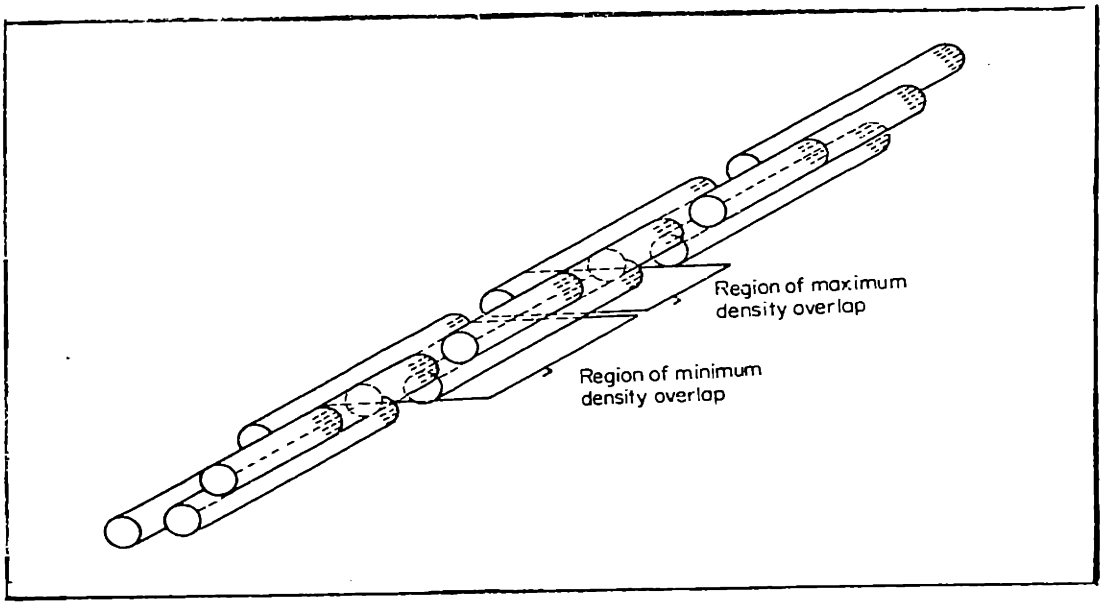


Fig. 2.4. Illustration of proposed packing of tetramolecular units to form microfibrils. From Veis et al. (1970).

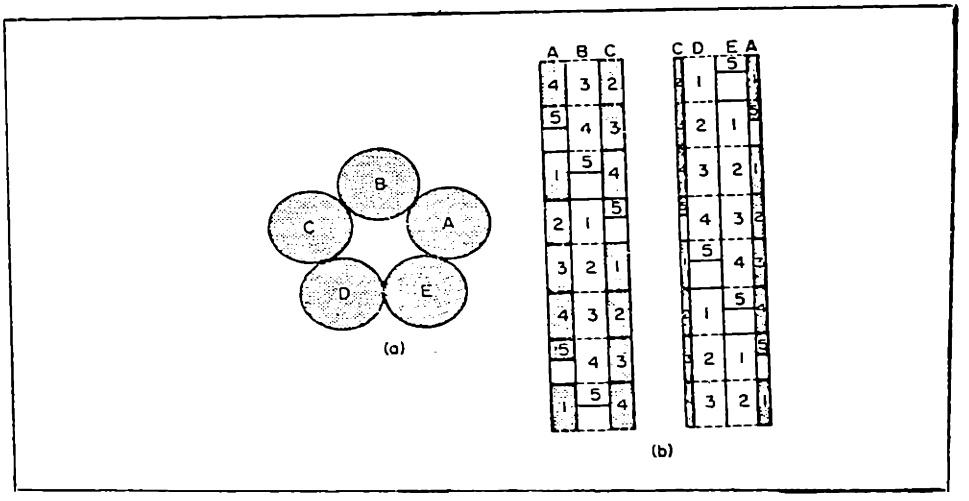


Fig. 2.5. Illustration of proposed structure of filament composed of five chains of collagen molecules in regularly staggered array. The filament is shown viewed (a) in cross section and (b) perpendicular to its length from two opposite directions. From Smith (1968).

microfibrils which are composed of five chains of collagen molecules (Fig. 2.5). Recent small angle x-ray diffraction studies of collagen fibers by Miller and Wray (1971) as well as the analysis of the problem on the molecular level by Segrest and Cunningham (1971) appears to favor the Smith model for the molecular arrangement of collagen.

Whatever the arrangement of the molecules are within the native fibrils, an intermolecular crosslinking process causes a progressive stabilization and loss of solubility of the fibrils. Veis and his co-workers have suggested that there are at least two chemically and structurally distinct sets of intermolecular crosslinks in mature insoluble collagen (Veis and Cohen, 1956; Veis and Anessey, 1965; Veis et al., 1967). One of these they have associated with the interchain links in γ_{222} components of collagen and the other with links in γ_{111}. They found that whereas the latter could form SLS aggregates, the γ_{222} components were unable to do so, but were easily renatured as native-type fibrils. From this, they inferred that $\alpha 2$ chains may be crosslinked in quarter stagger array and $\alpha 1$ chains may be joined with their lengths in register. Zimmermann et al. (1970) investigated this subject by studying SLS aggregates of pepsin treated collagen from lightly crosslinked tissue and they located several possible intermolecular crosslinks. They include bonds that link molecules head-to-tail with a small overlap and a small amount of less stable side-to-side bonds with large overlap. The latter were identified by the ability of one fraction to form fibrils and not SLS. They also reported additional bonds which join molecules at like ends (either N- or C- terminal) and suggested a scheme whereby they might be incorporated into intact fibrils. The nature of the intermolecular bonds

are yet to be characterized but Bailey et al. (1970) have presented evidence that they are based on semialdehydes derived from lysine and hydroxylysine.

The conversion of the rigid triple helical tropocollagen molecule to the randomly coiled macromolecule called gelatin can be brought about by a variety of means, such as treating it with chemicals or by application of heat. The temperature at which thermal denaturation of the collagen molecule occurs depends on the state of aggregation of the tropocollagen molecule, the nature of any diluent present, the amount of diluent and the extent of crosslinking between the molecules. For a dilute neutral salt solution of collagen, the denaturation temperature is about 37°C (Piez, 1967). The denaturation can often be detected by a dramatic lowering of the viscosity and optical rotation of the solution. In fibrillar form, collagen denatures at about 55°C when swollen in neutral salt solution. This can be detected by a shrinkage of the fiber to about a quarter of its original length and a loss of its characteristic x-ray pattern. The shrinkage temperature of the collagen fiber increases with intermolecular crosslinking of the collagen molecules (See Chapter 3). Either in the fibrillar form or in molecular dispersion, collagen, when completely dehydrated, denatures at about 215°C (Yannas, 1972). When collagen is even partially denatured, it susceptibility to enzymatic degradation increases tremendously (See Chapter 3).

In the connective tissue matrix, collagen is usually observed to exist as fibers of about 2 to 10 μ in diameter (Forrester et al., 1969; Yannas and Huang, 1972). Since the collagen fibrils that are observed with the electron microscope are usually about 500 to 1000 A° in diameter,

there must be some stabilizing forces other than intermolecular cross-linking which hold the fibrils together to form the larger fibers. A scheme has been proposed by Jackson and Bentley (1968) by which the fibrils may be held together to form larger fibers and by which the fibers can be further held together to form the macroscopic connective tissue. The scheme involves the PPS and GPS and is illustrated in Fig. 2.6. The carbohydrates of the PPS and GPS are pictured to be the links between the protein moieties and the collagen. The carobhydrates of the GPS, being short, can provide the necessary short range interaction between the fibrils to form the larger fibers. On the other hand, the MPS of the PPS, being long, can provide the necessary long range interaction between the fibers, which are generally randomly oriented and outstretched within the connective tissue matrix.

2.2.2. Elastin. Elastin is the insoluble rubberlike protein of the elastic fibers. It is found in most connective tissues in close association with collagen and reticular fibers and the ground substance. In skin, the elastic fibers are scattered throughout the epidermis, dermis and the subcutaneous tissue. The total amount of elastin in the skin is small, about 2 to 5% (Varadi and Hall, 1965; Weinstein and Boucek, 1960; Smith et al., 1962). Nevertheless, the presence of the elastic fibers is considered to be the major contribution to the elasticity of skin and is believed to be cause of the contraction of the skin when it is removed from the body (Harkness, 1971). Elastin exists in a substantial amount in some other tissues, as for example, 5-8% in lung (Pierce and Hocott, 1960; Briscoe and Loring, 1958), 30-45% in aorta (Kraemer and Miller, 1953; Ayer et al.,

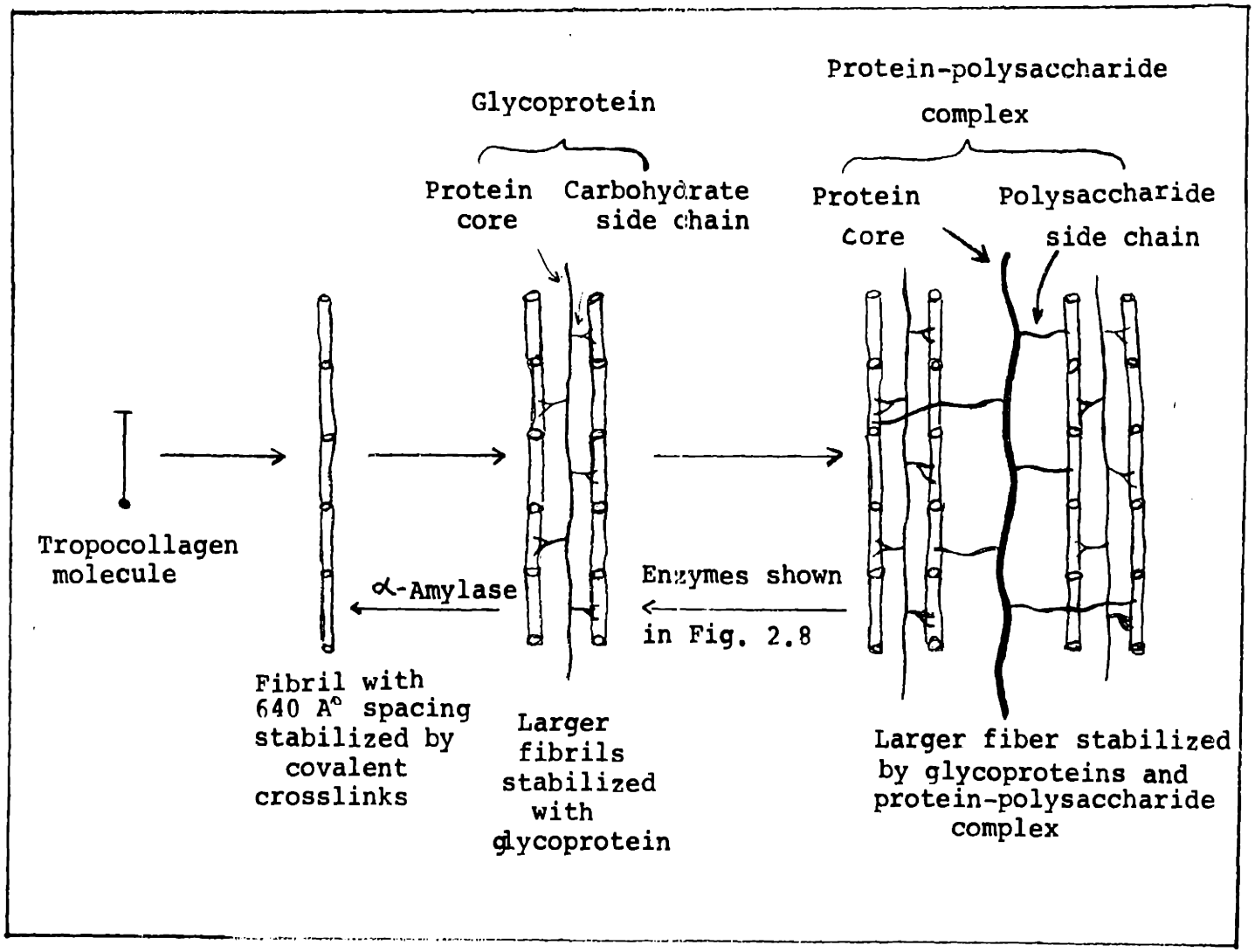


Fig. 2.6. The role of glycoproteins and protein-polysaccharides in the stabilization collagen fibrils. From Jackson and Bentley (1968).

1958; Neuman and Logan, 1950) and 70-80% in ligamentum nuchae (Bowes and Kenten, 1949; Neuman and Logan, 1950). When it is present in large concentrations (as in the ligaments), the tissue has a yellow color and is sometimes referred to as "yellow connective tissue".

Attempts in the last two decades to obtain pure elastin for physical and chemical characterization has been hampered by the insolubility of the protein and its intimate association with some components of the ground substance. The usual mild methods used to solubilize other proteins do not render elastin soluble so that drastic conditions have been used to isolate and purify elastin. The practical definition for elastin, at the present state of knowledge, appears to be the protein material that remains after all other components of the connective tissue have been removed. Removal of these components have been achieved by such drastic methods as boiling the tissue in neutral salt solution (Partridge, et al., 1955; Miller and Fullmer, 1966), treatment with hot acidic or basic solutions (Lansing et al., 1952; Lloyd and Garrod, 1946), or selective removal of the components with enzymes (Hospelhorn and Fitzpatrick, 1961; Steven and Jackson, 1968). In spite of these drastic methods, or perhaps because of them, the amino acid compositions of elastin prepared by different methods do not agree very well (Petruska and Sandberg, 1968).

Even when purified to the maximum extent possible, characterization of the elastin remains a problem because of its insolubility. The completely elastic behaviour of the protein indicates that it is a fully crosslinked network so that attempts to solubilize it will have to involve cleavage of peptide bonds. Solubilization methods such as boiling the insoluble elastin with mild acids and alkalis (Partridge et al.,

1955; Robert and Poullain, 1963) and treatment with elastolytic enzymes (Thomas and Partridge, 1960; Partridge, 1962; Miyada and Tappel, 1956; La Bella, 1961; Takahashi et al., 1970) have been used. Owing to the non-specific cleavage of peptide bonds by these various solubilizing agents, attempts to reconstruct the original elastin network from the characterization of the solubilized products remain a major problem. This problem may be solved by the recent discovery (Smith et al., 1968) that copper deficiency in swine results in the inhibition of formation of crosslinks in collagen and elastin. A soluble protein precursor of elastin, "tropoelastin" has been isolated and partially characterized. (Sandberg et al., 1969 a, 1969 b).

The amino acid composition of the insoluble elastin so far characterized shows certain general characteristics that are of worthwhile mention. As in collagen, glycine accounts for one-third the total amino acid residue in elastin. The unusually high concentration of proline (11%) is also similar to that found in collagen. At this point, however, the similarity ceases. The amount of hydroxproline (1.5%) found in elastin is considerably lower than that found in collagen (9%). The low amounts of polar amino acid residues found in elastin contrasts greatly with that found in collagen: the basic amino acids (arginine, lysine, hydroxylysine and histidine) accounts for only 0.7% (compared to 9% in collagen) the acidic amino acids (glutamic and aspartic acids) account for 2.0% (compared to 12% in collagen); while the hydroxyl amino acids (serine, threonine and tyrosine) account for 2.0% (compared to 5% in collagen). The amount of non-polar amino acids in elastin is consequently high: alanine accounts for 23% (compared to 11% in collagen) while the

lipophilic amino acids (leucine, isoleucine, valine and phenylalanine) account for 24% (compared to 5% in collagen). The low concentration of the polar amino acids in elastin (5%) probably explains its limited solubility in polar solvents. The amino acid composition of the soluble "tropoelastin" (Sandberg et al., 1969 a, 1969 b) is similar to that of the insoluble elastin except for one aspect. The composition of lysine in the tropoelastin (4.8%) is much higher than that of the insoluble elastin (0.8%). This correlates very well with the mechanism proposed by Thomas et al. (1963) in which lysine is involved in the crosslinking of the elastin.

The nature of the crosslinks in elastin and in collagen has been a subject of great interest in the past decade. Since it has been exhaustively reviewed elsewhere (Partridge et al., 1966; Piez, 1968; Franzblau et al., 1970), only a brief account of it will be given here. The pioneering work was done by Partridge et al. (1963) who isolated from an acid hydrolysate of elastin two polyfunctional amino acids which they designated as desmosine and isodesmosine. On the basis of their structure and the nature of the peptide which they contained, it was proposed (Thomas et al., 1963) that they were involved in the crosslinking of elastin. Franzblau et al (1970) have extended this idea by showing that other structures, based on lysine are capable of forming crosslinks in elastin. The first step in the crosslinking process is the oxidative deamination of lysine by lysyl oxidase to form α-aminoadipic-δ-semialdehyde (Pinell and Martin, 1968). Two or more of the lysine aldehydes can then combine by aldol condensation to produce the cross-links (Piez, 1968; Franzblau et al., 1970).

The rubber elastic behaviour of elastin has commanded the interests of researchers for nearly a century (For a review, see Ayer, 1964). The thermodynamic theory of rubber elasticity, when it became available (Meyer et al., 1932) was naturally applied to the elastic tissues (Meyer and Ferri, 1937; Wohlisch et al., 1943; Hoeve and Flory, 1958; Mukherjee and Hoffman, 1971). The molecular weight between cross-links, M_c, computed from the analysis of the stress-strain curve of the elastic fibers appears to be dependent on the nature of solution in which the measurement was made: in water of glycol-water mixture, M_c was found to be 3,400 (Hoeve and Flory, 1958) whereas in formamide or formamide-water mixture, M_c was 7,000 (Mukherjee and Hoffman, 1971). This was explained (Mukherjee and Hoffman, 1971) by the fact that the abundant non-polar amino acid groups in elastin results in hydrophobic interactions in the polar solvents (water and glycol-water mixture). This would give an apparent increase in crosslinking. The use of formamide-water mixture, which has a solubility parameter close to that of the hydrophobic peptide sequences, would result in an abolition of such hydrophobic interactions. The M_c value of 7,000 obtained in this mixture agrees well with the amount of crosslink that would be expected from the conversion of the lysines present in the elastin to crosslinks. The explanation of hydrophobic interactions in elastin also agress with the work of Robert and Poullain (1963) and Kornfeld-Poullain and Robert (1968) who found that the alkaline hydrolysis of elastin was facilitated by the presence of lower alcohols. From the analysis of this data, they concluded that the effect is due to the penetration of the hydrophobic centres by the water-alcohol mixture.

Conformational studies of elastin solubilized from insoluble elastin have recently been made, using circular dichroic studies. The studies have been hampered by the limited solubility of the elastin which results in the presence of a coacervate phase in solution (Urry et al., 1969; Gotte et al., 1970). The results obtained do not agree. Urry et al. (1969) found an α-helicity of about 50% in elastin whereas Gotte et al. (1970) found an α-helicity of only 10%. The x-ray diffraction studies of the insoluble elastin (Cox and Little, 1961; Ramachandran and Santhanam, 1957), however, do not reveal any helicity. The technique recently reported by Yannas et al. (1972) for the conformational studies of solid phase collagen may possibly be used to resolve these apparent discrepancies (See Chapter 3).

Recent ultrastructural studies of elastin have brought into attention the intimate association of elastin with the ground substances of the connective tissue. Ross and Bornstein (1970) have demonstrated that elastic fibers from arteries, tendons and elastic ligaments of mature animals actually contain two morphologically different components. An amorphous central component was found to be surrounded by microfibrils of about 100 to 200 Å in diameter. The staining properties of the two components were different. Amino acid analyses of these two components appear to suggest that the amorphous component is elastin while the microfibrils are made up of glycoprotein.

2.2.3. Reticulin. The reticular fibers of the skin are small in amount, making up to about 0.38% of the original dry weight of collagen (Highberger, 1936). The fibers are characterized histologically by their ability to be stained black by silver nitrate solutions (a characteristic

which distinguishes them from collagen fibers). Chemically, however, reticulin is indistinguishable from collagen. The amino acid analysis of reticulin (Windrum et al., 1955) is very similar to that of collagen except for a rather smaller quantity of proline and larger amounts of hydroxyproline, hydroxylysine and lipophilic amino acids. A considerable amount of fatty acids (10.9%) was found to be firmly bound to the molecule. The presence of about 4.2% of neutral sugar in the reticulin also suggest that it may be complexed with a lipo-glyco-protein.

Robb-Smith (1958) classified the reticulins into collagenous and non-collagenous. The noncollagenous reticulins have been identified in ovarian stroma; they are hydrolysed by trypsin and resistant to collagen attack. The collangenous reticulins include the basement membrane, the adult stroma and the newly synthesized collagen fibrils which are soluble in neutral salt solutions or citrate buffers. These fibers, as expected are hydrolysed by collagenase. In view of the work to be reported in this thesis which shows that the presence of MPS or GPS can interfere with the collagenase degradation (Chapter 4), the author feels that this classification may be simply one of separating the collagen which are strongly associated with ground substances from those which are loosely or not associated with the ground substances.

2.2.4. Mucopolysaccharides and Protein Polysaccharides. The term mucopolysaccharide was coined by Meyer (1938) to describe "hexosamine containing polysaccharides of animal origin". The majority of these polysaccharides are important constituents of the amorphous component of animal connective tissues, the so-called "ground substance". Here, for

the most part, they exist as "complexes" with varying amounts of protein. With the exception of hyaluronic acid, which is usually associated in tissue with relatively small amounts of protein, the MPS are conceived to exist as a central protein core with pendant polysaccharide chains (Fig. 2.6). Each pendant polysaccharide chain is of molecular weight about 10,000 to 50,000 (i.e. about 40 to 150 saccharide units) while the molecular weight of the protein portion between two adjacent pendant groups may be about 1,000 to 5,000 (i.e. about 10 to 50 peptide units). The MPS together with the protein core is termed protein polysaccharides.

The total amount of MPS in skin is very low, about 0.1% to 0.4% of the dry weight (Pearce, 1965). In some other connective tissues, the amount of MPS are higher, as for example, umbilical cord has about 4-7% MPS (Hadidian and Pirie, 1948); cornea has about 1-3% (Meyer et al., 1953; Anseth and Laurent, 1961); tendon has about 0.5-1% (Meyer et al., 1956; Levine, 1957); bone has about 0.1-0.2% (Meyer et al., 1956; Rogers, 1951). Cartilage on the other hand has about 30-40% MPS (Davidson and Woodhall, 1959; Strandberg, 1950). The MPS and PPS are presumed to play an important role in stabilizing and supporting the fibrous and cellular elements of the tissue, in contributing to the load-bearing properties of the tissue and in maintaining a controlled environment within the connective tissue. They may also play a role in the fibrillogenesis of collagen (Wood, 1964) and elastin (Ayer, 1964).

By definition, each of the MPS is characterized by the presence of substituted, glycosidically bound hexosamine moieties which are invariably either D-glucosamine (2-amino-2-deoxy-D-glucose) or D-galactosamine (2-amino-2-deoxy-D-galactose). Up to the present time

there has been no conclusive demonstration of hybrid polymers containing both hexosamine types of significant quantities. Within the polymer chain of the MPS, the hexosamine residues are conceived as alternating in a regular manner with either hexuronic acid or hexose moieties. The resulting polymers assume linear configurations although the possibility of branching cannot be excluded in certain instances. Each of the MPS exhibit polyanionic characteristics which result from the presence of the carboxyl groups of the constituent group, or of ester-bound sulfate residues, or both. Accepted structures show these anionic elements to be distributed regularly along the polymer chains. Table 2.2 summarizes the repeating units of the various MPS of the connective tissue while Fig. 2.7 illustrates the structural features of some of these MPS. The distribution of the various MPS in mammalian connective tissues is summarized in Table 2.3.

Hyaluronic acid (HA) is one of the most important MPS of the connective tissues. It was first isolated by Meyer and Palmer (1934) from the vitreous humor of bovine eyes. It is widely distributed in different connective tissues, synovial and vitreous fluids of animals. Its chemical structure as shown in Fig. 2.7 was established by Meyer (1947) and Jeanloz and Flowers (1962). Recent x-ray studies of oriented hyaluronic acid show that the chains of the polymer are capable of forming a double helix, each helix having a periodicity of 33.7 A° (Dea et al., 1973; Atkins and Sheehan, 1973). It was also shown (Atkins and Sheehan, 1973) that the several other conformations of the hyaluronic acid chains are possible, depending on its environment. Laurent (1970) has recently reviewed the structure of HA and come to the conclusion that it exists

TABLE 2.2

MAJOR CONSTITUENTS OF ACID MUCOPOLYSACCHARIDES

(From Dodgson and Lloyd, 1968)

Polysaccharides	Hexosamine	Other major monomer	*N*-Acetyl groups	Presence of: *O*-Sulphate groups	Sulphamate groups
Hyaluronic acid	D-Glucosamine	D-Glucuronic acid	+	−	−
Chondroitin	D-Galactosamine	D-Glucuronic acid	+	−	−
Chondroitin 4-sulphate	D-Galactosamine	D-Glucuronic acid	+	+	−
Chondroitin 6-sulphate	D-Galactosamine	D-Glucuronic acid	+	+	−
Dermatan sulphate	D-Galactosamine	L-Iduronic acid	+	+	−
Keratan sulphate	D-Glucosamine	D-Galactose	+	+	−
Heparin	D-Glucosamine	D-Glucuronic acid	−	+	+
Heparan sulphates	D-Glucosamine	D-Glucuronic acid	+	+	+

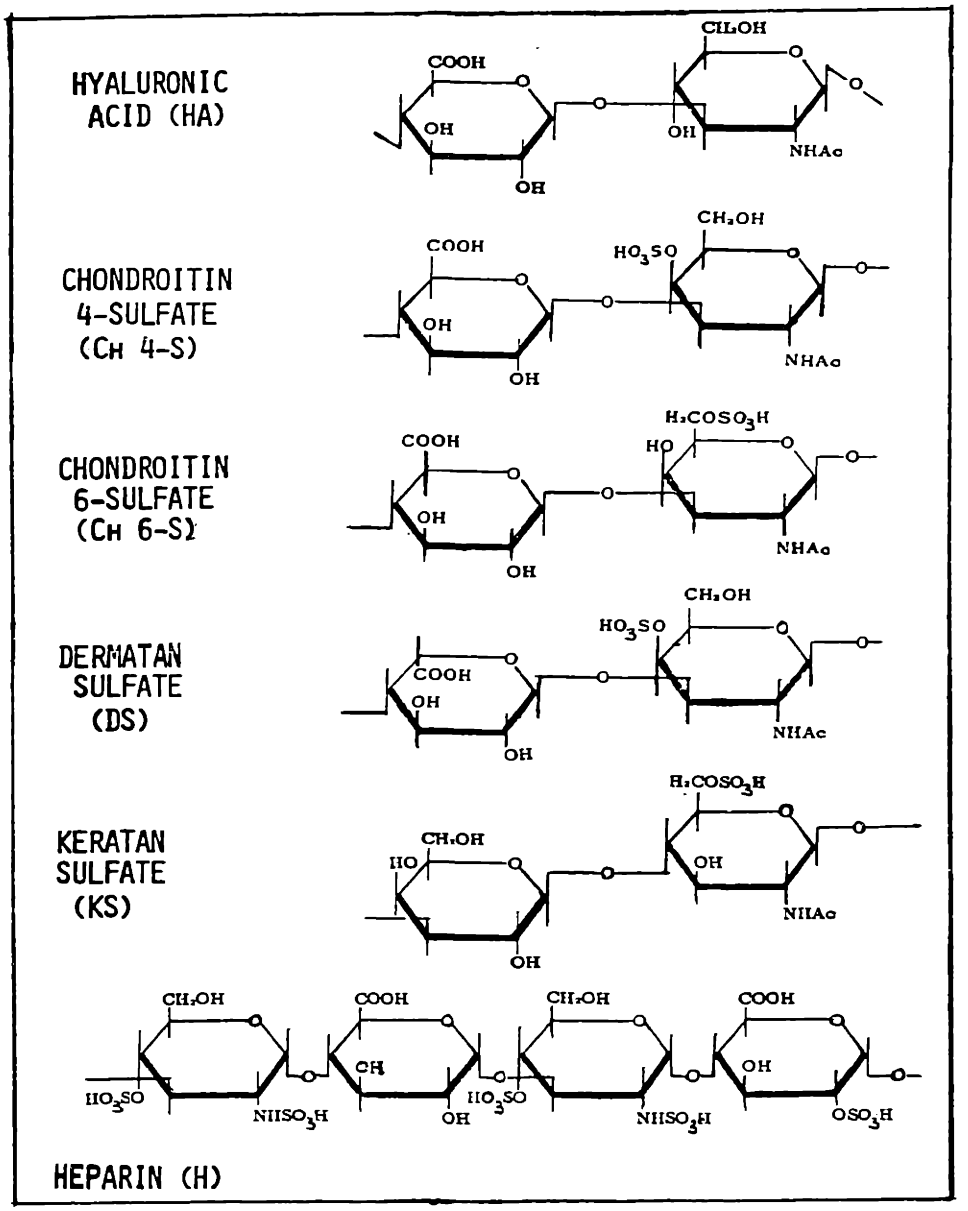


Fig. 2.7. Chemical structure of some acid mucopolysaccharides.

TABLE 2.3

DISTRIBUTION OF ACID MUCOPOLYSACCHARIDES IN

MAMMALIAN CONNECTIVE TISSUES

(From Dodgson and Lloyd, 1968)

Tissues	Polysaccharides							
	Hyaluronic acid	Chondroitin	Chondroitin 4-sulphate	Chondroitin 6-sulphate	Dermatan sulphate	Keratan sulphate	Heparin	Heparan sulphate
Umbilical cord	+	−	−	−	±	−	−	−
Synovial fluid	+	−	−	−	−	−	−	−
Cartilage	−	−	+	+	−	+	−	−
Bone	−	−	+	+	−	+	−	−
Tendon	+	−	−	+	+	−	−	−
Ligamentum nuchae	+	−	+	−	+	+	−	−
Skin (dermis)	+	−	±	±	+	−	+	+
Aorta	+	−	+	−	+	−	+	+
Vitreous humour	+	−	−	−	−	−	−	−
Cornea	−	+	+	−	−	+	−	−
Sclera	−	−	+	+	+	−	−	−

in the connective tissues and synovial fluid substantially free from proteins. The physical functions of HA in the tissues have been discussed by both Fessler (1960) and Ogston (1971). Solutions of HA resist limited compression (Fessler, 1960) and flow of solvent through the solution and reduce the rate of diffusion of both small and large molecules (Ogston and Sherman, 1961). The large solvent volume of HA partially excludes other molecules from its solution, reducing the actual fluid volume of the body (Ogston and Phelps, 1961). HA forms a three-dimensional network in dilute solution which reduces the sedimentation of other large molecules and particles (Laurent and Pietruszkiewicz, 1961). The role of the HA in the lubrication of joints by synovial fluids has recently been studied (Dowson, 1967; Linn, 1968).

The chondroitin sulfates (4- and 6-) are the major components found in cartilage (Schubert and Hamerman, 1965). Chondroitin 4-sulfate (Ch 4-S) was first isolated by Fischer and Boedeker (1961) from cartilage. Its chemical structure (Fig. 2.7) was obtained through the studies of Levene (1925), Wolfrom and Juliano (1960) and Davidson and Meyer (1954). Chondroitin 6-sulfate (Ch 6-S) was first isolated by Meyer and Palmer (1936) from umbilical cord. Its chemical structure (Fig. 2.7) was elucidated by Davidson and Meyer (1954). Recent studies on oriented sample of Ch 4-S (Atkins and Laurent, 1973) and Ch 6-S (Arnott et al., 1973 b) show that polymer chains of both MPS are capable of forming a three-fold helix with a unit height of 9.6A° or an eight-fold helix with a unit height of 9.8 A°. The structure of the carbohydrate-protein linkage of both Ch 4-S and Ch 6-S has recently been found (Roden, 1970). The groups involved in the linkage are Glucuronic acid-Galactose-Galactose-Xylose-

Serine, where the Glucuronic acid forms part of the polysaccharide chain while the serine forms part of the protein chain.

Dermatan sulfate is normally present in skin, tendon and blood vessels. It was first isolated from pig skin by Meyer and Chaffee (1941) and its chemical structure (Fig. 2.7) was characterized by Stoffyn and Jeanloz (1960). X-ray studies of oriented dermatan sulfate fibers (Arnot et al., 1973 a) indicate that it can adopt a two-fold helix (with unit height 9.6 A°), a three-fold helix (with unit height 9.5 A°) or an eight-fold helix (with unit height 9.3 A°). The structure of the carbohydrate-protein linkage in PPS of DS has been determined (Fransson, 1970) and found to be similar to that of the chondroitin sulfates, described in the last paragraph.

Keratan sulfate was first reported in bovine cornea (Meyer et al., 1953) where it constitutes about half of the MPS content. Its chemical structure was solved substantially by Hirano et al., (1961). Variations in the degree of sulfation have been found for keratan sulfates isolated from different sources (Cifonelli et al., 1967). The PPS of keratan sulfate appears to contain appreciable amounts of chondroitin sulfates (Roden, 1970). The nature of the carbohydrate-protein linkage for KS is unknown.

Heparin was first isolated from liver by McLean (1916). In spite of the large amount of structural investigation done on heparin, its primary structure is not fully known. The probable structure of heparin is as shown in Fig. 2.7. However, variations in the position of the sulfate group and of the glycosidic linkages are possible (Lindahl, 1970). The presence of residual amino acids in heparin extracted by various

procedures (Green et al., 1961, Lindahl et al., 1965) and the isolation of a carbohydrate-serine compound, consisting of equimolar amounts of serine and xylose, from hydrolysates of commercial heparin preparations (Lindahl and Roden, 1965) strongly suggested that the polysaccharides could occur in the native state covalently crosslinked to a protein moiety in a way analogous to the chondroitin sulfates and dermatan sulfate discussed above. Serafini-Francassini et al. (1970) have presented both chemical and histological evidence for the existence of a PPS of heparin.

Heparan sulfate was first isolated from beef liver by Jorpes and Gardell (1948). Its structure has yet to be solved. Based on the finding that it contains equimolar quantities of glucosamine, uronic acid, acetyl and sulfate residues, it has been proposed (Linker and Sampson, 1960) that heparan sulfate is a block copolymer of disaccharides in which the glucosamine moiety carries O-sulfates and of disaccharides having N-acetyl glucosamine moiety only. Lindahl (1970) has presented evidence that heparan sulfate may occur in native state covalently cross-linked to a protein moiety as in the chondroitin sulfates and dermatan sulfate discussed above.

2.2.5. Glycoproteins. The glycoproteins of the connective tissue matrix are conceived to be made up of a protein core with short pendant carbohydrate groups as shown in Fig. 2.6. They are usually observed to be very closely associated with the fibrous components of the matrix. They generally occur in very small quantity in connective tissues: in skin they account for about 0.2% of the dry weight (Pearce, 1965). In spite of this small amount, the glycoproteins in connective tissues have been attributed to play an important role in the stabilization of the fibrous component

of the tissue (Partington and Wood, 1963); in the antigenicity of tissue implants (Radhakrishnamurthy et al., 1964; Robert et al., 1968), in the resistance of the tissue of collagenase degradation (Quintarelli and Dellovo, 1970) and in the fibrillogenesis of collagen (Anderson and Jackson, 1972).

Due to the lack of a source for large quantities of connective tissue glycoproteins, characterizations of these materials have not been as comprehensive as the glycoproteins of blood (Gottschalk, 1966). Chemical analyses of the few connective tissue glycoproteins that have been prepared in reasonable purity (Robert et al., 1970 b); Anderson and Jackson, 1972) show that they have characteristic chemical compositions of glycoproteins: they contain a high quantity of protein (60-90%), a small amount of hexosamine (2-6%),a similar amount of hexoses (1-6%) and very small quantity of fucose (0.4%) and sialic acid (0.2 to 1.0%). Anderson and Jackson (1972) separated two glycoprotein fractions from bovine Achilles tendon and showed that one glycoprotein was essentially free of collagen while the other contains 8% collagen. Both fractions interacted strongly with acid soluble calf skin TC and it was concluded that they may have some function in the molecular organization of the tissue.

The immunological studies of the glycoproteins and other components of the connective tissue by Robert et al., (1970 a) are relevant to our attempts to design a material for skin replacement. One of the criteria for a suitable material is that it should be non-antigenic. Robert et. al. (1970 a) showed that of the components, collagen, PPS and GPS, the latter appeared to be the major tissue and

species-specific antigenic component of the connective tissue. The antigenicity of the glycoprotein was increased by limited proteolysis. This is interpreted to be caused by the creation of more "antigenic sites" by the scission of the glycoprotein.

2.3. Connective Tissue Cells.

A number of excellent reviews on the structure and functions of connective tissue cells have recently been written (Branwood, 1963; Fullmer, 1965; Ross, 1968; Dorfman, 1970). A survey of these reviews reveals that while the structure of these cells are relatively well understood, the functions which they perform remain, in many cases, unknown. The difficulties involved is common to any attempts to interpret the functions of living cells through fixed histological sections. For example, the presence of a certain component of the tissue in a certain cell (as evidenced by histological staining or by the use of labelled molecules) has often been presented as proof that the cell is responsible for the biosynthesis of that particular component. This, of course, may not necessarily be the case since it is possible that the component might have been absorbed by the cell from the extracellular environment through the processes of pinocytosis (engulfment of substances in solution) and phagocytosis (engulfment of large particles).

In spite of difficulties involved, some progress has been made in the interpretation of the functions of the connective tissue cells. The overall picture is that the cells are responsible for the biosynthesis of "young" connective tissue matrix and for the breakdown and

removal of "old" connective tissue matrix. These two processes are continually taking place even in the healthy tissues, but in situations such as wound healing and embryonic development, they are greatly accelerated. In this section, a general description of the cells and the role they play in the biosynthesis of the connective tissue matrix will be discussed. The next section (Section 2.4) will deal with enzymes responsible for the breakdown of the connective tissue matrix and the role that the cells play in this important process.

2.3.1. Fibroblasts. The fibroblasts are the most abundant cells found in normal skin. The vigorous activity of the cytoplasm of the cell gives it a rather undefined shape although it is generally described as either elongated or star-shaped in appearance. The shape of the cell generally reflect the environment in which the cell is located. This includes: (1) the presence and orientation of pre-existent extracellular matrix; (2) forces upon the tissues resulting in lines of stress in given directions; or (3) various restrictions and limitation to cell movement by adjacent structures (Fell, 1932; Glucksmann, 1938; Bassett, 1962). Two types of fibroblasts can be distinguished. The name fibrocyte was given (Porter, 1964) to the immobile, inactive or resting stage of the fibroblast. These cells have poorly developed nucleus and endoplasmic reticulum and are long, slender and smaller than the "blast" or active cell (Ross, 1968, p. 49). When activated by tissue injury or estrogen therapy, they are converted to mobile, active cells with rough endoplasmic reticulum and Golgi body, and accompanied by the appearance of collagen fibrils in the extracellular matrix surrounding the cells (Ross, 1968, p. 50).

Early observations of this kind were the first indication that these cells may be responsible for the production of connective tissue fibers, as their name imply. More recently, there are also evidence that they may be responsible for the production of some of the ground substances.

Recent studies suggest that the endoplasmic reticulum of fibroblasts is responsible for the biosynthesis of collagen or of a collagen precursor. The presence of an extensively rough endoplasmic reticulum is generally indicative of cells that are actively synthesizing proteins (Campbell, 1960; Porter, 1961). Closer examinations of these subcellular systems show that it is studded with aggregates of ribosomes (polyribosomes) which are oriented in the form of curves or spirals (Ross and Benditt, 1964; Goldberg and Green, 1964). The polyribosomes can be disrupted by collagenase (Fernadez-Madrid, 1967) suggesting that they were at least partially stabilized by the presence of the newly synthesized chains of collagen molecules. By reversibly interrupting the production of polyribosomes with chemicals, Ross and Benditt (1964) and Baglio and Farber (1965) were able to illustrate that the appearance of collagen fibrils in the extracellular matrix coincided with the presence of polyribosomes on the endoplasmic reticulum of the fibroblast.

The use of autoradiographic studies, coupled with the improvement of resolution of the electron microscope for the past few decades have greatly advanced our knowledge on the role of the various organelles of the fibroblasts in the biosynthesis of collagen and some of the protein polysaccharides. The autoradiographic studies involve injecting into the experimental animals or cultured tissues labelled precursor substances

required for the synthesis of the material studied and tracing their path by histological studies of the cells with the optical or electron microscope as well as with chemical analyses. Using such methods, the biosynthesis of proteins (with ^{14}C- or ^{3}H-labelled amino acids), mucopolysaccharides (with ^{14}C-labelled hexuronic acids, hexosamine and hexose), sulfated mucopolysaccharides (with ^{35}S-labelled sodium sulfate) and collagen (with ^{3}H-labelled proline) can be followed. Ross and Benditt (1965) have successfully employed this method to study the biosynthetic pathway of collagen and the proteins of the protein polysaccharides. Based on the concentration of the $^{3}[H]$ proline in the various organelles of the fibroblast, they postulate the following pathway: the tropocollagens are synthesized within the polyribosomes of the endoplasmic reticulum and passed into the peripheral vesicles where they are secreted directly out of the cell and eventually aggregate to form the fibrils of collagen. The protein moities of the protein-polysaccharides are first synthesized within the polyribosomes and then sent to the Golgi body where they are combined with the MPS (which are manufactured in the Golgi body - see next paragraph) to form protein-polysaccharides. These are eventually secreted out of the cell, again through the vesicles of the peripheral cytoplasm.

The biosynthesis of the MPS has received large amount of attention in the past two decades. These studies have shown that the fibroblasts or cells related to fibroblasts are responsible for the synthesis of hyaluronic acid (Grossfield et al., 1957; Hedberg and Moritz, 1958; Castor 1959), chondroitin sulfates (Grossfield et al., 1957, Holtzer et al., 1960, Lash et al., 1960) and dermatan sulfate (Berenson et

al., 1958). The use of autoradiographic studies on the biosynthesis of sulphated MPS (using $S^{35}O_4$) have been particularly vigorous in the last decade. These studies have shown that the radioactive sulfate is first located within the cells and later spreads outward in bone (Belanger, 1954), developing mouse connective tissue (Glucksman et al., 1956), rat skin and chick embryo (Mancini et al., 1961) and cartilage (Belanger, 1954; Engfeldt and Westerborn, 1960; Okada, 1960; Dziewiatkowski, 1962). Detailed examination of the sequence of events in chick embryo suggest that the labelled sulfate first appeared within the cytoplasm near the nucleus and then spread into the rest of the cytoplasm, eventually appearing in the intercellular matrix (Mancini et al., 1961). The resolution of the optical microscope in this study was apparently insufficient to indicate the organelles involved in the synthesis. The use of high resolution electron microscope on chondroblasts (Godman and Lane, 1964; Fewer et al., 1964), wound fibroblasts and tissue culture fibroblasts (Ross, 1968, p. 24) suggests that the labelled sulfate radicals are localized in the Golgi body prior to their incorporation into extracellular chondroitin sulfate. Other studies on intestinal globlet cells (Peterson and Leblond, 1964) and intestinal columnar cells (Neutra and Leblond, 1966) also implicate the Golgi body in the production of the polysaccharides. It is these observations, coupled with the observation that some of the [^{3}H] proline pass through the Golgi body that led Ross and Benditt (1965) to the postulation, outlined above, that the incorporation of the MPS to the protein to form protein-polysaccharides occur in this organelle. A review on the role of the Golgi body in the production of the protein-polysaccharides is given by

Revel (1970).

The synthesis of collagen and some of the MPS by the fibroblast has thus been sufficiently well studied. However, this does not imply that the fibroblast is exclusively the cell that can produce these materials. In fact, a number of instances have been found where cells other than the fibroblast have been found to synthesize collagen as well as some of the MPS. The early studies of Reed and Rudall (1948) indicated that the epidermal cells of the earthworm cuticle are capable of forming collagen fibers, although they do not possess the banded structures characteristic of connective tissue collagen. Later studies by Hay and Ravel (1963) and Mercer (1961) indicated that during amphibian development, the basement membrane (which is made up of a loose network of collagen fibers surrounded by ground substances) can be formed by the epithelial cells. A review on the synthesis of the basement membrane by epithelial cells is given by Pierce (1970). Kallman and Grobstein (1965) explored the ability of connective tissue cells and epithelial cells, grown in vitro, to form collagen by the introduction of labelled proline. They found that whereas the connective tissue cells were capable of producing isotopically labelled collagen molecules, the epithelial cells were not. They felt that their observations refuted the proposition that epidermal cells produce collagen. Instead they proposed that the epithelium with its associated basement membrane have a directive effect on the aggregation and polymerization of tropocollagen into fibrils. Ross (1968) has reviewed the evidence for the production of collagen by the smooth muscle cells and came to the conclusion that, while the evidences are circumstantial, they indicated that the "smooth

muscle cells may be related to the synthesis and secretion of extracellular collagen."

There is also evidence that cells other than the fibroblast can produce some of the mucopolysaccharides. In fact, Asboe-Hansen (1957; p. 12) had claimed that the mast-cell is the only cell type in which MPS can be found. This was based mainly on the evidence that the mast-cells have cytoplasmic granules that are stained metachromatically by basic dyes such as toluidine blue (a method generally used to detect the presence of acid mucopolysaccharides). On the other hand, fibroblasts are not stained by metachromatic dyes. This was later explained by Okada (1960) and Mancini et al. (1961) to be due to the fact that the MPS in the fibroblasts are probably not sufficiently polyermized within the cell to show metachromasia. The mast cells will be discussed in greater detail in Section 2.3.2.

Besides the mast cells, the lymphocytes under the influence of estrogens may also produce MPS (Muir and Marshall, 1961). Muir (1964) expressed the possibility that since some of the mononuclear cells of the circulation can turn into cells indistinguisable from fibroblasts (Petrakis et al., 1961) the capacity to synthesize MPS may be innate to certain cells of the blood.

While the synthesis of collagen and some of the MPS have been studied extensively, the synthesis of the other components in the connective tissue matrix remain obscure. The reticular fibers, since they are chemically indistinguishable from the collagen fibers, have been assumed to be produced by the fibroblasts. The elastic tissue is generally considered to be produced by the fibroblasts since it is never

seen without the presence of collagen and it occurs in the vicinity of fibroblastic type of cells in the electron micrographs of skin (Charles, 1961) and lungs. (Low, 1961). In the aortic media, collagen and elastic fibers are found associated with smooth muscle cells (Haust, et al., 1965; Buck, 1962; Parker and Odland, 1966) which can be construed as fibroblastic in type (Ayer, 1964). Because no elastic fibers have been identified in the fibroblast, it has been suggested that a soluble form of the molecule or its precursor is synthesized in the endoplasmic reticulum of the cytoplasm and is then aggregated to a recognizable form outside the cell (Ayer, 1964). As mentioned above, a soluble protein precursor of elastin (tropoelastin) has recently been isolated (Sandberg et al., 1969 a, b).

Apart from the postulation by Ross and Benditt (1965) which implicates the Golgi body of the fibroblast as the sites of combination of the non-collagenous proteins with the MPS, very little is known about the biosynthesis of the protein-polysaccharides. Virtually nothing is known about the synthesis of the connective tissue glycoproteins. Early studies (Harkness et al., 1954; Bowes et al., 1957) have implicated the fibroblast in the biosynthesis of the non-collagenous proteins but the relationship of these proteins to the protein-moieties of the PPS and GPS remain to be established.

The origin of the fibroblasts is a subject which has received large amount of attention recently (Allogower, 1956; Dunphy, 1963; VanWinkle, 1967). Much of the evidence seems to favor the view that fibroblasts are derived from resting fibrocytes or undifferentiated mesenchymal cells located within the connective tissues (MacDonald, 1959;

Grillo, 1963; Dunphy, 1963; Dixon, 1960; Block, 1960; Ross, 1970). Some evidence, however, supports the view that they may be derived from blood cells (monocytes) which normally migrate into the connective tissues during wound injuries (Gillman and Penn, 1956; Allgower, 1956; Allgower and Hulliger, 1960, Petrakis et al., 1961; Gillman and Wright, 1966). There is some evidence that the fibroblasts may also be derived from macrophages (Baker et al., 1961; Muir and Goldberg, 1961). The recognition that smooth muscle cells may be capable of producing collagen and elastin in aortic media (Buck, 1962; Haust et al., 1965; Parker and Odland, 1966) has led to the suggestion (Branwood, 1963) that both the fibroblasts and the smooth muscle cells are derived from the same primitive reticular cells or differentiated endothelial cells.

2.3.2. Mast Cells. The mast cells are characterized by the presence of a large number of cytoplasmic globular granules which are stained metachromatically by basic dyes such as toluidine blue. This observation, coupled with the absence of metachromasia by the other cells had led Asboe-Hansen (1957, p. 12) to postulate that the mast cells are the only cell in which MPS can be found. Recent studies, however, have shown that most of the MPS are synthesized in the fibroblast (see Section 2.3.1). It is now generally accepted that metachromasia exhibited by the mast cell is mainly due to the presence of the anticoagulant MPS, heparin. Early studies (Holmgren and Wilander, 1937; Jorpes et al., 1937; and Jorpes, 1946) had indicated that the mast cells are an important source of heparin. This conclusion received further support when Oliver et al.. (1947) isolated heparin from a dog mast-cell tumor. During the last

decades, the heparin content of mast cells has been further substantiated by the work of a great number of authors, and this has been reviewed recently by Ringertz (1965). In addition to heparin, the mast cells have been shown to contain heparin sulfate (Jorpes and Gardell, 1948; Meyer et al., 1957; Cifonelli and Dorfman, 1960) and hyaluronic acid (Asboe-Hansen, 1955; Ringertz and Bloom, 1960).

The ability of the mast cell to snythesize and store heparin has been demonstrated by autoradiographic studies (Asboe-Hansen, 1953; Jorpes et al., 1953; Montagna and Hill, 1957; Mancini et al., 1961). Heparin is generally identified by its electrophoretic mobility and its degradation by enzymes from heparin-adapted Flavobacterium (Korn, 1959). Most evidence points to the cytoplasmic granules of the mast cells as the site of synthesis of heparin. Hagen et al., (1959) were able to isolate these granules and demonstrate the presence of heparin in them. Recently, Serafini-Fracassini, et al., (1970) were able to demonstrate the presence of PPS of heparin in mast cell granules using histological as well as biochemical evidences.

The ability of the mast cells to liberate heparin and other MPS into the surrounding connective tissue and the role they play in the formation of the connective tissue ground substances is still subject to debate. While several instances of release of heparin by the mast cells have been demonstrated (Paton, 1958 and Riley, 1959) by the administration of a great number of agents into the cells, Ringertz (1965) doubts that the disruption of the mast cell granules by such means represents a physiological mechanism of secretion. There appears to be little evidence that heparin, when released from the mast cells enter the blood

circulation with consequent manifestation of its anticoagulant properties (Riley, 1959).

Asboe-Hansen (1957, p. 142) found an increase in the number of mast cells in the metachromatic areas of healing wounds in rabbit skin. It has been suggested (Riley, 1959) that the role of the mast cells is to store and release the MPS for the connective tissue. A carbohydrate precursor substance, probably formed by fibroblasts become a temporary component of the ground substance. At the beginning of fibrillogenesis, excess ground substance is broken down, rebuilt and stored in the granules of the mast cells. This could later be released and ingested by the cells of the connective tissues. However, the autoradiographic studies of Montagna and Hill (1957) and Mancini et al. (1961) show that although the mast cells took up labelled sodium sulfate, the radioactivity did not spread from the cells. Studies by Green and Day (1960) further showed that cells from mast cell tumours did not secrete heparin into the medium or absorb heparin from the medium. From these observations , Muir (1964) concluded that it is doubtful that the mast cells play any significant role in the formation of the connective tissue MPS.

Another important role of the mast cells appear to be the storage and release of histamine (Riley and West, 1952, 1953; Riley, 1959). This chemical is associated with itching skin diseases (Asboe-Hansen, 1951). It can be released from the granules of the mast cells by the administration of a large variety of agents, and, unlike heparin it has been shown to enter into the blood circulation upon disruption of the mast cells. (Riley, 1959).

2.3.3. Leucocytes. The white blood cells, or leucocytes are cells which migrate from the blood circulation into the connective tissue during tissue injury. Some of them are differentiated from the mesenchymal cells of the connective tissue and have remained there. These can sometimes enter the circulation (Bloom and Fawcett, 1962, p. 95). Two classes of leucocytes can be distinguished. The first are cells with single, well defined nucleus and non-granular cytoplasm and these include the lymphocytes and the larger monocytes. The second class are cells with irregular nuclei which are more or less subdivided into lobes and these are the polymorphonuclear (PMN) leucocytes or granulocytes. The PMN leucocytes can be further divided into acidophils (eosinophils), basophils and neutrophils (heterophils), depending on whether they are easily stained by acidic, basic or neutral dyes respectively. The monocytes, the lymphocytes and the neutrophilic PMN leucocytes are generally the cells associated with inflammation during the wound healing of connective tissues (Ross, 1970).

All the leucocytes are capable of phagocytic activity. The mechanism by which the phogocytosed materials are digested intracellularly has been a subject of great interest for the past decade. This mechanism, known as the "Lysosome Concept", has been reviewed adequately (de Reuck and Cameron, 1963) and only a brief outline of the concept will be given here. The best-understood example at the moment is the phagocytic process in neutrophil leucocytes obtained from the rabbit peritoneal cavity. These cells are equipped with a large variety of acid hydrolytic enzymes contained in cytoplasmic granules which are

called lysosomes. The granules are enclosed in a "unit" membrane which effectively separates the hydrolases from the remaining cytoplasm. When killed bacterial or yeasts are added to suspensions of neutrophils, the latter cells send out pseudopodia which encompass the material to be ingested. An invagination and pinching off of the cell membrane occurs, resulting in enclosure of the phagocytized material within the cell cytoplasm but separated from it by a membrane. This vacuolar structure is called a phagosome. Phase-contrast motion photography shows that this process is followed by an apparent "explosion" of the cytoplasmic granules into the vacuoles (Hirsch, 1962). Lockwood and Allison (1963) and Zucker-Franklin and Hirsch (1964) showed that this step invclves a fusion of the granule membrane with the vacuole membrane, followed by incorporation of the smaller into the larger membrane with concomitant discharge of the granule contents into the vacuole and enlargement of the vacuole. The necessity for the enclosure of the vacuole with the cell membrane appears to be due to the fact that most of the lysosomal enzymes are active only in an acid pH (Woessner, 1965). There is a distinct acid reaction which may be as low as pH 4.7 (Sprick, 1956) in phagocytic vacuoles.

There is evidence, however, that the neutrophilic PMN leucocytes may be capable of releasing the lysosomal enzymes extracellularly. Ross (1970) observed, in the healing of human skin wound, that the initial inflammatory response consisted largely of an exudate of PMN neutrophilic leucocytes. If the wound was sterile, the cells displayed little to no phagocytic activity. Instead most of them become fragmented and their granules were released into the extracellular

millieu. It was suggested that this extracellular release of the lysosomal enzymes could play a role in the initial digestion of some of the debris in the wound so that it may be subsequently ingested and removed by the monocytes and macrophages.

2.3.4. Macrophages. Two types of macrophages can be distinguished in the connective tissue. The "fixed macrophages" are immobile, resting cells which appear like fibroblasts. However, they have a slightly more granular and usually vacuolated cytoplasm with the nucleus more compact and smaller than that of the fibroblast. They are usually found in the richly vascular area of the connective tissue. When activated by inflammation of the tissue, they become free and ameboid. Whether they are "fixed" or "free", the macrophages are capable of phagocytosing particles that are introduced into the tissue. Organic particles are digested intracellularly, but inorganic ones may remain within the same cell or be passed on to others. When particles are too large to be engulfed, several cells may gather around them and become fused into multinucleated, plasmodium-like masses called foreign-body giant cells.

Macrophages arise from a variety of cells such as fibroblasts, lymphocytes and skeletal muscles (Montagna, 1962, p. 141). They can often be observed in vitro. The addition of blood serum of normal rabbits to explants of 18-day-old chick embryos induces the formation of large number of macrophages (Pomerate et al., 1949). The serum appears to contain a thermolabile, alcohol-and acetone-insoluble macrophage-promoting factor. The appearance of macrophages can also be induced in

tissue culture by irritants, intoxicants and infections. A high number of macrophages appear spontaneously in cultures of skeletal muscle or of subcutaneous connective tissue (Chevremont, 1948). The liquid media of these cultures was found to be rich in choline. If choline was extracted from the media, the transformation of the muscle fibers to macrophages ceases; when it was added to the media, the transformation began. The transformation was inhibited when choline oxidase, which selectively destroys choline, was added to the cultures. It was concluded that choline was liberated by the cultured tissues and that it was necessary for the transformation to macrophages.

The primary functions of the macrophage appear to be the removal of harmful microorganism and foreign particles which may have penetrated into the connective tissue and the disposal of loose blood, dead cells and tissue debris during injury (Tompkins 1955; Brucher, 1962). During an inflammatory response, they enter the lesion after the initial influx of polymorphonuclear leucocytes and lymphocytes. They can be seen to be in an active state of phagocytosis and the large vacuolar structures within the cytoplasm are thought to contain the remnants of ingested matter (Ross, 1970). The mechanism by which intracellular digestion occurs in the macrophages is probably similar to that which occurs in the phagocytic process of leucocytes (See Section 2.3.3). It involves the cytoplasmic granules that are generally found in phagocytic cells, including the macrophages (Cohn and Wiener, 1963). These granules, which are analogous to the lysosomes of liver and kidney (Cohn and Hirsch, 1960) contain a complete battery of acid hydrolytic enzymes capable of degrading a large variety of substances. A review

of these enzymes has recently been made (Woessner, 1965). Some of these enzymes, relevant to degradation of the connective tissue matrix will be discussed in Section 2.4.

The possibility that the macrophages may play an important role in the immunological response of cells had been suggested by Novikoff (1963). He noted that the work of Fishman (1961) suggested a macrophage requirement for the synthesis of antibody. He further noted that other studies have indicated that when some antigens are partially degraded a number of antigenic sites "open up" so that more antigenicity is exhibited by the partially degraded protein compared to the undegraded one. Novikoff (1963) speculated that the macrophages may play the role of partially degrading the macromolecules involved so that they can in turn be transmitted to the antibody-producing cells. Recent studies (Laskin and Lechevalier, 1972) have confirmed the important role of macrophages in the immune response of cells.

2.4. Enzymatic Degradation of the Connective Tissue Matrix.

The mechanism of enzymatic degradation of the connective tissue matrix is probably the least understood of the processes that occur within the matrix. While several enzymes capable of degrading the various components in the matrix have been isolated, they have often been obtained through bacterial culture or they have been located in tissues which are not obviously connected with the connective tissues. Even in the few cases where the enzymes have been located in the connective tissues, the mechanism of their degradation and the cellular

site of their synthesis remain obscure. The extent of knowledge of the enzyme system involved in the degradation of a particular component in the connective tissue matrix appears to coincide with the extent of knowledge of the chemistry and structure of that component. Hence, while the enzymes involved in degrading collagen, elastin and PPS are relatively well characterized, those that are involved in degrading reticulin and glycoproteins remain unexplored.

2.4.1. Degradation of Collagen. As collagen is the major component in the connective tissue matrix, the mechanism by which it is degraded is fundamental to an understanding of orderly growth and remodelling, aging and pathological connective tissue resorption. In spite of the fact that collagen is obviously degraded under these circumstances, early workers were confronted with the perplexing problem that native collagen,both in fibrillar form as well as in solution, is extremely resistant to degradation by proteolytic enzymes commonly found in animal and human tissues. Attempts to isolate in animals an enzyme capable of specifically attacking the native collagen helix under non-denaturing conditions at physiologic levels of pH, temperature and salt concentration were uniformly unsuccessful.

The isolation by Mandl et al. (1953) of a collagenase from the bacterium Clostridium histolyticum, capable of degrading collagen under the conditions noted above suggested the possible existence of true animal collagenases. It was not until about a decade later, however, that the first animal collagenase from the tissue cultures of metamor-

phosing tadpole was demonstrated by Gross and Lapiere (1962). Subsequently, specific collagenases have been isolated from a number of animal and human tissues (Eisen et al., 1970; Lazarus, 1973). Most of the enzymes were not extractable but had to be produced by tissue culture (Fullmer and Gibson, 1966; Fullmer et al., 1966; Evanson et al., 1967; Eisen et al., 1968; Riley and Peacock, 1967; Jeffrey and Gross, 1967; Jeffrey and Gross, 1970). Recently, a number of extractable collagenase have been isolated from crab hepatopancreas (Eisen, 1967; Eisen and Jeffrey, 1969); granules of human PMN leucocytes (Lazarus et al., 1968; Robertson et al., 1972); macrophages and giant cells (Salthouse and Matlaga, 1972); tadpole tails (Harper et al., 1971; Eisen et al., 1971); and tissue homogenates of human skin (Eisen et al., 1971). In the case of the tadpole tails (Harper et al., 1971; Eisen et al., 1971), the collagenase exists as an inactive zymogen which can be activated by a heat-labile, non-dialyzable cofactor found in collagenase-free, tadpole tail-fin culture medium (Harper et al., 1971). In the case of the skin homogenates, Eisen et al. (1971) showed that the collagenase present is enzymatically inactive unless an inhibitor is chromatographically separated from the enzyme. None of the collagenase isolated so far have been pure enough, or available in sufficient quantity, to allow physicochemical characterization.

A variety of techniques have been used for the detection of collagen degradation. They can be classified into two types: One which employs solutions of collagen and the other which employs reconstituted collagen fibrils. The solution assay measures the viscosity of the

collagen as it is degraded by the enzyme. It was one of the earliest techniques used for the study of kinetics of collagen degradation by collagenase (Gallop et al., 1957). The advantages of this assay are that it is rapid and that it can be used in conjunction with other techniques such as optical rotation studies, acrylamide gel electrophoresis and electron miscroscopy. The optical rotation measures any changes that may occur in the helical structure of the collagen as it is degraded by the enzyme. The acrylamide gel electrophoresis enables the reaction products to be separated and thus ultimately can be used to reveal the site of attack by the enzyme. If the reaction products are not denatured, they can be reconstituted to give fragments of the SLS form, and examination of these fragment with the electron microscope often reveal the site of attack on the collagen by the enzyme. The combination of these techniques have been used very fruitfully for the characterization of the animal collagenases and for locating the site of attack on the collagen by the enzymes. The disadvantage of using solutions of collagen is that temperatures below the physiological temperature of 37°C have to be employed. This is necessary since tropocollagen molecules usually become denatured at around 37°C and if this happens, the collagen is susceptible to attack by other proteolytic enzymes while the attack of the collagenase on it is no longer specific. The use of non-physiological temperatures, on the other hand, cast doubts on the extrapolation of the results obtained to in vivo degradation.

This difficulty can be circumvented by the use of reconstituted collagen fibrils. Two variations of techniques can be used in this case.

In the first method a thin film of collagen fibrils is formed at the bottom of a Petri dish and small pieces of tissue in culture medium are incubated at 37°C upon it. If collagenase is formed by the tissue then lysis of the gel, as evidenced by a transparent halo around the tissue, will occur. Gross and Lapiere (1962) used this technique to demonstrate the first animal collagenase. In the second method, radioactive collagen fibrils are incubated with the material to be assayed at 37°C and then separated from the incubation mixture by centrifugation or filtration (Nagai et al., 1966). The radioactivity in solution reflects peptide reaction products and solubilized collagen molecules. A third method for the study of enzymatic degradation of fibrillar collagen will be described in Chapter 3. A comparison of the three methods will also be made there.

Based on the site of attack on collagen by the various collagenases isolated so far (as evidenced by visualization of SLS fragments from the degraded collagen), two classes of animal collagenases can be classified. The first class attack the molecule at a single locus one quarter of the distance from the C-terminal end (Fig. 2.2.D) and severing three peptide bonds, one in each of the α-chains. The second class of enzyme, which after first having attacked the molecule at this same specific point, continues to remove, stepwise, 8%, then an additional 5% of the large fragment from the C-terminal end. Collagenases from tadpole tissues (Sakai and Gross, 1967; Kang et al., 1966), rat and human skin (Eisen et al., 1968), mammalian wound epithelium (Grillo et al., 1969), human rheumatoid synovial tissue (Evanson et al., 1967), bone (Shimizu et al., 1969) and human leucocytes (Lazarus et al.,

1968) are of the first class and enzymes from rat uterus (Jeffrey and Gross, 1967, 1970), regenerating newt limb (Dresden and Gross, 1970), and crab hepatopancreas (Eisen and Jeffrey, 1969) seem to be of the second class. The possibility of contaminating proteolytic enzymes in the second class cannot, however, be eliminated.

The widespread occurence of animal collagenases make it unlikely that these proteins have a single cellular origin. In an extensive survey by Riley and Peacock (1967), 238 human tissues were examined for collagenolytic activity and the results showed that of 130 tissues with epitheluum, 81 were positive and 40 were negative, whereas of 108 tissues without epithelium, only 2 were positive and 106 were negative. Such observations, coupled with the more direct study of Eisen and Gross (1965), which demonstrated the production of tadpole tailfin collagenase exclusively by epithelial cells, would suggest a predominantly epithelial source for collagenolytic enzymes. On the other hand, the collagenases of synovial fluid (Evanson et al., 1967; Harris et al., 1968), granulocytes (Lazarus et al., 1968; Daniels et al., 1969), macrophages (Salthouse and Matlaga, 1972) and bone (Fullmer and Lazarus, 1967; Shimizu et al., 1969) are probably mesenchymal in origin. In some instances, enzymes of both epithelial and mesenchymal origin are present, as in human skin wounds (Grillo, et al., 1969), human gingiva (Fullmer and Lazarus, 1969), rabbit (Donoff et al., 1971) and guinea pig (Grillo and Gross, 1967) skin wounds.

While studies of the kind so far described are interesting they are not helpful in explaining the mechanism by which collagen is

degraded *in vivo*. Several points have to be made in order to illustrate the nature of the problem. Firstly, collagen exist in tissue mainly as an insoluble fiber. Almost no work has been done on the enzymatic degradation of insoluble fiber. The substrates used for the collagenases so far are either collagen in solution or reconstituted collagen fibrils, which under appropriate pH and salt concentration can be dissolved. The method described in Chapter 3 is specifically designed to study the enzymatic degradation of insoluble collagen fibers. Secondly, the extensive and insoluble nature of the collagen fibers makes it very unlikely that the initial step in the degradation of the collagen fiber be intracellular. While the theory of intracellular digestion (Lysosomal Theory--See Section 2.3.3) has been developed to quite an advanced level and substantiated in many instances, theories for extracellular degradation remain in the speculative level. Thirdly, collagen exists in the connective tissue in close association with the various components of the ground substances. The effect of these components on the susceptibility of the collagen to collagenase have hardly been considered. The work to be reported in Chapter 4 of this thesis will hopefully shed some light in this matter.

Several theories on the *in vivo* enzymatic degradation of collagen have been postulated over the past decade. These have been reviewed extensively by Perez-Tamayo (1973). They can basically be divided into three classes: one is based on intracellular digestion, another is based on extracellular degradation and the third is based on the combination of the first two. The main proponent of the intra-

cellular theory is Woessner (1962, 1968) who has isolated a lysosomal enzyme called Cathepsin D which is capable of degrading collagen. Since the enzyme has optimum activity at pH 3.2, the requirement for intracellular digestion is necessary. Bazin and Delaunay (1970) have isolated from granular tissues a lysosomal enzyme which they called "collagenolytic cathepsin", capable of degrading collagen. They also propose an intracellular mechanism for the degradation of the collagen. The main problem with this theory is, as mentioned above, the extensive and insoluble nature of the collagen fibers which would make it difficult for them to be phagocytosed.

The theories for extracellular digestion can be subdivided into two classes, based on the alleged source of collagenase. On one hand, the collagenase involved is believed (Dingle, 1969) to be Cathepsin D, which is lysosomal in nature. The lysosomal enzymes are believed to be secreted by the cell with the result that the proteolysis occurs extracellularly. This requires, however, a local acid pH in the extracellular region. On the other hand, the discovery of collagenases which have optimum activities at physiological pH (listed at the beginning of this section) would argue against the lysosomal source of the collagenase. It is believed (Gross, 1964; Kang et al., 1966; Nagai et al., 1966) that these collagenases, produced by cells of epithelial or mesenchymal origin in a yet unknown way, attack the collagen extracellularly, resulting in fragments which will denature under physiological conditions. Once denaturation occurs, the fragments can be further attacked by the collagenase and other proteolytic enzymes

found in the extracellular matrix.

The third class of theory on *in vivo* degradation of collagen (Dingle *et al.*, 1971; Woessner, 1971; Nordwig, 1971) attempts to reconcile all the evidences presented for both intracellular and extracellular degradation. It is believed (Lazarus, 1973) that the first step involved is the secretion of an inactive form of collagenase. This is followed by the activation of the enzyme, either by proteolytic attack of the molecule, as in the case of the tadpole (Harper *et al.*, 1971) or by the dissociation of an inhibitor, as in the case of human skin (Eisen *et al.*, 1971). The collagenase then specifically cleaves the molecules located on the outer diameter of the collagen fiber. At the site where the scission occurs, local denaturation probably occurs, making the chain ends susceptible to further attack by the collagenase or by other proteolytic enzymes. As this process continues, fragments of the collagen molecules separate from the fiber exposing fresh collagen molecules beneath it (Harris *et al.*, 1970). These fragments may then be phagocytosed by the macrophages or other phagocytic cells. In the meantime, the freshly exposed collagen in the fiber will be attacked by the activated collagenase and the cycle continues. It can be stopped by the secretion of a serum protein which has been shown (Eisen *et al.*, 1970; Evanson, 1971) to be an effective inhibitor for collagenase. The theory just outlined is necessarily vague because of the limited information presently available. Nevertheless, it is probably the most plausible mechanism so far proposed for the degradation of collagen and for its control.

2.4.2. Degradation of Elastin. Studies on elastase, the enzymes that specifically degrade the elastic fibers, were initiated by Eijkman (1904) who isolated the first bacterial elastase and by Balo and Banga (1949) who isolated the first animal elastase from ox pancreas. The first pure crystalline elastase was isolated by Lewis et al. (1956) from porcine pancreas. Much of the recent studies on elastase have been focused on the elucidation of the primary, secondary and tertiary structures of the elastase and this has been reviewed extensively by Hartley and Shotton (1971). Physiocochemical studies of the enzyme and of its reaction with elastin have been reviewed by Loeven (1963), Hall (1971) and Mandl (1961).

The porcine pancreatic elastase is a protein with a molecular weight of about 25,000 (Lewis et al., 1956). It is a linear polymer, made up of 240 peptide residues and crosslinked by four disulfide bridges (Brown et al., 1967). Its complete amino acid sequence has been determined by Shotton and Hartley (1970) and found to be similar to that of α-chymotrypsin, which has similar enzymatic activity as the elastase. Furthermore, the tertiary structure of the elastase was found (Shotton and Watson, 1970) to be similar to α-chymotrypsin, giving rise to the postulated generalization by Hartley and Shotton (1971) that enzymes possessing similar enzymatic mechanism and exhibiting a high degree of similarity in amino acid sequencing have very similar tertiary structures. Nevertheless, the ability of the elastase to degrade elastin is not shared by chymotrypsin or trypsin, the other pancreatic proteases. (Partridge and Davis, 1955). On the other hand, elastase is capable of degrading proteins other than elastin (Lewis et al., 1956).

Three forms of elastases have so far been identified. The first form, called the proteolytic elastase component by Loeven (1963) exhibits normal elastolytic activity, converting insoluble elastin to soluble peptides. The second form, called proelastase, is the inactive precursor of the first form and was first demonstrated by Grand and Robbins (1957). Activation of proelastase to elastase is induced by tryptic cleavage of a single peptide bond (Gertler and Birk, 1970). A third form of elastase, called the mucolytic elastase component (Loeven, 1963), shows only slightly elastolytic activity but has a synergistic effect on the first form of elastase, enhancing greatly its proteolytic activity. Banga and Balo (1960) suggested that this component removes the ground substances that are known to be intimately associated with the elastin and hence makes it more susceptible to attack by the proteolytic elastase. They showed that treatment of elastic fibers with the mucoid elastase resulted in a loss in strength of the fiber. The hypothesis is further supported by the work of Yu and Blumenthal (1958) who showed that pretreatment of the elastin with aortic mucopolysaccharides render it resistant to attack by the proteolytic elastase. The authors suggested that the fibers were physically coated with the mucopolysaccharide or that a chemical union of this material with the material surrounding the fibers took place, so that the enzyme is excluded from access to the susceptible chemical bonds. It is interesting to note that the work to be reported in Chapter 4 suggests that a similar mechanism appears to operate in preventing collagenase from attacking collagen when it is coated with acid mucopolysaccharides.

The insolubility of the elastic fibers and the presence of intimately associated ground substances present problems for the determination of enzymatic degradation by elastase. The assay methods that have been used involve measuring the amount of insoluble substrate which is solubilized by the enzyme by determining either the weight of the residual undigested elastin or the amount of solubilized protein. Radioactive elastin has been used (Robert and Robert, 1970) to determine the amount of solubilized protein. One mechanochemical method, devised by Ridge and Wright (1965) for studying the effect of elastase on skin and used by Loeven (1967) for studying the degradation of elastic fibers, is of special interest to the work to be reported in Chapter 3. In this method, the elastic tissue was immersed in the enzyme solution, maintained at 37°C and was periodically extended to measure the modulus of the material. A decrease in modulus was indicative of enzymatic degradation. Loeven (1967) found that a 30% decrease in the modulus coincided with a 5% loss in weight of the fiber, indicating that the mechanochemical method is probably more sensitive than the gravimetric method. In Chapter 3 of this thesis we describe an independently discovered mechanochemical method for the study of enzymatic degradation of insoluble collagen fiber by collagenase.

Early studies on the mechanism of interaction of the elastase with elastin were hampered by the impurity of the enzymes used and presence of ground substances that are associated tightly with the elastin. Most of these studies (Banga, 1955; Hall, 1955; Loeven, 1960) suggest that the synergistic effect of mucoid elastase, described earlier, is due to its removal of ground substances. Loeven (1960) showed that when the pro-

teolytic elastase was used alone, the hexosamine-containing material was released only when nearly 50% fo the elastin has been dissolved. If, however, both the proteolytic and mucolytic elastases were used, the hexosamine was released immediately after the start of the enzyme reaction. Recent studies on a relatively purer form of elastin has shown that there are specific sites of attachment on the elastin by the elastase (Robert and Robert, 1970). Hall and Czerkawski (1961) showed that both carboxyl and hydroxyl groups are essential in the substrate for the close association of enzyme on substrate while Bagdy et al. (1960) showed that similar requirements were necessary for the enzyme. Hall (1971) has studied extensively the requirement of calcium for the binding of the elastase to the elastin. Inhibitors of elastase have been found in serum proteins by Walferd and Schneider (1959) and Tolnay and Bagdy (1959).

It must be pointed out that all of the studies reported so far have been on bacteria or pacreatic elastases. There has been no report on the presence of elastase in the connective tissues. The possible turnover of elastic fibers in these tissues is unknown. The mechanism by which the elastic fiber is degraded in the connective tissue is virtually unknown but the studies made with the pacreatic elastases suggest that it may be fairly similar to the degradation of collagen fibers. The presence of a proelastase which can be activated by proteolytic degradation; the necessity of the enzyme to degrade an insoluble fiber; the requirement of calcium for the enzyme to bind to the fiber; the existence in serum proteins of inhibitors for the enzyme; and the resistance of mucopolysaccharide-treated substrate to the enzyme are features which are common to the degradation of collagen by collagenase. The effect

of mucopolysaccharides on the degradation of elastin by elastase may be important in arteriosclerosis, where changes in the composition of the mucopolysaccharides have been found (Balo, 1963).

2.4.3. Degradation of Reticulin. Little attention has been paid to the enzymatic degradation of the reticular fibers because of their limited availability and because of their similarity in composition to collagen (Section 2.2.3). Robb-Smith (1958), however, has classified the reticulins into collagenous and non-collagenous, depending on their susceptibility to collagenase or trypsin degradation. The non-collagenous reticulins, found in ovarian stroma, are resistant to collagenase attack but are hydrolysed by trypsin. The collagenous reticulins, found mainly in the basement membrane and in young connective tissues are degraded by collagenase. As mentioned in Section 2.2.3, this author feels that the reticular fibers are probably collagen fibers associated with variable amounts of ground substances. When the collagen fibers are intimately associated with these substances, (as in the ovarian stroma), they are resistant to collagenase attack (see Chapter 4), and when they are loosely associated with the ground substances (as in the young tissues and basement membrane) they are susceptible to collagenase attack.

2.4.4. Degradation of Protein Polysaccharides. Due to the chemical complexity of the protein polysaccharides, a variety of enzymes are theoretically capable of degrading it. These are shown diagrammatically in Fig. 2.8. Proteases (A) can degrade the protein core into peptide

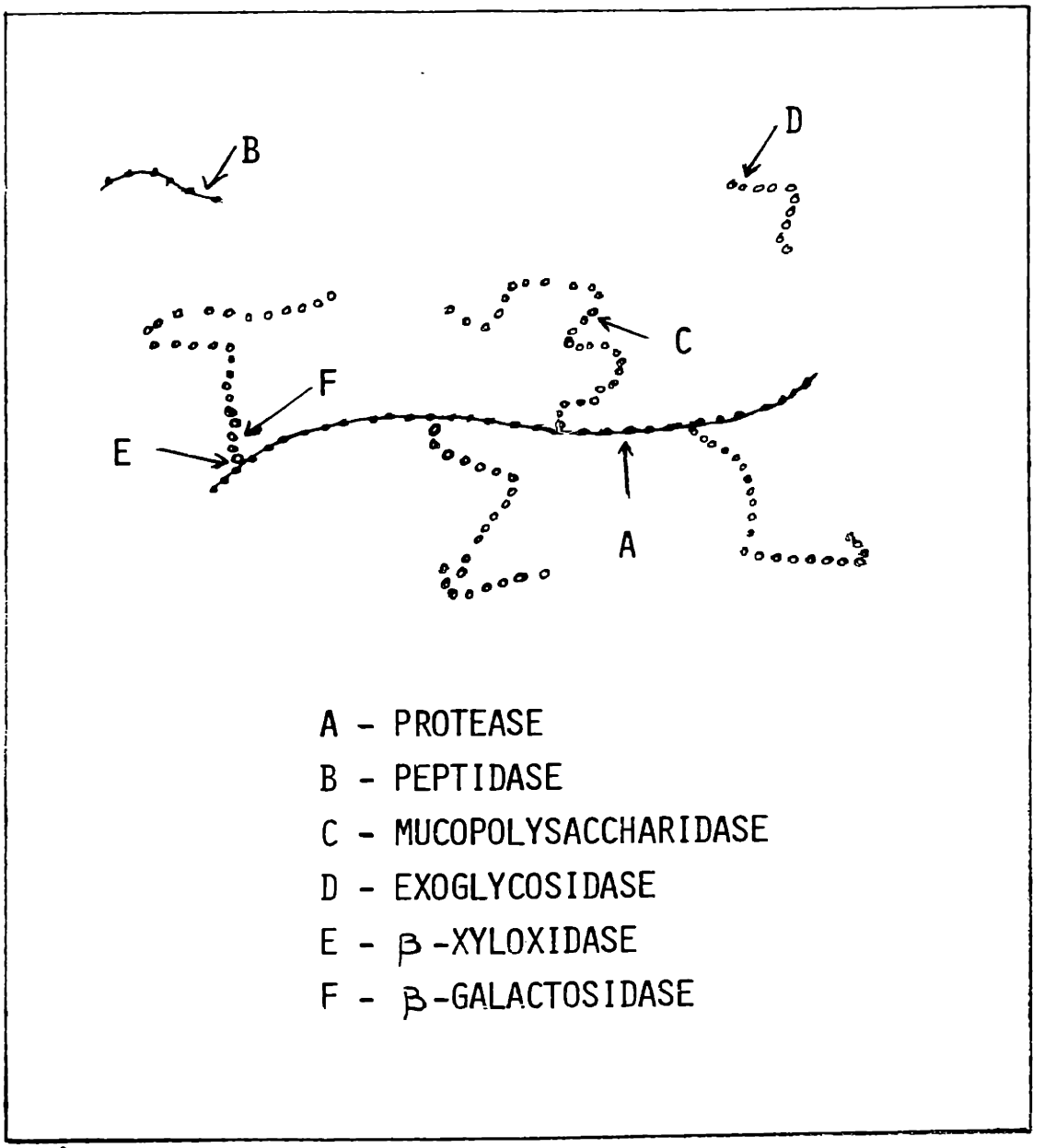


Fig. 2.8. Illustration of the probable enzymes that can attack a protein-polysaccharide molecule. The points of attack by the enzymes are indicated.

fragments with varying number of polysaccharide chains still attached to the core. Peptidases (B) can further degrade the peptide fragments into amino acids. Mucopolysaccharidases (C), which are endoglycosidases, can cause random splitting of the mucopolysaccharide chains producing oligosaccharides. These oligosaccharides can be further degraded by exoglycosidases (D) into the monosaccharides. The carbohydrate-protein linkage of the protein polysaccharide can conceivably be "unlinked" by β-xylosidases and β-galactosidases. To date, all of these enzymes have been isolated but the β-xylosidases (Patel and Tappel, 1969) and the β-galactosidases (Vaes and Jacques, 1965) obtained are only capable of acting as exoglycosidases i.e., they attack only a terminal non-reducing sugar and, hence, will not act while the polysaccharide chains are still in a polymerised form.

The mucopolysaccharidases (MPS-dases) are the most well characterized of the enzymes depicted in Fig. 2.8. These can be basically divided into two classes, according to their source. MPS-dases of bacterial origin were the earliest to be isolated and have been used to great advantage to elucidate the chemical structure of the MPS outlined in Section 2.2.4. However, they are unlikely to play a significant role in the *in vivo* degradation of the connective tissue. MPS-dases of mammalian source are increasingly being isolated in the past decade and their role in the degradation of the MPS of the connective tissue are beginning to be realized.

Bacterial MPS-dases for the degradation of hyaluronic acid, chondroitin 6- and 4-sulfate (Linker *et al.*, 1960); heparin and heparan sulfate (Silbert, 1966); dermatan sulfate (Hoffman *et al.*, 1960) and keratan sulfate (Rosen *et al.*, 1960) have been isolated and used for

the structural studies of the individual mucopolysaccharides. As their role in the *in vivo* degradation of the connective tissue is doubtful and they have been reviewed thoroughly elsewhere (Meyer, 1971) they will not be described here.

Of the mammalian MPS-dases isolated, the testicular hyaluronidase is the most well characterized. The enzyme has been found to be an endo-β- N-acetylhexosamidase, attacking β-linked N-acetylglucosamine groups at random within the hyaluronate chain to produce mainly the tetrasaccharide (Rapport *et al.*, 1951). It attacks hyaluronic acid, chondroitin 4- and 6- sulfates and, to a limited extent, dermatan sulfate. The latter case is due to the presence of glucuronic acid which has been shown (Fransson, 1970) to replace occasionally the iduronic acid usually found in dermatan sulfate. It has no effect on heparin, heparan sulfate or keratan sulfate. The enzyme has an optimum activity at the acid pH of 4.5 and can be inhibited by various bivalent and trivalent metals, other MPS and many other compounds (Weissman, 1955; Bernfeld *et al.*, 1961). Hyaluronidases which have similar characteristics as the testicular hyaluronidases have been isolated from rat skin (Cashman *et al.*, 1969) tadpole skin (Silbert *et al.*, 1965), rat liver (Hutterer, 1966), rat lung and kidney (Bollet *et al.*, 1963) and alveolar macrophages (Goggins *et al.*, 1968). However, the tadpole skin enzyme can degrade Ch 4-S but not Ch 6-S (Silbert *et al.*, 1965) while the liver enzyme is capable of degrading heparan sulfate (Hutterer, 1966). It is to be noted that none of the skin enzymes isolated so far are capable of degrading dermatan sulfate

which is the prime component of the MPS of skin.

The degradation of the MPS other than HA, Ch 4-S and Ch 6-S have been studied to a much lesser extent. The limited attack of the DS by testicular hyaluronidase and the attack of heparan sulfate by liver hyaluronidase have been discussed above. There is some evidence in mammalian tissues of enzymes capable of depolymerising heparin (Day et al., 1962; Green, 1963), but the mechanism for the degradation remains obscure. Apart from these scattered reports there has been little description of mammalian enzymes capable of degrading these compounds. The nature of the degradation of these compounds in vivo remains unknown.

The oligosaccharides resulting from the degradation of MPS by MPS-dases can be further attacked by β-glucuronidase (which acts on terminal glucuronic acid) and β-N-acetyl-hexosamidases (which acts on terminal N-acetylhexosamine). β-Xylosidases (Patel and Tappel, 1969) and β-galactosidases (Vaes and Jacques, 1965) have been found in mammalian tissue, suggesting that they might be involved in the degradation of the oligosaccharide linkage between protein and polysaccharide. None of these glycosidases, however, are endoglycosidases and, hence, will not be able to cleave within an intact polysaccharide or oligosaccharide. They are found in many tissues as lysosomal enzymes acting at acid pH, which would suggest that at least the later stages of the degradation of MPS occur intracellularly.

The possibility of the protein moiety of the PPS being degraded by proteases was demonstrated by Thomas (1964) who injected papain into rabbit ears. This procedure resulted in a rapid loss of

cartilage rigidity accompanied by mobilization and excretion of MPS in urine. This demonstrated that proteases can release large quantity of MPS from connective tissue in vivo. The lysosomal enzymes appear to be involved in the in vivo degradation of the protein-polysaccharides. It was shown (Ali, 1964; Barrett, 1966) that cathepsins of bone and cartilage are capable of degrading the protein moiety of PPS. Ali and Evans (1969) have identified Cathepsin D as the major component involved in the breakdown. Further degradation of the peptides resulting from breakdown have also been shown to be effected by the lysosomal enzymes (Ali, 1964; Quintarelli et al., 1968).

The overall mechanism for the breakdown of the PPS is still unknown. The acid pH requirement of all of the enzymes involved point to the lysosomes playing a major role in the breakdown. Whether the initial stages of the breakdown occur extracellularly (by the secretion of the lysosomal enzymes) or intracellularly (by phagocytosis of PPS) is unknown. The presence of large quantities of the MPS-dases in tissues which are relatively free of MPS (spleen, liver) and the absence of MPS-dases in tissues which contain substantial quantities of MPS (aorta, cornea and umbilical cords) remain a puzzling question.

2.4.5. Degradation of Glycoproteins. As the chemical structure of the connective tissue glycoproteins have hardly been elucidated (Section 2.2.5), studies on their enzymatic degradation remain rather scarce. The early studies of Harkness and Harkness (1952) and Partington and Wood (1963) indicated that removal of a non-collagenous protein from connective tissues by treatment with trypsin resulted in a

loss in mechanical strength of the tissue. Based on our present knowledge of the connective tissue matrix, the trypsin can either attack the protein moiety of the PPS or of the glycoprotein or both. It is difficult at the present moment to distinguish proteases for these two protein moieties since our knowledge of their structures is limited. There is, however, one instance where the lysosomal enzyme, Cathepsin D, was reported (Bazin and Delaunay, 1970) to be capable of degrading the protein moiety of PPS but not of the GPS. This was cited as the reason for the continuing accumulation of GPS but not of the PPS in granular tissues where there is a high level of cathepsin activity (Bazin and Delaunay, 1966).

Steven (1970) has presented evidence that treatment of connective tissues with a crude preparation of α-amylase resulted in the removal of most of its ground substances. The demonstration that the preparation did not possess proteolytic activity and was not capable of degrading mucopolysaccharides may lead one to the conclusion that it may contain an enzyme capable of cleaving the carbohydrate portion of the glycoproteins. Quintarelli and Dellovo (1970) have followed this line of reasoning and presented histological evidence to show that the α-amylase did remove the glycoproteins present in the connective tissue. They further demonstrated that the glycoproteins form a "coat" surrounding the collagen fibrils, making them resistant to collagenase attack. When this coat was removed, the collagenase attack was apparently increased. The work to be presented in Chapter 4 of this chapter also support this finding.

An enzyme which plays an important role in the degradation of blood glycoproteins is the neuraminidase which has been reviewed extensively by Gottschalk and Bhargava (1971). The enzyme is an exoglycosidase (Drzeniek and Gauhe, 1970) which attacks the α-ketosidic linkage of terminal N-acyl neuraminic acids, a class of monosaccharides commonly known as sialic acids. Since the amount of sialic acid present in connective tissue GPS is very small (usually less than 1%), it is questionable whether this enzyme will play any significant role in the in vivo degradation of the glycoproteins.

The overall mechanism for the degradation of the glycoprotein is obviously far from being understood. A set of enzymes corresponding to the ones available for the degradation of the PPS (Fig. 2.8) can conceivably exist. To date, there is little definitive evidence for the presence of any of these enzymes.

2.5. Wound Healing.

The subject of wound healing has attracted the attention of researchers for over a century. The skin has been the organ used most frequently for the study of wound healing because of its ready accessibility for observation and experiment. Much of the earlier work in this subject has been reviewed extensively by Arey (1936). The development of the powerful electron microscope and other biochemical techniques in the last two decades have contributed greatly to our understanding of the subject. Several reviews (Chen and Postlewait, 1964; Gillman, 1968; Schilling, 1968; Ross, 1970) and books (Slome,

1961; Whipple, 1963; Hartwell, 1955; Montagna and Billingham, 1964) have been written on it.

In describing the processes that occur in the healing of wounded skin, it is customary to treat separately the regeneration of the epidermal cells (Section 2.5.1) and the repair of the underlying dermal connective tissue (Section 2.5.2). While such a separation is convenient, it must be remembered that both events occur simultaneously. As the dermis is undergoing various inflammatory responses and tissue repair, the epidermal cells are actively dividing and migrating into the wound until epithelial continuity has been reestablished. The interaction between the regenerated epithelial cells and the newly formed connective tissue has only been studied to a small extent and will be discussed in Section 2.5.3.

2.5.1. Epidermal Regeneration. The migration of epithelial cells into a wound occurs within 24-48 hours of the injury (Gillman et al., 1955). The initial migration is considered to be in the form of a thin, single layer of cells (Pinkus, 1954; Ross, 1970) parallel to the surface of the skin. Odland and Ross (1968) observed that the cells appear flattened but remain attached to each other by desmosomes. The cells probably originate from the basal layer (Russell and Billingham, 1962; Ross, 1970) although some workers (Gillman and Penn, 1956; Schotte and Smith, 1959) claim that they are derived from the stratum spinosum. Hair follicles adjacent to the wound lose their normal structure and contribute to the epithelialization process and so may sweat glands and sebaceous glands and their ducts.

Depending on the extent of injury, various parts of these appendages may be truncated but the remnants can often be the source of epithelial cells. In burned skin where the whole dermis is not destroyed, hair follicles and sweat glands are said to provide multiple foci for epithelial regeneration (Gordon et al., 1946; Sevitt, 1957). However, in burns of pig skin, Bennett and Keller (1963) found that only hair follicles produced new epithelium, none apparently being forthcoming from sweat ducts. The epidermal cells that are derived from skin appendages appear to be indistinguishable from those originating from the surface epidermis (Lobitz et al., 1954).

It is important to note that the migrating epithelium does not move over the top of the tissues that constitute the wound floor; rather it passes through the tissue. According to Gillman and Penn (1956), the epidermal cells burrow underneath the blood and fibrin clot that collects between the cut edges of the wound near their surfaces, and this extra-vasated material does not, therefore, serve as a support for epidermal cells, as is stated in most textbooks of pathology. Several mechanisms have been proposed for the penetration of this sheet of moving epithelial cells through the tissue. Viram et al. (1964) concluded that in rabbit incisions, the presence of a band of PMN leucocytes that separated intact from necrotic tissue acted as a guide for migrating epithelium; the cells always moved between the polymorphs and the underlying intact dermis. Odland and Ross (1968) have provided evidence that the moving epithelial cells themselves are capable of phagocytic activity. Gibbins (1968) has also studied the phenomenon of phagocytosis by epidermal cells in regenerating

epidermis of rat skin wounds and has isolated lysosomal enzymes from the cells. Ordman and Gillman (1966) found that the migrating epithelium in human and pig skin wounds was capable of exerting a lytic influence upon fibrin clot that lay in the path of the advancing cells, but the precise nature of such a fibrinolytic enzyme remains to be elucidated. The observation by Eisen and Gross (1965) that the epithelial cells of metamorphosing tadpole tailfin are capable of producing collagenase and hyaluronidase may also be important in explaining the penetration of the epithelial cells through the connective tissue. However, Lazarus and Fullmer (1969) found only minimal collagenolytic activity in the epidermis of normal human skin, much of the activity being located at the dermis. It should be interesting to investigate the situation with wounded human skin since it may be possible that only under such circumstances will be epithelial cells be activated to produce collagenase.

Incised wounds are epithelialized within 3-5 days (Ordman and Gillman, 1966). The rate at which excised wounds are epithelialized depends on the size of the excision. Using rabbit ear chamber, van den Brenk (1956) calculated that the average rate of epithelial regeneration was 0.2 mm/day for wounds averaging just over 5 mm in radius. The pattern of repair for excised wound is illustrated in Fig. 2.9. The wound is initially covered with fluid-exudate and blood clot which serve as a temporary protective cover for the underlying tissue (Fig. 2.9a). Such a protection helps to minimize the extent of further tissue death that would result from dehydration (Hadfield, 1963; Ordman and Gillman, 1966). Epithelial cells migrate from the

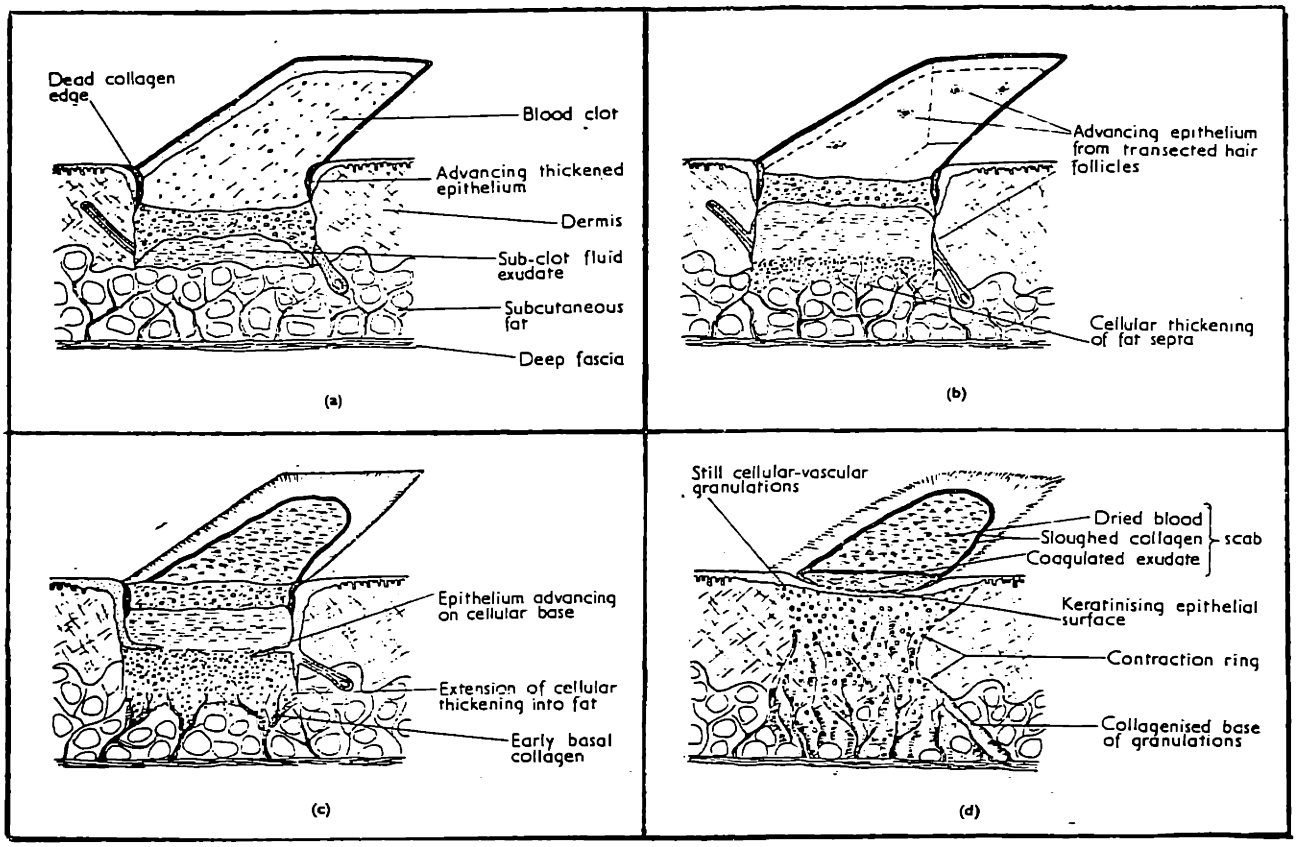


Fig. 2.9. Diagrammatic representation of the various structures injured by an excised wound and their contributions to the repair process. Note especially in (b) and (c) the new epithelium growing from injured cutaneous appendages and that these are distributed in all of the cut dermal faces comprising the collagenous "walls" of the excised wound. These diagrams also indicate the relative contributions, from various sources, of new connective tissue which comes to fill the wound "space" and the progression of collagenization and contraction of this new connective tissue. See text for detailed description. From Gillman (1968).

edges of the surrounding undamaged epidermis as well as from the truncated hair follicles (Fig. 2.9b). The base of the wound, in the meantime, forms a granulation tissue (an accumulation of loose connective tissue containing fibroblasts and other infiltrating cells). The epithelial cells penetrate this tissue just below the sub-clot fluid exudate to form a flattened sheet of advancing cells, As the granulation tissue grows, this sheet of cells together with the fluid exudate and blood clot are raised up (Fig. 2.9c). When the epithelial cells from the opposing edges of the wound meet, epidermal continuity is reestablished and the cells start redifferentiating into the various cell types present in the epidermis. The blood clot, the coagulated exudate and the sloughed collagen from the tissue all together form the scab (Fib. 2.9d) which eventually falls off. The uppermost part of the epithelium, in the meantime, is keratinized while the granulation tissue is degraded and transformed to fibrous tissues (see Section 2.5.2).

While cells are migrating into the wound from the wound margin and epidermal appendages, considerable changes occur in the epidermal cells at the surrounding sites. The cells become enlarged and the basal layer and lower layer of the stratum spinosum may become columnar (Gillman and Penn, 1956). The migrating basal cells become flattened and lose their appearance of differentiated basal cells (Odland and Ross, 1968). All these changes in shape indicate considerable modification of the factors involved in maintaining the contiguity of cells one with another. The exact nature of the changes and the

mechanism involved are unknown.

Studies with labelled thymidine injected into animals undergoing wound healing indicate that the cells at the wound margin also undergo increased mitotic activity (Bullough and Laurence, 1960; Hell, 1963; Block et al., 1963). Although earlier workers contended that mitosis does not occur in epithelial cells that are in the act of migrating, Joseph (1960) and Joseph and Towsend (1961) reported the presence of mitotic activity in migrating epithelial cells in immobile rabbit-ear skin during healing of excised areas. Bullough (1962, 1964) has presented evidence to explain the increased epidermal mitotic activity in wound tissues. He believes that normal epidermal cells secrete within themselves some substance (called "chalones") which inhibits and, hence, controls epidermal mitotic activity, when the epidermal cells are damaged, they cease to produce this inhibitor, which may also diffuse away to be lost or destroyed in the cavity of the wound. While this hypothesis may explain the increased mitosis during epidermal migration, it does not explain why the mitosis (and migration) ceases when the wound is epithelialized. The idea of "contact inhibition" has been used (Abercrombie, 1965) to explain the limitation of movement of the cells when the wound is epithelialized but how far this phenomenon, which has been observed in vitro studies of cells, apply to the epidermal healing in vivo is unknown.

2.5.2. Connective Tissue Repair. The initial response to an injury of the connective tissue is an acute inflamation. This inflammatory response consists largely of an exudate of leucocytes from the blood

vessels. During the first 24 to 48 hours, the PMN neutrophilic leucocytes are the predominant type of cell in the wound (Ross and Odland, 1968). If the wound is sterile, the cells display little to no phagocytic activity. Instead some of the neutrophils, become fragmented and their granules are released into the extracellular matrix (Ross, 1970). Since these granules are known to contain lysosomal enzymes (Hirsch, 1965), it was suggested that the enzymes play a role in the initial digestion of some of the debris in the wound so that it may be subsequently ingested and removed by the second group of inflammatory cells which appear in large numbers within 48 hours (Ross and Odland, 1968; Janoff, 1968). These are the mononuclear leucocytes which consist largely of monocytes and lymphocytes.

The monocytes display the greatest capacity for phagocytic activity within the wound. They are responsible for the removal and digestion of most of the extracellular material in the initial clot. They enter the wound between 24 and 96 hours and differentiate into the phogocytic macrophages. They then actively engage in the ingestion of fibrin, serum protein and cell debris. Evidence has been provided (Ross and Odland, 1968; Volkman and Gowans, 1965) that both the neutrophils and the monocytes are derived from the blood vessels.

The last cell types that appear in the wound are the fibroblasts and the endothelial buds which revascularize the wound site (Ross, 1968). The fibroblasts become prominent from about the third day onwards and, concomitant with their appearance, formation of the granulation tissue occurs. Some of the latter matures and remains to

effect the definitive repair, but much of it eventually disappears, together with most of the cellular invaders. In clean incised wounds that have been well sutured, the amount of granulation tissue is minimal; this constitutes "primary healing" or "healing by first intention". In excised wounds or in wounds where primary healing has been complicated by infection, "secondary healing" or "healing by second intention" occurs, and, in this, the formation of the granulation tissue is a prominent event.

The origin of the fibroblasts which appear at the wound site has attracted the attention of researchers for a long time. This has been discussed in Section 2.2.1. The role of the fibroblasts in the production of the connective tissue fibers and ground substances in the granulation tissue was also discussed in that section. The mechanism by which the growth of the tissue is initiated and by which it is halted is not known. The role of the mast cells, which have been found in increased number in the metachromatic areas of the healing wounds (Asboe-Hansen, 1957) remains obscure.

The growing granulation tissue has been described (Gillman, 1968) to undergo the following changes: 1) a progressive increase in new (extracellular) tropocollagen, reticular and finally collagen fibers; 2) closer packing into larger bundles, of the originally fine collagen and reticular fibers; 3) alterations in inter-fibrillar crosslinking associated with the diminution in inter-fibrillar ground substance, these processes, together ultimately leading to alterations in the architecture, staining reactions and solubility of the new collagen as it increases in amount and then matures; 4) a progressive

decrease in the size of the nuclei and cytoplasmic mass of the originally actively secreting fibroblasts which are then said to become tenuous, spindle-shaped cells compressed between the now thick, wavy, interlacing bundles of new collagen fibers; 5) a diminution of previously prominent and numerous new vessels. All these changes together slowly produce avascular, relatively "inert" scar tissue which binds together the sides of an incision, or by contraction, draws together (in a variable way depending on the size and site of the wound) the edges of an excised wound.

The gradual disappearance of the granulation tissue, starting about 10 days after its appearance, is a subject of great interest to researchers concerned with the enzymatic degradation of the connective tissue matrix (see Section 2.4). Due to the chemical complexity of the matrix, the number of enzymes theoretically required for its breakdown is quite considerable. To date, collagenase and hyaluronidase (Eisen et al., 1968), cathepsin (Bazin and Delaunay, 1970) and β-glucuronidase (Raekallio and Levonen, 1963) have been isolated from human and animal skins. The mechanism of degradation of these enzymes remain unknown. The mechanisms by which the degradation is triggered and halted is still and unsolved problem. The fate of the cells in the diminishing granulation tissue remain largely unknown although Reynolds et al. (1963) have presented evidence for cell breakdown in such a tissue.

The contraction of excised wounds, which often lead to scarring and deformity, has been studied extensively for a long time, but the exact mechanism by which it occurs remains unknown. The

regenerating epithelium growing over the wound floors apparently does not play any significant role in contraction (Billingham and Russell, 1956; Watts et al., 1958). The role of dehydration of the scab in producing contraction remain uncertain. While some workers (Billingham and Russel, 1956; Cuthbertson, 1959) believe that the drying of the scab can exert a contractile force on the edges of the wound, others (Church and Warren, 1968) question it. The role of collagen fibers present in the granular tissue has been found to be minimal (Abercrombie and James, 1957). An increasing amount of evidence, (Grillo et al., 1958; Church and Warren, 1968) have been produced to support the view that the cells within the tissue (probably, fibroblasts) are responsible for the contraction. The exact mechanism by which this is achieved and the site at which it occurs (wound center or wound periphery) are major items for further study.

The formation of scar tissues in wound healing is another subject which has been studied extensively but whose exact nature is not very well understood. Gillman (1968) distinguishes two types of healing: regeneration, the complete reconstitution of injured tissue or organ with consequent full restoration of the original architecture; and repair, the replacement of part or all of the injured tissues, with varying degrees of architectural distortion as one of the inevitable consequences. Both processes are initiated by acute (or non-proliferative) inflammation, which rarely involves local multiplication of cells but rather is characterized by the focal and usually temporary interstitial accumulation, in the injured site, of cellular

and non-cellular components, normally retained in the circulating blood (as described earlier). However, at a certain point in time and histogenesis, a separate series of events is triggered which determines whether resolution of the acute inflammation followed by regeneration, with full architectural reconstitution, will occur, or whether the more prolonged and potentially more serious scarring will occur. The latter response, termed "sub-acute" or "chronic inflammatory response" is generally marked by the deposition of new collagen in aberrant locations, resulting in such conditions as scarring, arteriosclerosis and nephrosclerosis. Chronic (or proliferative) inflammation is invariably associated with local multiplication of cells (Gillman, 1968). The mechanisms underlying the apparently sudden and critical transition from "acute" to "chronic" inflammation remain unknown. Yet is is precisely these mechanisms which determine whether the injured tissue will heal by repair and scarring or by regeneration, with full architectural reconstitution.

2.5.3. Dermal-Epidermal Relationships. During the first few days of healing the migrating epithelial cells have an exceedingly tenuous attachment to the underlying tissue and can easily be pulled off, e.g. by wound dressings or elevated by slight air pressure through a syringe needle inserted beneath them (Billingham and Reynolds, 1952). At the later stages of healing, attachment of the epidermis to the dermis, through the basement membrane, becomes secure. The mechanism by which this attachment occurs has been more recently studied.

Singer and Salpeter (1961) observed that when epidermal continuity was reestablished, the basal cells began to form an extensive rough endoplasmic reticulum, indicating active protein synthesis. At the same time a new basement membrane began to be formed adjacent to the cells. Since no connective tissue cells were seen in the vicinity when the basement membrane was formed, it was concluded that the basal cells were responsible for the formation of the basement membrane. A number of recent studies (Hay and Revel, 1963; Pierce et al., 1967; Johnson et al., 1969) have confirmed the notion that the basal cells of the epithelium are capable of producing the fibrous proteins and ground substances found in the basement membrane (see Section 2.3.1).

The studies of Gillman et al. (1955) and Gillman and Penn (1956) on incised wounds have drawn attention to the way in which actively mitotic epidermal cells can invade the underlaying connective tissue. Both at the wound margins and over the healing site, groups of cells dip down into the connective tissue forming "pseudo" rete pegs by the tenth to fifteenth day. They were called "pseudo pegs" because they ultimately disappear. The significance of these structures is unknown. However, the formation of epithelial downgrowths must imply a reciprocal acceptance of the epithelium by the connective tissue.

Contrary to former beliefs, a number of workers using various animals, have claimed that new hair follicles and sebaceous glands can be formed from new epidermis (Gillman and Penn, 1956; Mikhail, 1963; Billingham, 1958; Joseph and Townsend, 1961). The recent work of Joseph and Dyson (1966) on the replacement of tissue following full-

thickness defects in rabbit's ear showed that many new hair follicles formed over the wounded area. All stages of follicle formation were observed: from small epithelial downgrowths to follicles with sebaceous glands. Stenback et al. (1967) have also shown that new hair follicles can develop in rabbit skin wounds.

2.6. Design of Materials for Skin Replacement.

Two classes of materials can be considered for skin replacement: synthetic polymers and biopolymers. While both classes of materials have been equally used for biomedical purposes, certain advantages can be cited for the use of the biopolymers:

(1) The richer chemistry of these materials generally provides more flexibility in attempts to modify them. Such modifications as crosslinking, graft polymerisation, complexing with metals or other biopolymers, and blocking of certain undesirable chemical groups have been widely used in biopolymers for medical purposes.

(2) The intrinsic ability of certain biopolymers (such as collagen) to form fibrous materials by such simple means as precipitation often facilitate the technological processing of these materials. Structures in the form of fibers, membrane, sponge, tubings and tapes can usually be prepared with ease from these materials.

(3) The compatibility of these materials with the tissue are usually superior in terms of ability to be permeated by the tissue cells, ability to allow ingrowth of the surrounding tissue and the ability to form chemical bonding with the tissue.

(4) The susceptibility of the biopolymers to enzymatic degradation may be an advantage in certain medical uses. For example, in the use of collagen sutures for surgery, the necessity to remove the sutures after healing of the wound is eliminated.

(5) The semipermeable nature of some biopolymers may be an advantage in providing certain selective permeation of molecules of the body fluid--a function which is sometimes provided by the natural tissue.

The main disadvantages of biopolymers are their potential immunological reaction with the tissue, their susceptibility to bacterial infection during preparation, difficulties involved in their sterilization and their generally poor wet strength. The synthetic polymers have been used mainly to circumvent some or all of these difficulties. The other approach is to combine both the synthetic and biopolymers in order to derive the advantages of both materials. However, such an approach often leads to a structure which also incorporates the disadvantages of both the materials.

The approach that we have taken, in an attempt to design materials for skin replacement is (as stated in Section 2.1.5) to produce materials which have properties close to that of the connective tissue matrix. In attempting to achieve this goal, we have examined the chemical and structural characteristics of each of the component of the matrix and the functions which they perform in it (Section 2.2.). We have examined the nature and functions of the connective tissue cells (Section 2.3) in order to understand the type of cells that the

implanted materials may encounter and we have examined the possible mechanisms by which these materials can be degraded (Section 2.4). Finally, we have examined the phenomenon of wound healing (Section 2.5) in order to understand the sequence of events that occur when a wound is inflicted so that we may be able to design materials which can cope with these changes.

It appears logical that in order to produce materials which have properties close to that of the connective tissue matrix, one or more of the components of the matrix, extracted from suitable sources, should be used. The choice of the specific components of the connective tissue matrix to be used for skin replacement is governed by several considerations, namely: (1) their availability, (2) their physical properties, (3) their chemical properties and (4) their biological properties. These will be treated separately in the following sections.

2.6.1. Availability of Materials. It should be clear from Section 2.2 that of all the components of the connective tissue matrix, collagen, elastin and protein polysaccharides (and their associated MPS) are the only materials that can be extracted and purified in sufficient quantity to be used for the preparation of materials for skin replacement. Methods for extracting and purifying collagen have been studied exhaustively for the past two decades and have been recently reviewed (Chvapil et al., 1973). The preparation of both soluble and insoluble

elastin have been reviewed critically by Partridge (1970) and Perez-Tamayo and Rojkind (1973). It is still questionable as to whether elastin, which is completely free of the intimately held ground substances, can be prepared. For the extraction and purification of the individual PPS (and their associated MPS), the original literatures describing their isolation (see Section 2.2.4) and the review by Gardell (1965) may be consulted. Extraction procedures for reticulin (Highberger, 1936; Windrum et al., 1955) and the glycoproteins (Anderson and Jackson, 1972; Radhakrishnamurthy et al., 1964; Robert et al., 1970 b) have been described but they do not produce materials of sufficient quantity and purity to be of any practical use.

2.6.2. Physical Properties. The physical properties that are required of the materials for skin replacement are as follows:

(1) The material must possess good mechanical strength. This is important since the material must be able to withstand the various surgical operations (such as suturing) which may be required to secure the material to the wound site. The material must also be able to withstand various tensile, compressive, shear and abrasive stresses that are normally encountered by skin. A material with a high tensile, tear and shear strength is, therefore, desirable.

(2) The material that is in direct contact with the tissue must be sufficiently porous and have an adequately hydrophilic surface. This is important in order to promote ingrowth of tissue, capillaries and cells. The material must be porous enough to allow the diffusion of nutrients into these structures.

(3) The overall structure of the material must be such that it will be sufficiently impermeable to moisture in order to prevent a rapid loss of water from the body to the environment. This requirement is in direct conflict with (2) above and can only be resolved by using a multilayered structure, made up of a porous, permeable material at the bottom where it is in contact with the underlying tissue and a relatively impermeable material on the top where it is in contact with the environment.

The components of the connective tissue matrix which provide the major portion of the mechanical strength are the fibrous proteins. Pure elastin, however, tends to be weak and highly extensible and may not be able to provide the necessary strength. The reticular fibers, being young collagen fibers, tend to be loosely packed and scattered over the connective tissue. They are probably too weak to be of any practical use. The collagen fibers, on the other hand, have high tensile strength and relatively low extensibility. The high tensile strength is due to the ability of the collagen molecules to be oriented to form long fibers and their ability to form intermolecular crosslinks (Section 2.2.1). By artificially crosslinking the collagen fibers with chemical agents such as formaldehyde, a series of materials with varying crosslink densities and tensile strengths can be obtained. These characteristics have made collagen one of the most popular biomedical materials to be used (Chvapil et al., 1973).

Another advantage of using collagen fibers is the ease with which porous structures can be made out of them. If the fibers are

sufficiently large, the simple process of dispersing them randomly and laying them out on a mat can produce a structure with "holes" between the fibers which are large enough to allow the flow of body fluid and nutrient. The only disadvantage in using collagen, from the physical point of view, is that crosslinking often leads to a material which is brittle and lacking flexibility. The addition of a small quantity of highly charged mucopolysaccharide to the collagen has been demonstrated to remedy this problem (see Chapter 4).

The MPS by themselves are very weak materials, even when crosslinked to the maximum extent possible (Borgna, 1973). It appears unlikely that the presence of a small amount of protein in the PPS can enhance this weakness in strength of the MPS to any significant extent. This is to be expected since the primary role of the MPS and PPS in connective tissues is not to provide mechanical strength. The hydrophilic nature of these materials and their ability to act as molecular "sieves" in the tissue may, however, be put to good use when they are incorporated with collagen fibers (Chapter 4).

2.6.3. Chemical Properties. The chemical properties which are desirable in the materials for skin replacement are (1) the ability of the material to be crosslinked with ease; (2) the ability of the materials to exchange ions; and (3) the possibility of modifying the chemical groups in the material. The primary goal of chemically modifying the materials is to improve both their physical and biological properties.

Of the components of the connective tissue matrix, collagen and MPS are the two materials which appear to be most amenable to chemical modifications. The crosslinking of collagen by such agents as aldehydes, chromium salts, sulfonyl and bifunctional phosphonium halides, methylol triamines and their derivatives and carbodiimides, and by ionizing and U.V. radiations have been reviewed extensively by Chvapil et al. (1973). In addition to increasing the mechanical strength of the fibers, some of these crosslinking reactions result in blocking of some groups in the collagen, resulting in materials which are less antigenic and more biologically acceptable (Chvapil et al., 1973).

The MPS have polymer chains which are quite similar to those of the commercial polysaccharides, particularly, cellulose. Hence, the various methods that are known for crosslinking, graft polymerization, complexing with ions and chemically blocking groups of these polysaccharides (Arthur, 1970; Tesoro and Willard, 1970) can conceivably be used for the MPS. To date, only crosslinking of the MPS (Brogna, 1973) has been achieved.

The cationic nature of the collagen and the anionic nature of the MPS enable the two materials to interact very strongly. Such interactions have been extensively studied and will be reviewed in Chapter 4. It will also be shown in that Chapter that the interaction of these two materials leads to a number of improvements in both the physical and biological properties.

The lack of polar groups in the elastin molecule and the consequent hydrophobic nature of the molecule makes it difficult for chemical modifications to be made. It cannot be crosslinked by formaldehyde (Partridge, 1970) and possesses very little reactive groups which can be modified (Ayer, 1964). Finally, the glycoproteins and reticular fibers are insufficiently characterized to permit any unambiguous chemical modifications.

2.6.4. Biological Properties. The biological requirements for materials considered for skin replacement are: (1) they must be non-toxic; (2) they must be non-antigenic; (3) they must be of such chemical nature as to permit bonding (covalent or ionic) with the surrounding tissue; (4) they must not interfere with the various stages of wound healing, namely epithelialization, formation of granulation tissues, and connective tissue repair; (5) they must not be too easily degraded.

These requirements are probably the most difficult to meet compared to all the others that have been discussed. The only way to determine whether a material will satisfy these requirements is to implant them in experimental animals and to observe histologically the response of the tissue to the material. To date, only collagen has been sufficiently studied to permit an appraisal. Most clinical investigations done on the implantation of collagen in animals agree that, other than the problem of degradation, all of the requirements listed above are satisfied by collagen (Chvapil et al., 1973). The work to be reported in Section 3.4 is primarily focussed on solving

the problem of enzymatic degradation of collagen.

The immunological properties of the components of the connective tissue matrix have been studied quite extensively. Chvapil et al. (1973) have reviewed exhaustively the various literature on the antigenicity of collagen and they concluded that collagen, if freed of its impurities is non-antigenic or, at most, weakly antigenic. Elastin, on the other hand, has been found to be antigenic by Mandl et al. (1970) and the site of antigenicity has been shown to be on the polar regions of the elastin. The antigenicity of the PPS has been studied (Loewi and Muir, 1965; Pankovich and Korngold, 1967) and the antigenic sites have been located to be in the protein moieties of the PPS. However, the MPS have been shown (Loewi and Muir, 1965; Di Ferrante, 1965) to influence the antigenic characteristics of the protein portion of the PPS. Robert et al. (1970 a) compared the antigenicities of collagen, PPS and glycoproteins and concluded that the glycoproteins possess the highest degree of antigenicity. Based on these studies it appears that, of the components of the connective tissue matrix, collagen and MPS are the least antigenic.

2.6.5. Summary. Taking into consideration all the factors that have been discussed above, two materials emerge as most likely candidates for skin replacement: pure collagen and collagen-MPS composite materials. The rest of the chapters in this thesis are focussed on studying the properties of these two materials and on assessing their potential use as skin replacements.

Chapter 3

COLLAGEN IN THE SOLID PHASE

Since the collagen to be used for skin replacement must necessarily be in the solid phase in order to provide any structural integrity, a number of methods have been developed to study the properties of the collagen in this phase. These properties can be classified into four types:

(1) Ultrastructure, which describes the state of aggregation of the collagen molecules within the fiber. In this chapter, structures of the collagen fiber above that of 500 - 1000 A° diameter fibrils (described in Section 2.2.1) are studied using the scanning electron microscope.

(2) Extent of crosslinking of the collagen fiber. This can be determined by analyzing the stress-strain curve of the denatured collagen. The effect of pH of solution and crosslinking of the fiber on the denaturation temperature was studied in order to establish conditions under which denaturation of the collagen can be avoided. This is important because our studies showed that denaturation of the collagen fiber, even partially, will lead to a material which is readily degraded by collagenase (Section 3.4).

(3) Helical content of the collagen. This can be studied using x-ray diffraction, infrared spectroscopy and optical rotation studies. The question as to whether the helical nature of the tropo-

collagen is changed when going from solution to solid phase was explored using optical rotation studies.

(4) Enzymatic degradation of insoluble collagen. This can be studied by measuring the force relaxation of the collagen fiber, held at fixed extension and immersed in solutions of collagenase. This method is analogous to that used by Tobolsky et al. (1944) for the study of oxidative degradation of rubbers.

3.1. Ultrastructural Studies.

3.1.1. Introduction. Most of the ultrastructural studies on collagen have been done on the collagen fibrils which are 500 - 1000 A° in diameter. The various models for the arrangement of the collagen molecule in the fibril have been discussed in Section 2.2.1. While these studies are interesting, it must be remembered that they are often done on fibrils which either have been reconstituted from solutions of collagen molecule or have been dispersed mechanically or chemically from connective tissues. None of these situations represents the true state of aggregation of the collagen in the native fiber. It was desirable that a method be developed such that the connective tissue could be studied at a high level of resolution with minimal disturbance of the integrity of the tissue.

The rapid development of the scanning electron microscope (SEM) in the past decade appeared to offer just such a method. The SEM provides a three dimensional view of the tissue at magnifications in excess of

100,000 and with minimal sample preparations. The technique has been used recently to study the stretching of the human dermis (Finlay, 1969), the healing of wounds in rat skin (Forrester and Hunt, 1969), the aging of human skin (Millington, 1970), the enzymatic degradation of human skin (Finlay et al., 1971), the surface changes on collagen sutures implanted subcutaneously in rats (Robert et al., 1973) and the fracture of rat tail tendon collagen (Yannas and Huang, 1972).

The work to be reported in this Section focusses on the structure of the collagen fiber above the 500 - 1000 A° fibrils. Previous studies by Kuntzel (1941) using phase-contrast optical microscopy on microtomed cross section of tendon fibers reveal round filaments of about 5 - 10μ within the fiber. The nature of the method, however, did not reveal the spatial configuration of these filaments. Later, on the basis of indirect evidence, several workers concluded that the macroscopic fibers are composed of smaller units arranged helically around the macroscopic fiber axis (Verzar, 1955, 1964; Banga et al., 1956). However, Diamant (1970), on the basis of optical polarization observations, concluded that such smaller units must be crimped and arranged parallel to the fiber axis. The results to be presented here appear to resolve some of these controversies concerning the existence and spatial configuration of structural units in collagen fibers above the 1000 A° level.

3.1.2. Experimental. Rat tail tendon fibers, about 200 μ diameter, were teased out of tails of 3-month-old Sprague-Dawley rats by the method of Dumitru and Garrett (1947). These fibers are covered with a noncollagenous (Kwon et al., 1964) reticular membrane which was

removed by immersion in 0.5M NaH_2PO_4 at 23°C over a two-hour period. Fibers which had been treated over a fraction of this period with NaH_2PO_4 were examined with the SEM in order to note various characteristics of the membrane as well as to confirm its removal (Figure 3.1). Several membrane-free fibers were subsequently treated at room temperature over varying periods of time with 0.05M acetic acid and were also examined with the SEM in order to discern intermediate steps in the dissolution process (Fig. 3.2.).

Significant improvement of resolution of the SEM was observed to occur upon dehydrating the membrane-free fibers to water content of a few percent. Freshly teased fibers contain about 65 wt-% but attain a water content of about 15% when exposed to laboratory atmosphere for about 1/2 hour. Depending on factors such as the age of the animal, the solubility of these fibers often exceed 90% of specimen weight as long as the water content does not fall below 1%. Highly dehydrated specimens were prepared by heating at 105°C at a pressure of 10^{-3} mm Hg for 48 hours; at the end of such a treatment the water content drops below 1% while the solubility of both gelatin (Yannas and Tobolsky, 1967) and collagen (Sung, 1972) specimens drop to zero, indicating that these specimens have been transformed into covalently crosslinked three dimensional networks. Nevertheless, the characteristics of the collagen triple helix remain unchanged after such a severe treatment, as shown by the wide-angle x-ray pattern of the fibers and by the optical rotatory dispersion of nonbirefringent collagen films (Yannas, 1972). Finally, determination of the amino acid composition before and after

Fig. 3.1. Scanning electron micrograph of rat tail tendon fibers treated with 0.5M NaH_2PO_4 for 1/2 hr. Various stages in the removal of the reticular membrane, which normally covers the crimped fibers, are shown. Scale 200 μ.

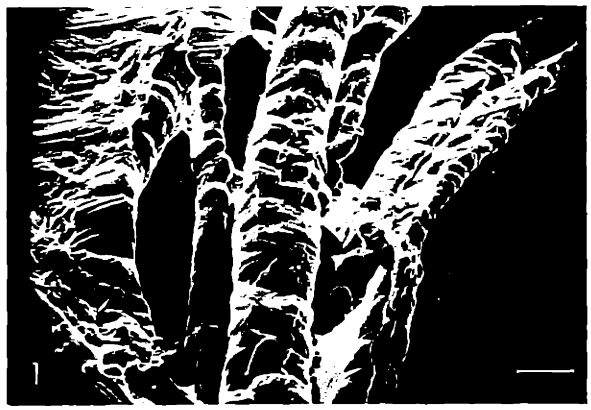
Fig. 3.2. Following removal of the membrane as in Fig. 3.1, this fiber was treated at one end with 0.05M acetic acid to illustrate this intermediate stage in the dissolution process. Scale, 100 μ.

Fig. 3.3. Fibrillar fracture of wet tendon (water content ca. 60%). Scale, 40 μ.

Fig. 3.4. Detail of specimen shown in Fig. 3.3 illustrating the nature of fracture surface obtained with a wet specimen. Scale, 4 μ.

Fig. 3.5. Fracture of an anhydrous tendon fiber (water content ca. 0.1%). Scale, 20 μ.

Fig. 3.6. Detail of fracture surface shown in Fig. 3.5 illustrating the parallel arrangement of collagen fibrils, about 0.1 μ in diameter. Scale, 2 μ.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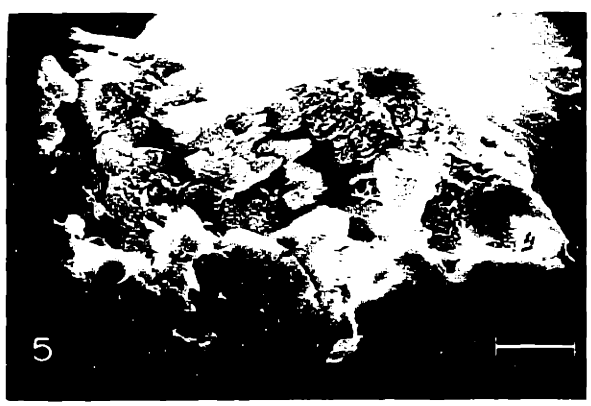
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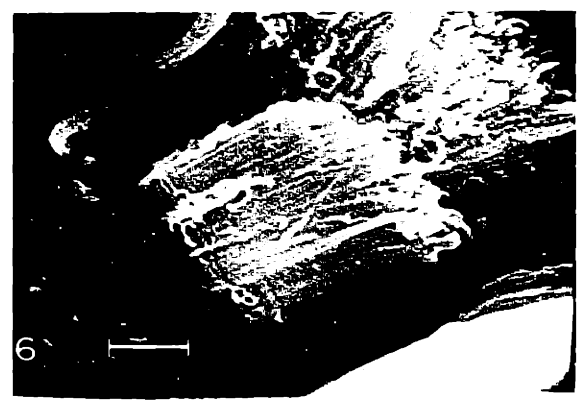
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dehydration under the conditions described above shows no change in the levels of all amino acids (Sung, 1972). These observations led to the conclusion that the sharp improvement in resolution obtained by dehydration was apparently gained at the expense only of increasing the density of crosslinks in the specimens.

Following adjustment of their water content to the desired value, tendon fibers were fractured in an Instron Tester (Instron Engineering Co., Canton, Mass.) in which the stress-strain curves at constant extension rate were also obtained for several representative specimens. Fractured specimens were examined with a Cambridge Stereoscan Electron Microscope (Cambridge Instruments Company Ltd., London) operating at an accelerating voltage of 20 kV. The moisture content of dehydrated specimens was determined by titration with the Fischer reagent, as described by Sung (1972).

3.1.3. Results. The removal of the reticular membrane from tendon fibers by 0.5M NaH_2PO_4 is illustrated in Fig. 3.1. Jordan-Lloyd and Marriott (1935) previously reported the restraining effect of the membrane on swelling of collagen fibers by various agents while Kwon et al. (1964) later described in great detail this effect as well as the decrease in shrinkage temperature of fibers which results upon removal of the apparently noncollagenous (Kwon et al. 1964) membrane. As a result of the mild treatment of the fibers in Figure 3.1, the membrane remained intact on certain fibers and on others was partly removed, exposing the collagen fiber below, while a few fibers were completely denuded. The latter appear to have an

ellipsoidal cross section and are crimped with a frequency of about 0.01 μ^{-1} (Figure 3.1).

Details of the dissolution of membrane-free tendon fiber in 0.05M acetic acid, a well-known collagen solvent (Piez, 1967) are shown in Fig. 3.2 where the fiber shown has been treated with solvent at the top end only. Prior to becoming molecularly dissolved, the collapsing fiber reveals aspects of its internal structure which is quite clearly made up of parallel filaments, averaging about 10 μ in diameter. There was no evidence in Fig. 3.2 that these filaments are helically wound around the macroscopic fiber axis as has been suggested by several investigators (Verzar, 1955, 1964; Banga et al., 1956) on the basis of evidence less direct than ours.

The conditions under which the specimens shown in Fig. 3.3 to 3.6 were prepared can be best described by reference to the stress-strain curve of wet and dehydrated tendon fibers shown in Fig. 3.7. The stress-strain curve of the wet fiber is made up of three regions. The region up to point A, where the stress rises very slowly with extension and where the deformation is immediately and completely recoverable, corresponds to the uncrimping or straightening out of the waviness of the fibers (Rigby et al., 1959). In the region A through B, the stress rises approximately linearly with strain and the deformation incurred is partly recoverable, the amount of irrecoverable deformation increasing with strain (Abrahams, 1967). This region probably corresponds to a combination of slippage and extension of the

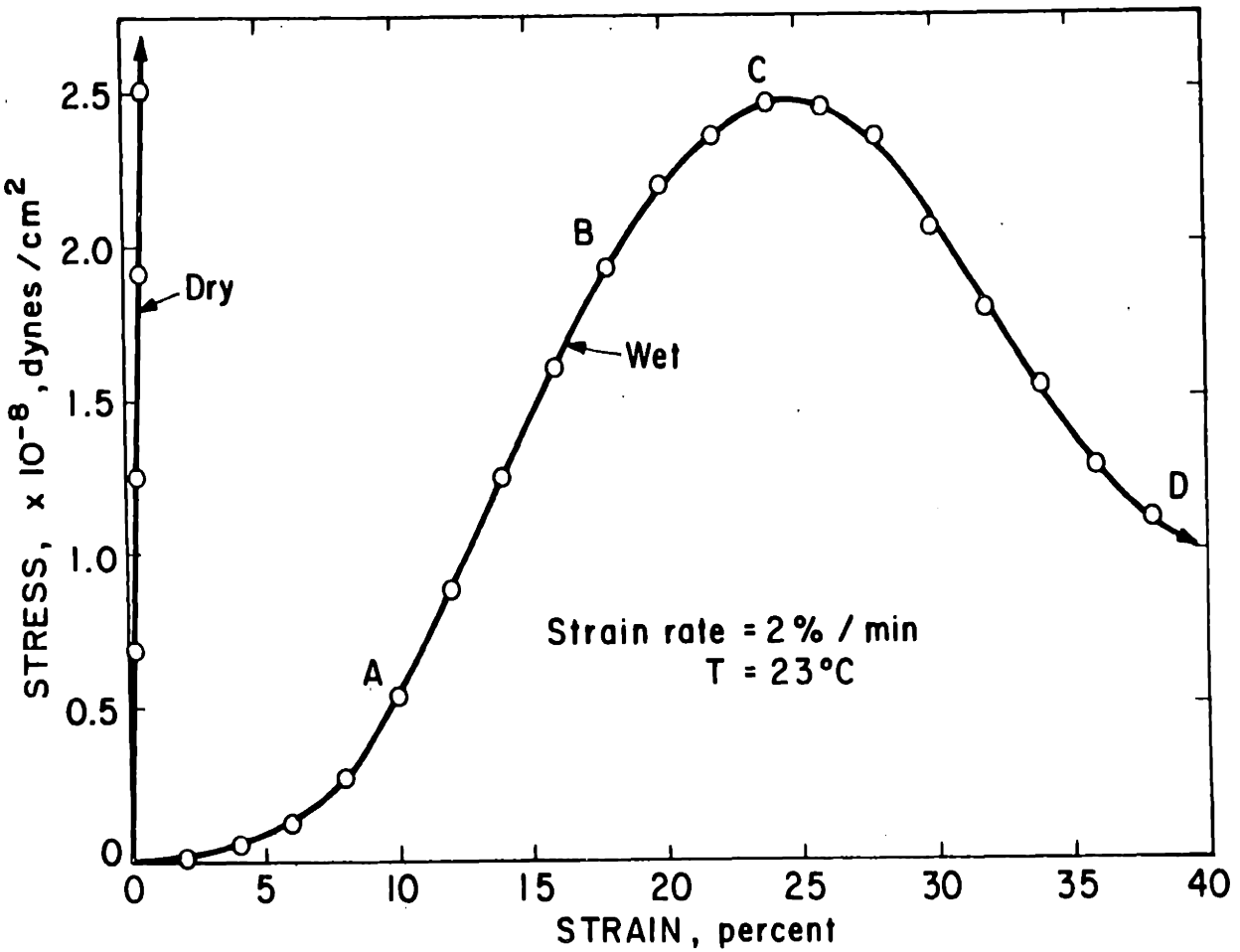


Fig. 3.7. Stress-strain curve of wet and dehydrated rat tail tendon fibers. Strain rate, 2%/min; temperature, 23°C. Fracture occurs beyond point D. The significance of the other symbols is explained in the text.

straightened fibers, the amount of slippage increasing with the strain. The occurrence of slippage in wet fibers is directly confirmed in the scanning electron photomicrographs in Figs. 3.3 and 3.4, the latter presenting a magnified view of a fracture site in the former. It is, in fact, clear that the slippage has involved primarily filamentous units about 10 μ in diameter (Fig. 3.3) and secondarily only the much smaller constituent fibrils with apparent diameters clustering around 0.1 μ (Fig. 3.4). Specimen failure, which occurs progressively beyond point C of the stress-strain curve (Fig. 3.7) appears, however, to involve both classes of constituent structures to an individual extent which cannot be clearly discerned in the photographs (Figs. 3.3 and 3.4).

While revealing the existence of apparently discrete structural units, the photographs of specimens fractured in the wet state (>60% water) were considerably less explicit than photographs of specimens fractured while in states of significantly lower hydration. With progressive removal of water, the 10-μ and 0.1 μ structural units, already evident with wet specimens (Fig. 3.3 and 3.4), were delineated with increasing sharpness until, at a moisture content of less than 1%, the internal structure of the original fiber was disclosed in the clearest manner (Figs. 3.5 and 3.6). In Figure 3.5 the ellipsoidal cross section of the tendon fiber is seen to be characterized by an axial ratio of roughly 2. The presence of the two structural units, the ca. 10-μ filament and the ca. 0.1-μ fibril, is confirmed, while it also becomes clear that the diameters of these units are distributed

rather than being monodispersed. Nevertheless, the ranges of variability of diameters are relatively narrow, being 5 - 10 μ and 0.1 - 0.2 μ, respectively, for each structural unit. Finally, there seems no doubt that both the ca. 10-μ filaments and the ca. 0.1-μ fibrils are arranged in strictly parallel configuration along the fiber axis rather than helically about it (Figs. 3.5 and 3.6).

3.1.4. Discussion. The results support directly Kuntzel's much earlier observation of filaments, 5 - 10μ in diameter, by phase-contrast optical microscopy of the microtomed cross section of rat tail tendon (Kuntzel, 1941) while refuting the model of helical arrangement of such filamentous units (Verzar, 1955, 1964; Banga et al., 1956). Furthermore, the ca. 0.1-μ diameter units clearly evident in Fig. 3.6 (and less clearly in Fig. 3.4) are most probably none other than the fibrils that have been first observed by Schmitt et al. (1942) with transmission electron microscopy and by Bolduan and Bear (1950) with small-angle x-ray diffraction. Assuming that the crimped collagen fiber extends as if it were an elastic body, Diamant (1970) estimated the diameter of the fundamental load-bearing unit for tendons from 3-month-old rats at 2100 A°, in substantial agreement with the above.

Two considerations support the view that the dehydration step introduced no artifacts which may invalidate the conclusions drawn above. First, the same structural units observed by fracturing the wet, almost completely soluble and, by all appearances, native specimen (Figs. 3.3 and 3.4) were observed also at all subsequent

dehydration levels; apparently, the cross-linking induced by dehydration prevented slippage of filamentous units thereby changing the mode of fracture (Figs. 3.5 and 3.6) without changing either the number of discrete and repeating structural units or their average diameter. Second, the two cylindrical units revealed by fractography have been previously observed independently by entirely different methodology: the ca. 10-μ unit by Kuntzel (1941) and the ca. 0.1-μ by Schmitt et al. (1942) and by Bolduan and Bear (1950) as described immediately above. Dehydration of the specimens did not, therefore, prevent observation of these two, previously observed, structural units of collagen. These considerations suggest the clear absence of discrete structural units either inside the range bounded by the ca. 0.1-μ and the 10-μ units or the range bounded by the 10-μ and the 200-μ units (Figs. 3.5 and 3.6) is a characteristic of native tendon rather than an artifact produced by dehydration.

Fig. 3.8 sums up our current understanding of structural levels in tendon fibers. The diagram amounts to a graphical tabulation of mean diameters of all repeating cylindrical units that compose the tail tendon fibers of a 3-month-old rat. This diagram is proposed as a supplement to the useful pictorial representation of the levels of order in collagen fibers presented by Bear (1952). The 15-A° unit shown in Fig. 3.8 is the well-known tropocollagen molecule described in Section 2.2.1. The tetramer unit of Veis et al. (1967) and the pentamer unit of Smith (1968) which correspond to structural units of 40-60 A° diameter have been discussed in

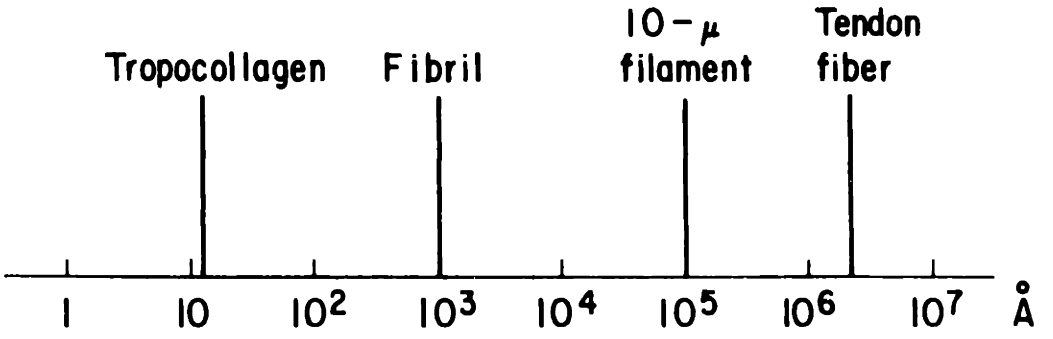


Fig. 3.8. Mean diameters of repeating structural units in rat tail tendon are indicated on this logarithmic scale. The present study covers the diameter range from ca. 10^3 A° (0.1μ) to ca. 2×10^6 A° (200μ).

Section 2.2.1 but have not been included in Fig. 3.8 because of their predominantly speculative nature. Earlier attempts to discover any possible structures between the level of the tropocollagen molecule and that of the native fibers (ca. 200 μ diameter) have been made by transmission electron microscopy of fibers which have been partially disintegrated by mechanical agitation or separated into smaller components by suitable solvents (Vanamee and Porter, 1951; Schwarz and Pahle, 1953; Wasserman, 1954). These studies have led to postulations of existence of various structural units between 50 A° and 2000 A°, but their usefulness in elucidating structural hierarchies is questionable in view of the fact that the sizes of the observed structures seem to depend on the extent of mechanical agitation and on the nature of the solvent used.

No doubt, however, exists about the discreteness of the ca. 0.1-μ fibril (Schmitt et al., 1942, Bolduan and Bear, 1956) which is depicted in Fig. 3.8. The work of Kuntzel (1941), supported directly by the present independent observations (Figs. 3.2 to 3.6), also points to the existence of a ca. 10-μ unit, also shown in Fig. 3.8 before reaching the level of the 200-μ tendon fiber. The present work leads us to the conclusion that the ca. 10-μ filamentous unit is the only discrete structural element in the range between the 0.1-μ fibril and the macroscopic tendon fiber which, therefore, appears to possess distinct structural discontinuities at several levels. The usefulness of the SEM for studying the ultrastructure of connective tissues is clearly demonstrated in the present study.

3.2. Denaturation and Crosslinking

3.2.1. Introduction. Two factors which influence strongly the rate at which collagen is enzymatically degraded by collagenase are the helical content of the collagen and the extent of its crosslinking (Section 3.4). Hence it is important to determine quantitatively these two parameters for collagen existing in the solid phase and also to determine the various conditions under which denaturation can be avoided. The latter conditions are particularly important when attempts are made to interact the insoluble collagen with mucopolysaccharides by varying such conditions as pH and temperature of the solution in which the collagen fibers are immersed (see Chapter 4). Careful monitoring of the conditions must be exercised in order to avoid a partially denatured collagen-MPS composite, which can be readily degraded by collagenase. The present section will describe the methods by which the extent of crosslinking in solid phase collagen can be determined and the conditions under which denaturation will occur. The next section (Section 3.3) will cover the various methods for determining the helical content of collagen.

3.2.2. Theory. The conversion of a crosslinked native collagen fiber to a denatured polymer network is depicted in Fig. 3.9. This conversion can be effected most easily by heating the native collagen fiber when immersed in a suitable fluid medium. When denaturation occurs, at a temperature called the shrinkage temperature, the fiber shrinks to about a quarter of its original length. At the same time, the denatured polymer network becomes highly swollen and acquires a high extensibility,

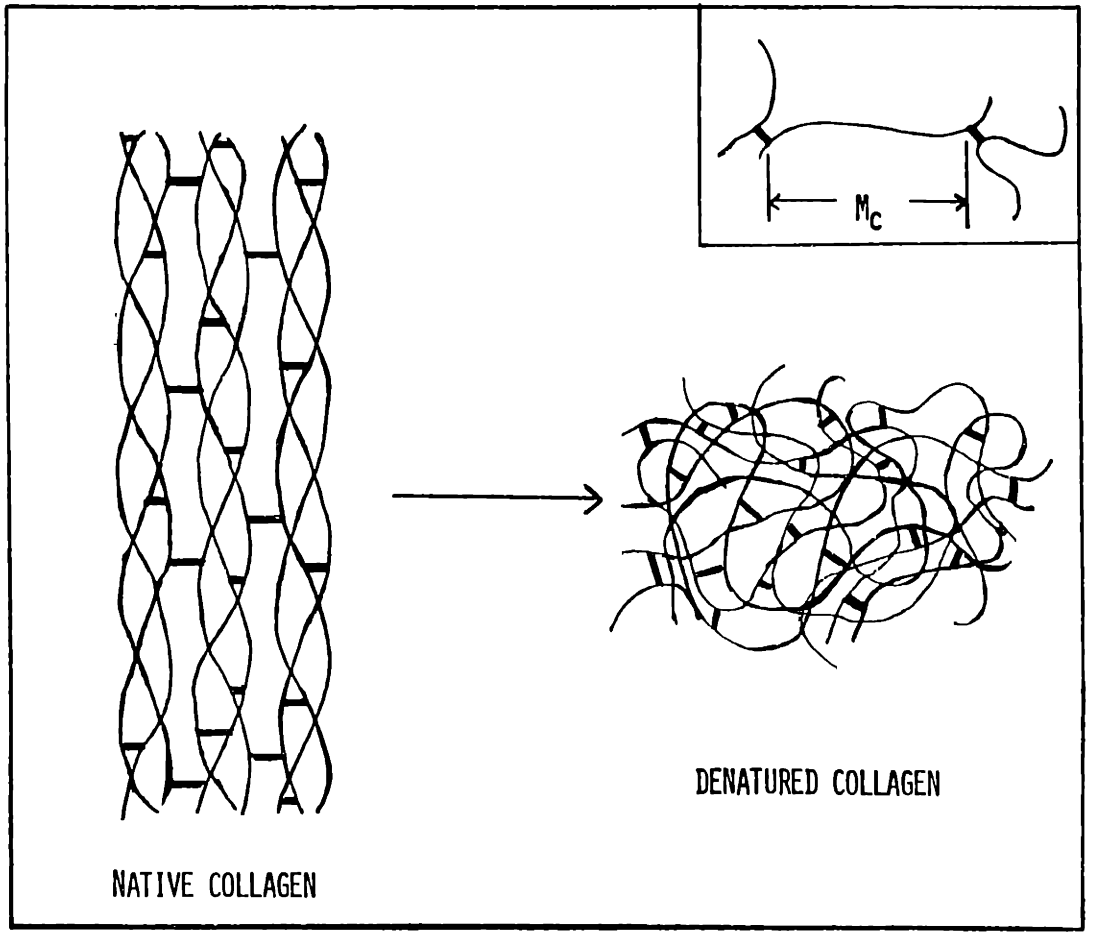


Fig. 3.9. Illustration of denaturation of a crosslinked collagen fiber. The meaning of the number avearge molecular weight of the chain (M_c) is illustrated in the inset.

characteristic of rubber-like materials (Wright and Wiederhorn, 1951).

The stress-strain behaviour of thermally denatured collagen was investigated by Wiederhorn and Reardon (1952) who concluded that under appropriate conditions of temperature, strain level and nature of solvents, the denatured collagen behaves like an ideal rubber. The concept of an ideal rubber has been treated excellently by Treloar (1958) and will only be reviewed briefly here. By thermodynamic considerations, the tensile stress required to deform a solid which does not flow may be given as a sum of two terms:

$$\sigma = \left(\frac{\partial E}{\partial l}\right)_{T,V} - T\left(\frac{\partial S}{\partial l}\right)_{T,V} \qquad (3.1)$$

where σ is the retractive force per unit area (i.e. stress), $(\partial E/\partial l)_{T,V}$ is the change of internal energy with length at constant temperature and volume, $(\partial S/\partial l)_{T,V}$ is the change of entropy with length at constant temperature and volume and T is the absolute temperature. An ideal rubber is one in which the energy term is negligibly small so that:

$$\sigma = - T\left(\frac{\partial S}{\partial l}\right)_{T,V} \qquad (3.2)$$

The evaluation of the entropy change with length is based on models of the polymer network in which the polymer chains are pictured to terminate at both ends in a junction with other chains (Fig. 3.9). The polymer chains are considered to be made up of molecular bonds that are freely rotating. For such a material, swollen in a fluid medium:

$$\sigma = -T\left(\frac{\partial S}{\partial l}\right)_{T,V} = \rho\frac{RT}{M_c} V_2^{1/3}(\alpha - 1/\alpha^2) \qquad (3.3)$$

where

σ = force per unit area of unstrained, swollen polymer.

α = the extension ratio (i.e. strained length/original length).

V_2 = volume fraction of the polymer (i.e. volume of dry sample/volume of wet sample).

ρ = density of the dry polymer.

T = absolute temperature.

R = gas constant.

M = number average molecular weight of the chain between junction points (see inset in Fig. 3.9).

The validity of Equation 3.3 has been verified for collagen that has been crosslinked by formaldehyde (Wiederhorn et al., 1953), by a variety of aldehydes and difunctional compounds (Cater, 1963) and by irradiation (Bailey et al., 1964) and was subsequently denatured. Most of the stress-strain measurements were done at high temperatures (60-85°C), the reason being that the denatured collagen does not obey Equation 3.3 at lower temperature. This is to be expected, since denatured collagen fibers are known to renature in solutions which are kept below their shrinkage temperature (Huang, 1971). Renaturation is generally associated with intermolecular interactions which will give rise to an increase in internal energy - a situation which is not allowed if Eq. (3.3) is to be obeyed.

samples using the method just described is a problem because of the technical difficulties involved in determining accurately the stress-strain curves of small samples. An alternative method was therefore developed to eliminate the necessity to obtain the stress-strain curves of the samples. This method is based on a theory developed by Flory and Rehner (1943) who showed that when a polymer is swollen to equilibrium by a solvent, the amount of swelling (as measured by the volume fraction of polymer, V_2) is dependent on the extent of crosslinking of the polymer (as measured by M_c) through the relationship:

$$\ln(1-v_2) + v_2 + \chi v_2^2 + (\rho V_1/M_c)(v_2^{1/3} - v_2/2) = 0 \qquad (3.4)$$

where

V_1 = the molar volume of the solvent.

χ = polymer-solvent interaction parameter.

ρ = density of the dry polymer.

The polymer-solvent interaction parameter, χ is characteristic of a specific polymer-solvent combination at a particular temperature. A number of methods are available for determining χ (see Flory, 1953) but in the present investigation, it is obtained by combining the study of the stress-strain behaviour of the denatured collagen fiber immersed in a fluid medium and the measurement of its extent of swelling in the same medium. From the stress-strain behaviour, a value of M_c for the collagen can be obtained through Equation 3.3. Using this value of M_c and the volume fraction of polymer, v_2, obtained from the swelling

measurement, χ can be obtained through Equation 3.4 if the molar volume of the solvent (V_1) and the density of the dry polymer (ρ) are known. Once the value of χ is known for the denatured collagen-solvent combination, the value of M_c for an unknown collagen sample can be easily determined through Equation 3.4 by measuring the volume fraction V_2 of polymer when it is swollen to equilibrium in the solvent. Such a correlation has been found for the denatured collagen used in this investigation and will be described below.

3.2.3. Experimental. The collagen fiber used in this investigation was prepared from bovine tendon by the method of Oneson et al. (1970) and was generously donated to us by R. K. Kronenthal, Ethicon, Inc., Somerville, New Jersey. The fiber is in the form of a tape of thickness ca. 0.0032 cm. and width ca. 0.13 cm. The total amount of impurities (protein polysaccharides, glycoproteins, various tissue and serum proteins) in the tape was found to be less than 0.2%-wt. (Oneson et al., 1970). The collagen fibers show no evidence of degradative change when examined with the electron microscope (Oneson et al., 1970) or by wide-angle x-ray diffraction and infrared spectroscopy in our laboratory (see Section 3.3). The fibers have been crosslinked by irradiation to give a M_c value of 28,900, as measured by the method to be described below.

Further crosslinking of the collagen tapes was achieved by immersing them in solutions of aldehydes for a variable time period. The aldehydes used were formaldehyde (Merck Chemical Co., N. J.), glyoxal (Eastman Kodak Co., N.Y.), and glutaraldehyde (J. T. Baker Chemical Co.,

N.J.). The concentration of each aldehyde was fixed at 0.025 moles and the buffer solution used for diluting the aldehydes was 0.19M citric acid-phosphate buffer, pH = 7.4. The temperature used for the crosslinking was 23°C. For each aldehyde, collagen tapes were immersed for 1, 2, 5, 18 and 36 hours. After immersion in the aldehydes, the collagen tapes were removed and rinsed thrice with the citric-phosphate buffer solution before being placed into the same buffer solution containing 0.2% of 1,3-cyclohexanedione-5,5-dimethyl (dimedone: Eastman Kodak Co., N.Y.), for 24 hours at 23°C with gentle stirring. Dimedone is a reagent which removes unreacted aldehydes from the collagen (MacFayden, 1945). After immersion in dimedone, the tapes were rinsed five times with distilled water and stored in citric phosphate buffer solution pH 7.4 at 4°C until they were required either for shrinkage experiments or for the measurement of crosslink density.

The shrinkage experiments were peformed in citric-phosphate buffer solutions with pH ranging from 1.2 to 7.4. The experiment consists of suspending the fiber in a test tube containing the buffer solution with a small load (ca. 0.5 gm) hanging on the free end of the fiber. The test tube containing the buffer solution and the suspended fiber was placed in a constant temperature bath where the temperature was increased incrementally. The length of the fiber was measured with a cathetometer for each increment of temperature. Measurement of the length was halted when the fiber had shrunken to an equilibrium length. By using a number of test tubes containing buffer solutions of varying pH, and measuring successively the lengths of fibers immersed in each of them as the

temperature of the buffer solutions are increased incrementally, it is possible to find the effect of pH on the shrinkage of collagen fibers which are all crosslinked to the same extent (Figs. 3.11 and 3.12). Furthermore, by fixing the pH of the buffer solution, collagen fibers of varying crosslink densities can be suspended and the procedure described above repeated to obtain the effect of crosslink density of the collagen on the shrinkage temperature (Fig. 3.13).

The crosslink density of the collagen was determined by first denaturing the collagen fiber in a citric acid-phosphate buffer solution, pH 7.4 kept at 95°C. The immersion time varies from 1 to 2 minutes, depending on the extent of crosslinking of the fiber (the longer time for the more crosslinked fiber). The fiber was then transferred to the critic acid-phosphate buffer solution, pH 7.4, kept at 80°C and allowed to equilibrate to that temperature for ca. 10 minutes. The stress-strain curve of the fiber was then obtained in the same buffer solution (kept at 80°C) using the apparatus shown in Fig. 3.10. The top end of the fiber was attached to the load cell of the Instron Tester (Instron Engineering Corporation, Canton, Massachusetts) whose output in grams was calibrated before every measurement and was displayed on a Perkin-Elmer strip chart recorder Model 165. The lower end was attached to the crosshead of the Instron through the clamping system shown in Fig. 3.10. The strain on the fiber is obtained by measuring its initial unstrained length, l_o with a cathetometer and recording the incremental elongation on the fiber, Δl by the distance moved by the Instron crosshead. The extension ratio, α in Eq. 3.3 is then given by $(l_0 + \Delta l)/l_0$. By stretching the fiber to a

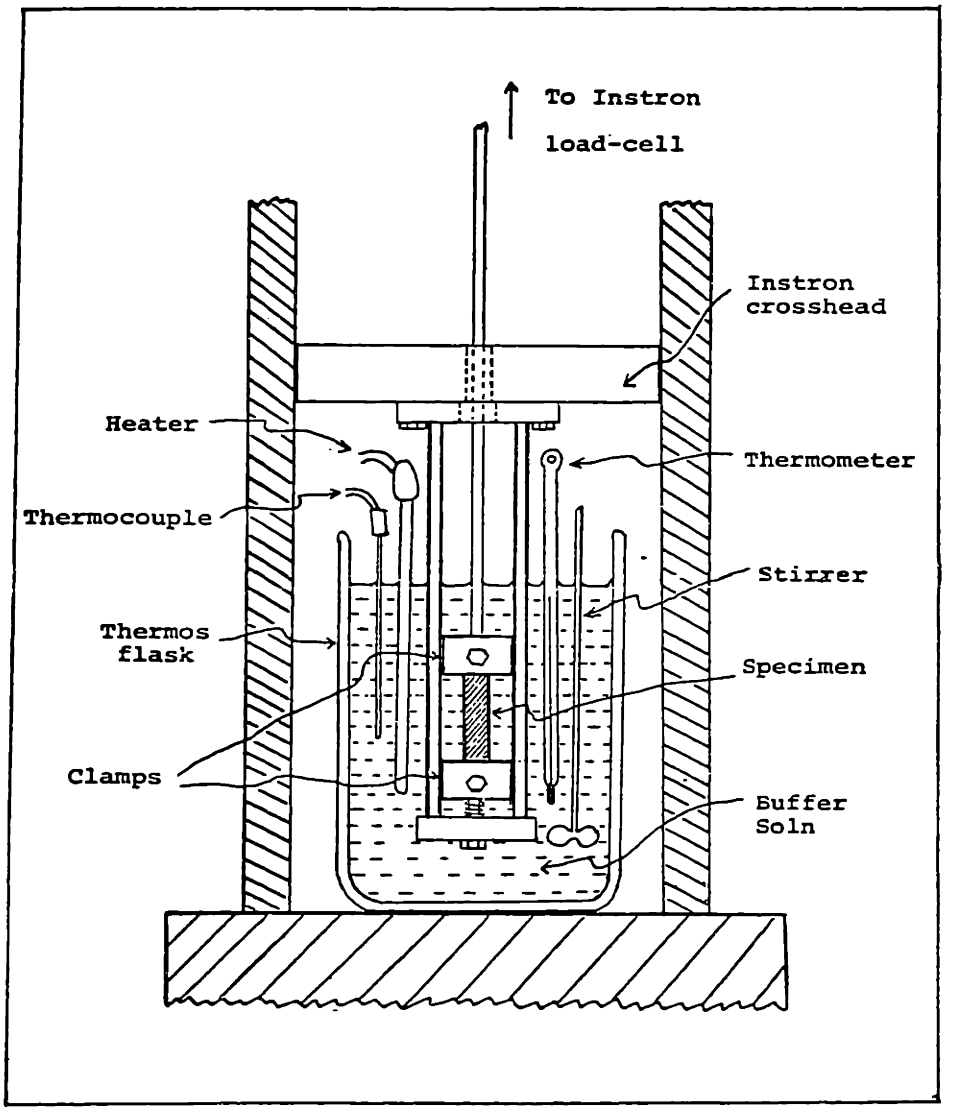


Fig. 3.10. Apparatus for the determination of stress-strain behaviour of a material.

series of strain levels and registering the equilibrium stress obtained at each level, the stress-strain curve of the fiber can be obtained, both on extension and retraction (Fig. 3.14).

After the determination of the stress-strain curve, the fiber was cut at both ends where it was clamped and the wet weight of the fiber, after blotting lightly with a filter paper to get rid of excess liquid, was determined. The fiber was then dehydrated at 105°C at a pressure of 10^{-3} mm Hg for 48 hours and its dry weight was obtained. The volume fraction, V_2, of collagen can be computed from:

$$V_2 = \frac{W_o \phi}{W - W_o (\rho - \phi)} \tag{3.5}$$

Where:

W_o = weight of dry sample.

W = weight of wet sample.

ρ = density of dry collagen.

ϕ = density of swelling medium.

The cross sectional area, A, of the unstrained swollen collagen sample can be obtained from the relationship:

$$A = \frac{W}{\phi l_0 + V_2 l_0 (\rho - \phi)} \tag{3-6}$$

where l_0 is the unstrained length of the collagen fiber. The stress on the specimen, σ in Eq. 3.3 is given by f/A where f is the load registered on the Instron recorder. The density of dry collagen was

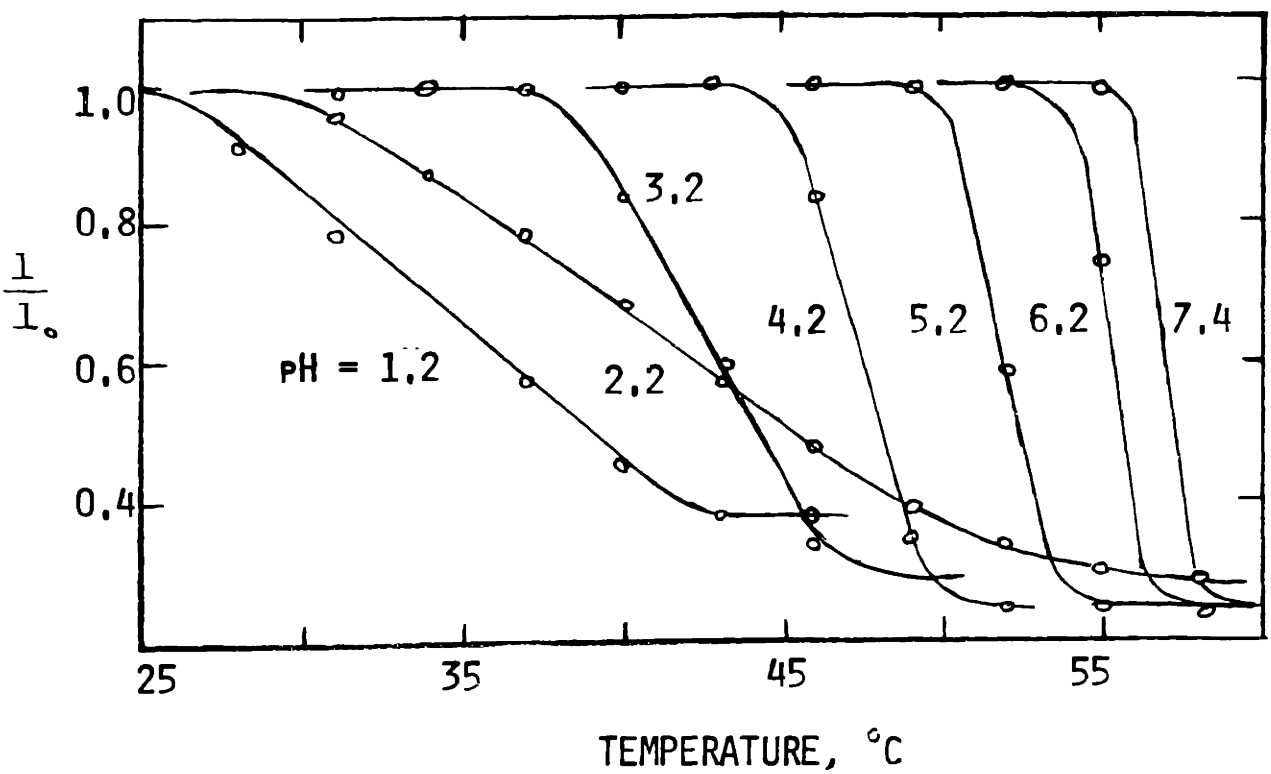


Fig. 3.11. Shrinkage of crosslinked collagen fibers. Length-temperature curves of crosslinked fibers immersed in 0.19M citric acid-phosphate buffer solutions at the indicated pH.

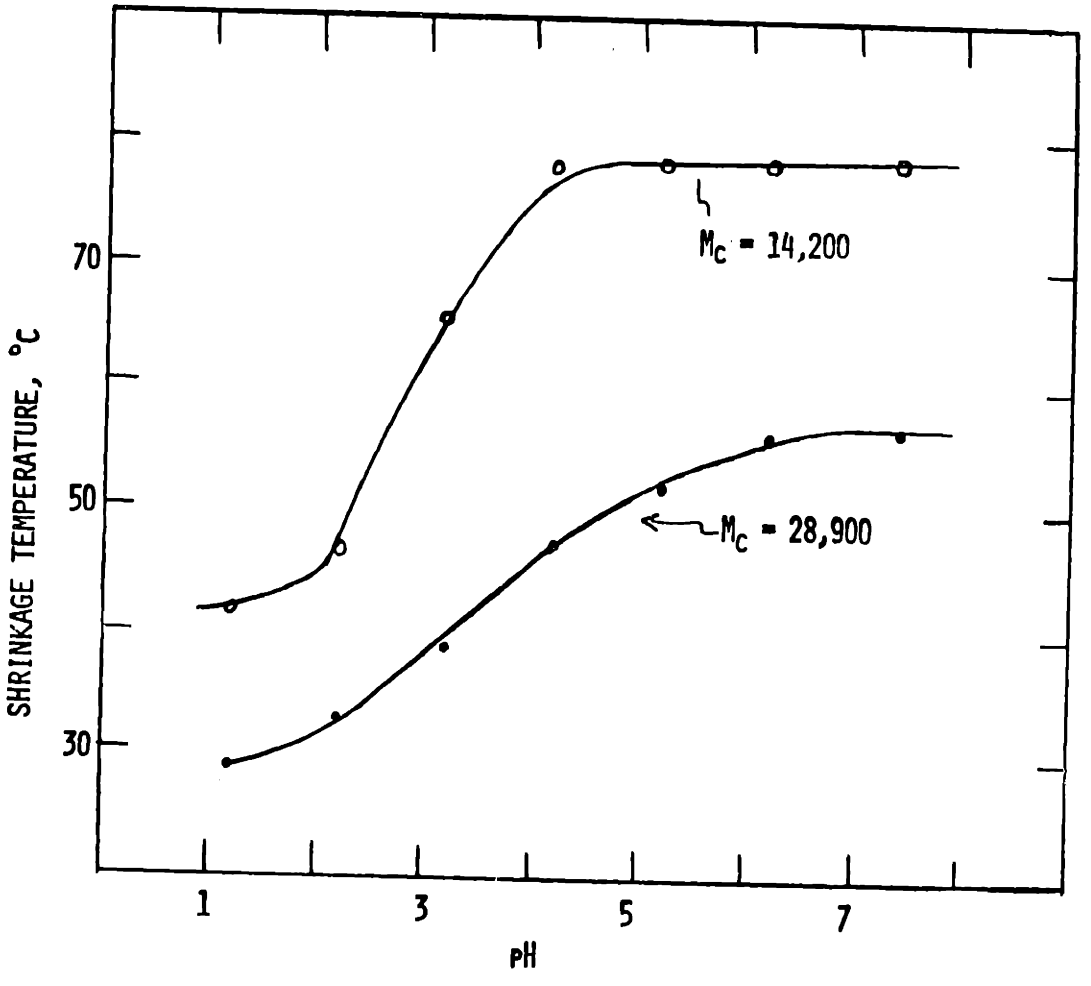


Fig. 3.12. Variation of shrinkage temperature with pH for collagen fibers, crosslinked to different extent. See Fig. 3.9 and text for the definition of M_c.

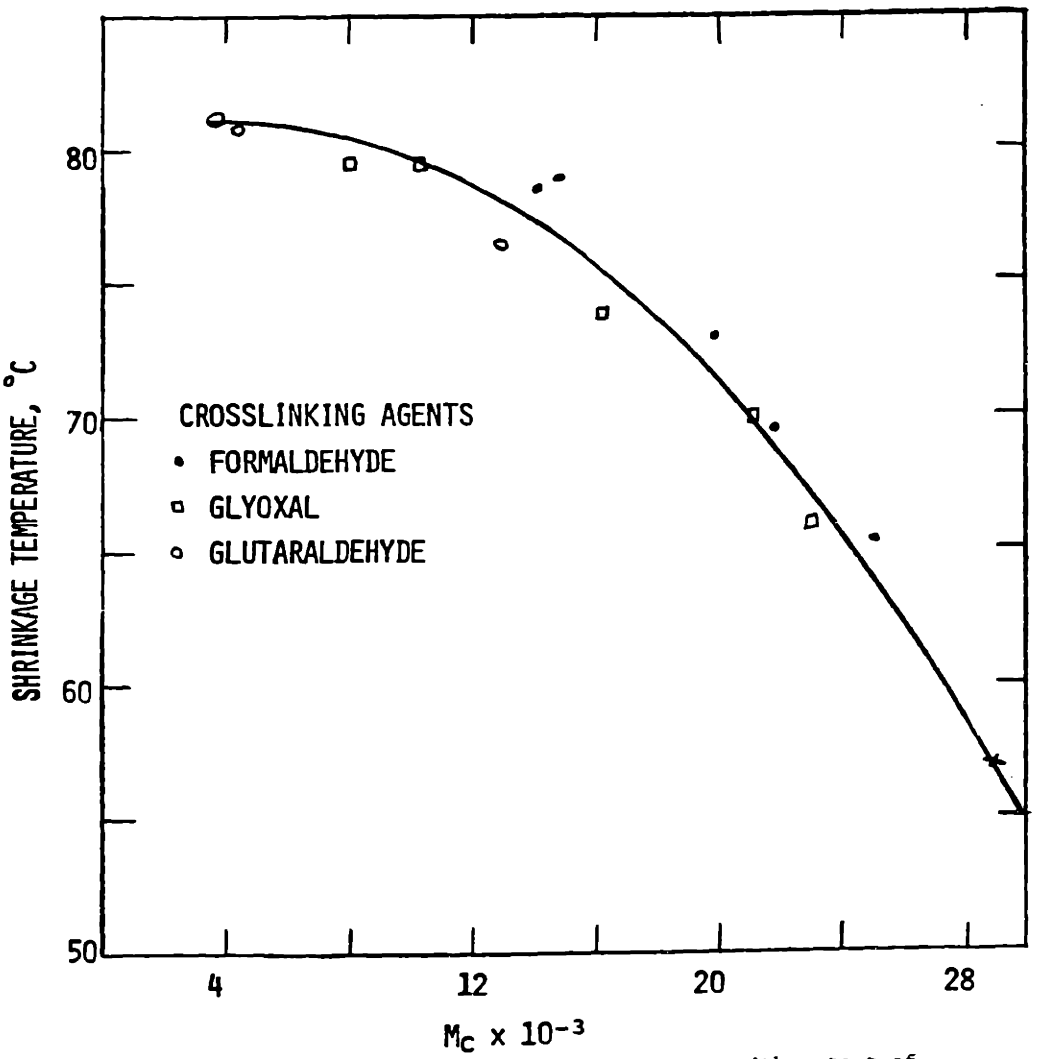


Fig. 3.13. Variation of shrinkage temperature with extent of crosslinking of collagen fibers. The various extent of crosslinking of the fibers were obtained by using different crosslinking agents, as indicated, and using various duration for crosslinking (see Fig. 3.15). The concentration of crosslinking agent used was 0.025moles in all cases and the crosslinking was conducted in a 0.19M citric acid-phosphate buffer, pH 7.4, at 23°C.

taken to be 1.3 gm/cc (Astbury, 1940) while the molar volume of the citric acid-phosphate buffer solution was taken to be similar to that of water 18 cc/mole) because it was dilute.

3.2.4. Results and Discussion. The shrinkage of the collagen tapes, shown in Fig. 3.11, is abrupt for pH > 3.2. At low pHs, the shrinkage is gradual as the temperature is increased beyond the shrinkage temperature. Similar results have been obtained by Ciferri et al. (1965) who studied the shrinkage temperature of quinone-crosslinked collagen tendons and concluded that the gradual shrinkage behaviour of the tendons at low pH is due to the high stresses resulting from the increased swelling at these pH levels. When the ionic strength of the solution was increased by the addition of salts, the high swelling at low pH was suppressed and consequently, the shrinkage of the collagen tendons became abrupt.

The effect of pH and crosslinking on the shrinkage temperature are shown in Figs. 3.12 and 3.13. The results show that the collagen helix is stabilized both by increasing the crosslink density of the collagen (i.e. decreasing the M_c) and increasing the pH (up to 7.4) of the solution in which the collagen is immersed. The effect of pH on the shrinkage temperature of the collagen can probably be explained by the polar nature of the collagen molecule (see Section 2.2.1). At low pH, the acidic amino acids of collagen (glutamic and aspartic acids) are ionized and interact strongly with the ions of the solution. As a result, a low thermal energy is sufficient to disrupt the collagen helix. Above pH 4 to 5(depending on the value of M_c) the shrinkage

temperature of the collagen reaches a maximum plateau. This coincides with the isoelectric point (i.e. no net charge) of collagen, as determined electrophoretically (Anderson and Eriksson, 1968) and mechanochemically (Yannas and Grodzinsky, 1973). The work of Ciferri et al. (1965) has shown that about pH 10, the shrinkage temperature decreases sharply because of the ionization of the basic amino acids of the collagen.

The decreased stability of collagen at low pH (Figs. 3.10 and 3.11) may be important in the intracellular degradation of the molecule under physiological conditions. The combined effect of low pH (ca. 4.5) and physiological temperature (37°C) in the phagosomes of the phagocytic cells (see Section 2.3.) is sufficient to denature the tropocollagen as well as fibrillar collagens which are lightly cross-linked (see Fig. 3.12). Once denaturation occurs, the molecular chains are susceptible to attack by the numerous acid proteases found in the lysosomes of the cells (Woessner, 1965). If extracellular degradation of insoluble collagen fibers is effected by the lysosomal enzymes (see Section 2.3.3), local denaturation of chain ends that have been cleaved by the "collagenolytic cathepsins" (Bazin and Delaunay, 1970) is also facilitated by the low local pH supplied by the lysosomal secretion.

The increased stability brought about by crosslinking (Fig. 3.12 and 3.13) is probably due to the formation of a molecular configuration which is more stable to thermal denaturation. Studies by Chirita and Chisalita (1971) show that serine and lysine are probably involved in the crosslinking of collagen by aldehydes. In vivo, lysine is involved in both intramolecular (Bornstein and Piez, 1966) and intermolecular (Bailey et al.,

1970) crosslinking of collagen. Since lysine is also involved in the interaction of the collagen with mucopolysaccharides (Podrazky et al., 1971), the effect of ageing, which generally involves crosslinking of the collagen is to reduce the collagen-MPS interaction. This probably explains the reduced amount of MPS that is generally found in aged connective tissues (Jeanloz and Balazs, 1965). However, since the MPS are thought to produce a stabilizing effect on the collagen helix (Jackson, 1954; Kuhnke, 1962) the loss of the MPS alone would result in a lowering of the stability of the helix. The in vivo crosslinking of collagen can therefore produce two opposing effects on the stability of the collagen helix: an increased stabilization due to the formation of a more stable molecular configuration; and a decreased stabilization due to a decreased interaction with the MPS present. Since the collagen helix of aged tissue is usually more stable (Rasmussen et al., 1965), the former effect must be predominant.

The stress-strain curve of the denatured crosslinked collagen, when plotted with the appropriate functions, as shown in Fig. 3.14 for glyoxal-crosslinked tapes, shows that the materials behave as ideal elastic rubbers (Equation 3.3) as long as the temperature is high (80°C). When the temperature at which the stress-strain curve of the collagen tape is determined falls considerably below the shrinkage of the material, Equation 3.3 does not hold and the retraction portion of the stress strain curve no longer coincides with the extension portion. This observation is in agreement with those obtained for collagen tendon crosslinked by a variety of difunctional compounds (Cater, 1963) and by irradiation (Bailey et al., 1964).

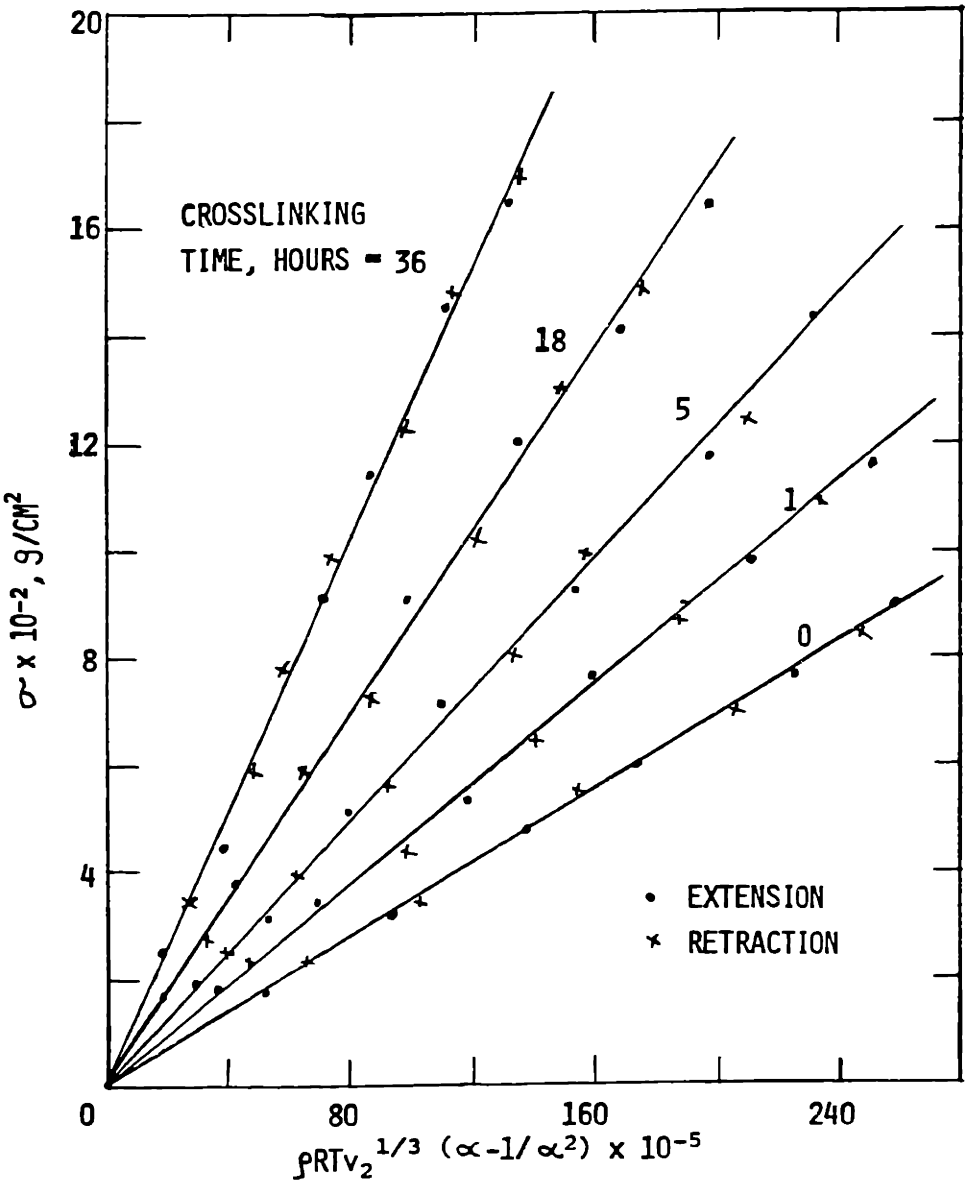


Fig. 3. 14. Stress-strain curves of denatured collagen fibers which had been crosslinked for various time period, as indicated, by 0.025 moles glyoxal (in 0.10M citric acid-phosphate buffer, pH 7.4) at 23°C. The stress-strain curves for both the extension and retraction portion coincide closely and can be seen to exhibit ideal rubber elasticity behaviour (see Equation 3.3). The temperature at which the stress-strain curves were obtained was 80°C.

The observation that the denatured crosslinked collagen fibers behave as ideal elastic rubbers only at temperatures above the shrinkage temperature of the fibers in agreement with previous observations made in our laboratory (Huang, 1971) that the denatured collagen fibers can renature when kept at temperatures below the shrinkage temperature. The increase in internal energy resulting from renaturation invalidates the use of Eq. 3.3, since the equation was derived with the assumption that the internal energy of the polymer contributes negligibly to the stress on the fiber. The requirement of high temperatures for estimating the crosslink density of the collagen is, however, a disadvantage since it raises the question as to whether scission of the collagen chains and crosslinks will occur due to hydrolysis. Cater (1963) has investigated this problem for collagen crosslinked by formaldehyde, glyoxal and glutaraldehyde and his results showed that a small amount of chain scission does occur for formaldehyde- and glyoxal-crosslinked collagen after prolonged immersion (7 hours) at 100°C. However, over the period required to obtain the stress-strain data (about 1/2 hour) and at the lower temperature of 80°C, it is unlikely that chain scission of the crosslinked collagen will cause substantial errors in the estimation of the crosslink density.

The slopes of lines in Fig. 3.14 yield $1/M_c$ according to Equation 3.3. The variation of M_c as a function of the time of immersion in the aldehyde solution allows one to study the kinetics of the crosslinking reaction, as shown in Fig. 3.15, which includes the results of crosslinking by formaldehyde, glyoxal and glutaraldehyde. Two features are apparent from Fig. 3.15. The maximum extent of crosslinking

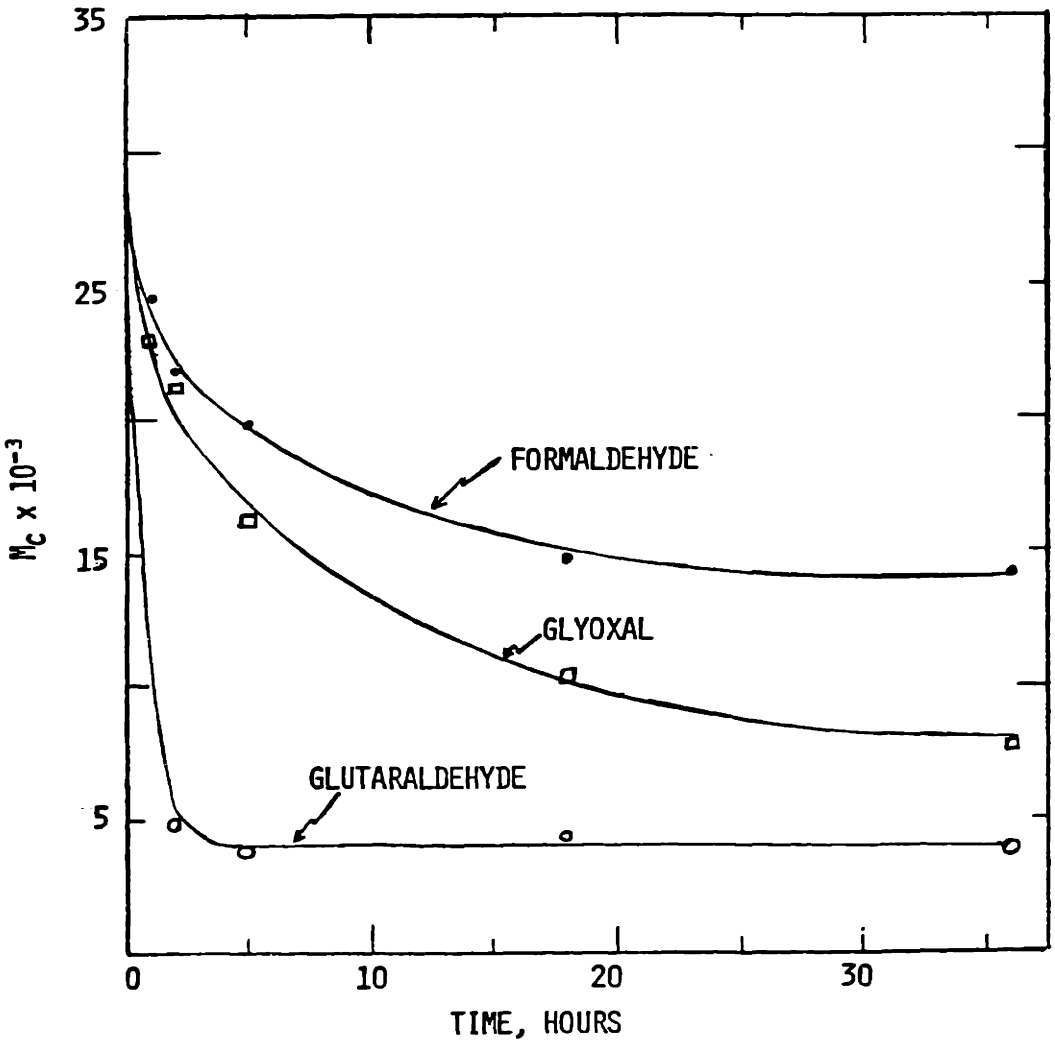


Fig. 3.15. The extent of crosslinking of collagen fibers, crosslinked by formaldehyde, glyoxal and glutaraldehyde for various time period. The concentration of crosslinking agent used was 0.025 moles in all cases and the medium for crosslinking was 0.19M citric acid-phosphate buffer solution at 23 C. The temperature used for determining M_c was 80 C.

by the aldehydes increases in the order: formaldehyde < glyoxal < glutaraldehyde. The rate of crosslinking, however appears to increase in the order: glyoxal < formaldehyde < glutaraldehyde. It is clear that of the aldehydes used in this investigation, glutaraldehyde is the most efficient and effective crosslinking agent.

The different extent of crosslinking obtained for the three aldehydes (Fig. 3.15) can probably be explained by the molecular lengths of the aldehyde. Glutaraldehyde, being the longest of the aldehydes used is probably capable of linking more pairs of amino groups than the other aldehydes. Glyoxal causes a higher extent of crosslinking than formaldehyde for the same reason. However the rate of crosslinking depends also on the reactivities of the aldehyde groups present in each of the molecules. The result obtained in this investigation (glyoxal < formaldehyde < glutaraldehyde) is in good agreement with the findings of Chirita and Chisalita (1971) who showed that a similar order of reactivities occur for reaction of the aldehydes with a number of amino acids which represent the largest part of the collagen structure (glycine, proline, serine, lysine and arginine).

The experimental correlation of the crosslink density (as measured by the stress-strain curve described above) and the volume fraction of polymer, v_2 (as measured by swelling measurements), for denatured collagen in citric acid-phosphate buffer solution, pH 7.4 at 80°C is shown as points on Fig. 3.16. By means of a computer program, a family of curves corresponding to Equation 3.4 with several different values of χ was obtained. It was found that a value of $\chi=0.52\pm0.04$

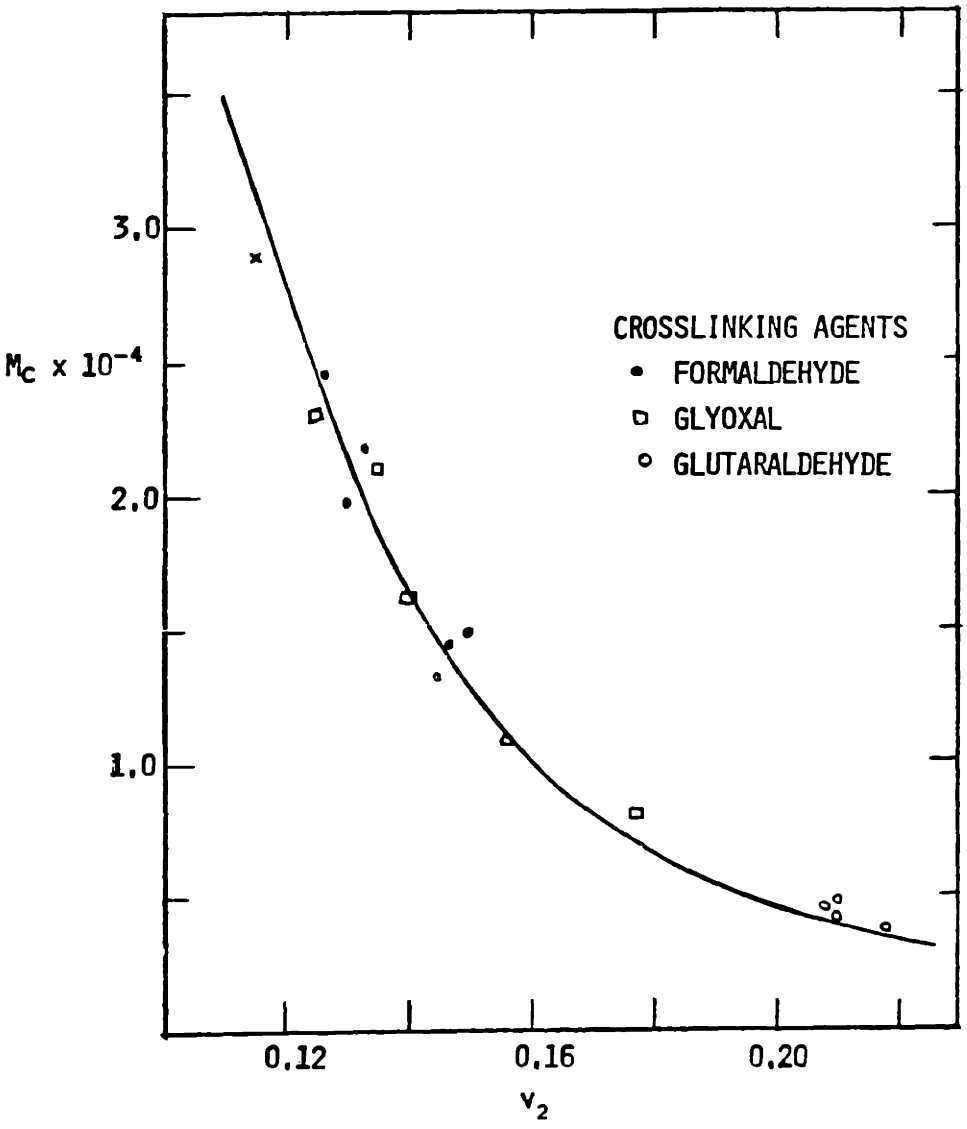


Fig. 3.16. Relationship between the extent of crosslinking of collagen fibers and the polymer volume fraction (v_2) of the denatured collagen when immersed in a 0.19M citric acid-phosphate buffer solution, pH 7.4 at 80°C. The points are experimental data obtained from collagen which had been crosslinked by the various crosslinking agent indicated. The curve is the theoretical curve corresponding to Equation 3.4 with a χ value of 0.52 $\pm$ 0.04.

gave the best agreement between experimental and theoretical results (see Fig. 3.16). The two results agree very well at points where the volume fraction of polymer, v_2 is low. At high v_2, the experimentally obtained v_2 tends to be higher than expected from theory. This can probably be explained by the fact that the high v_2 data was obtained from samples which were highly crosslinked. The shrinkage temperatures of such samples are higher or close to the temperature at which the experiments were conducted (Fig. 3.13). Consequently, renaturation of the collagen fibers may occur and this will result in an increased measured v_2 as well as an overestimation of M_c(Equation 3.3).

The applicability of the Flory-Rehner Equation (Equation 3.4) for denatured collagen (Fig. 3.16) allows the crosslink density of the collagen to be estimated by the relatively simple determination of the volume fraction of polymer in the denatured state. This method was used for the determination of M_c of samples that have been implanted in experimental animals but were too small to allow an accurate estimation of M_c by the stress-strain analysis described above (see Chapter 5).

3.3. Helical Content of Collagen.

3.3.1. Introduction. The extent to which collagen is denatured greatly influences its susceptibility to enzymatic degradation (see Section 3.4). It is therefore an important parameter to characterize if materials are to be assessed for their potential use as skin replacements. The triple-helical nature of collagen has been studied conventionally with wide-angle x-ray diffraction when it is in the fibrous form (Rich and

Crick, 1955, 1961; Ramachandran, 1967) and with optical rotation studies when it is dissolved in dilute solution (Carver and Blout, 1967; Von Hippel, 1967). However, no attempt has been made in these studies to estimate quantitatively the amount of helicity of collagen which may be partially denatured. In this section, the use of wide-angle x-ray diffraction, infrared spectroscopy and optical rotation studies for the estimation of helical content of collagen in the solid phase will be assessed.

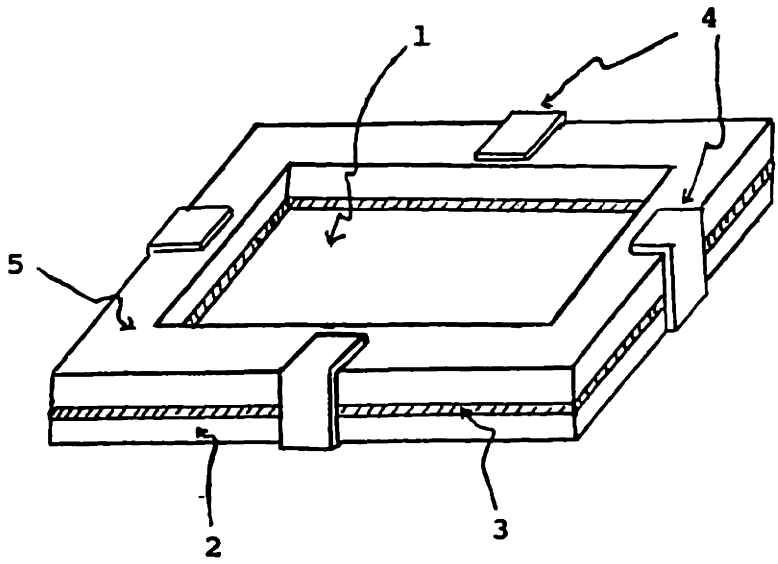
3.3.2. Sample Preparation. The samples used for the assessment of the various methods of estimating helical content of collagen were films of collagen and gelatin (of varying helical content) obtained by solution casting of the macromolecules. The source of the collagen was rat-tail tendon (RTT) and the method of extraction was similar to that of Dumitru and Garett (1947). The tails were obtained from freshly killed rats and preserved by freezing at -4°C. When required, the tendons were removed from the tail by means of a pair of wire-cutting pliers, using an operation similar to stripping insulated wires. The exposed tendons were cut and immersed in 0.5M NaH_2PO_4 for about three hours at room temperature. At the end of period, the reticular membrane surrounding the fiber was separated (Fig. 3.1) thus facilitating its dissolution by acetic acid (Fig. 3.2) in the next step. The tendons were briefly rinsed with distilled water (for about 5 secs.) before they were transferred to a 0.05M acetic acid solution. Solubilization of the tendon was carried out overnight at room temperature with mild agitation. The solution was then filtered through medium and coarse

glass frits to remove undissolved collagen and foreign materials. Purification of the solution was achieved by precipitation with 2M KCl followed by dialysis with three exchanges of distilled water at room temperature. The precipitate obtained was redissolved in 0.05M acetic acid and stored under refrigeration. Fully denatured gelatin is obtained from the solution of RTT collagen by heating the solution at 50°C for half an hour.

Casting of both the collagen and gelatin films was done on polymethylmethacrylate (Forest Products, Cambridge, Mass.) dishes made up of a smooth rectangular base on top of which is a rectangular frame; a thin rubber frame separated the two pieces and clamps were used to make the dish leak-proof (Fig. 3.17). This arrangement facilitated the removal of samples from the substrate. Film thicknesses ranging from 0.0001" to 0.01" were cast according to the requirements of the analytical methods used.

Solid phase collagen which is fully native was obtained by blowing air over a solution of RTT collagen placed in the casting dish at room temperature (see Sung, 1972 for details). The final stage of the casting (just before gel formation) was done is a vacuum desiccator with a low negative pressure provided by a water aspirator. This relatively quiescent condition allows films free from birefringence to be cast. This is important because the measurement of optical rotation is very sensitive to even a small amount of birefringence (Sung, 1972).

Solid phase gelatin which is fully denatured (hot-cast gelatin) was obtained by casting the fully denatured gelatin solution



1. SMOOTH CASTING SURFACE
2. PMMA BASE
3. RUBBER FRAME
4. CLAMPS
5. PMMA FRAME

Fig. 3.17. Casting dish for preparing collagen and gelatin films.

(obtained by the method described above) at 60°C. Gelatin films of varying degree of helical content were obtained by casting the fully denatured gelatin solution at lower temperatures (23°C and 4°C).

3.3.3. X-ray diffraction studies. The X-ray unit used in the present investigation was a Norelco X-ray Analytical Instrument (Phillips Electronics Instrument, N.Y.). The X-ray radiation was provided by a copper target, filtered through a nickel screen and generated at 35 kV with a current of 15 miliamperes. A transmission X-ray diffraction pattern (Laue pattern) was obtained with a beam collimator length of 6 cm and a beam cross section, 1mm in diameter. The photgraphic film used was of a high speed type (Kodak No-Screen, General Electric, Medical Department, Wellesley, Mass.). The exposure time was dependent on the thickness (e.g. 1 hour for 0.01" specimens).

The X-ray diffraction pattern of RTT collagen fiber is shown in Fig. 3.18A and an interpretation of its various layer lines is shown in Table 3.1. The distribution of intensities of the various layer lines is shown diagrammatically in Fig. 3.19. The two most important layer lines are the meridional reflection, corresponding to a residue repeat distance of 2.91°A and the equatorial reflection, corresponding to an intermolecular distance of about 11°A in relatively dry specimens. The X-ray diffraction pattern of the collagen film cast from solution at 23°C, obtained with the X-ray beam normal to the plane of the film is shown in Fig. 3.18C; the pattern of a collagen film cast as above but obtained with beam parallel to the plane of the fim is shown in Fig. 3.18D. The latter two photographs contain evidence that a film

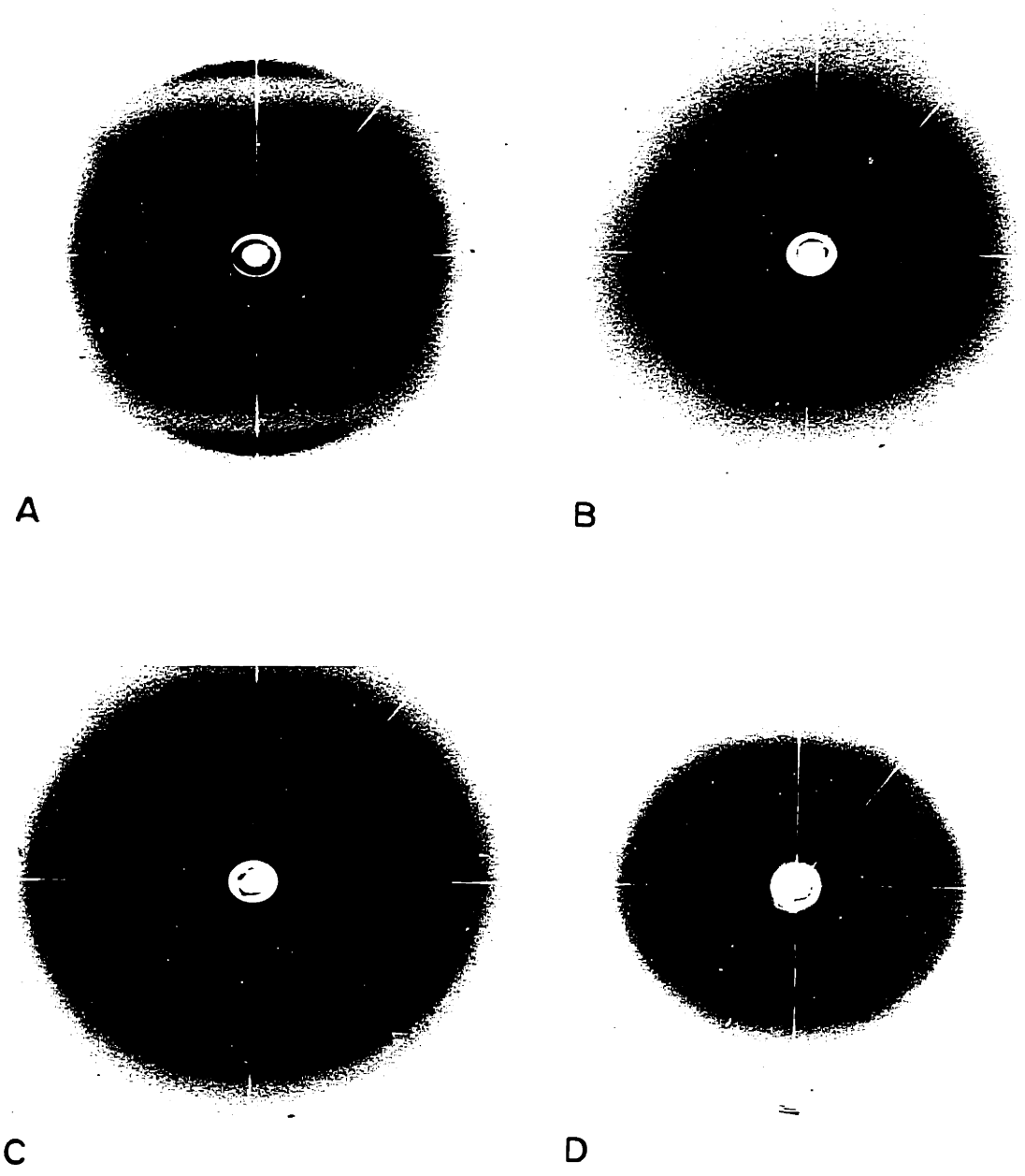


Fig. 3.18. Wide-angle X-ray diffraction pattern of several forms of collagen. A, native rat tail tendon; B, denatured rat tail tendon or hot cast gelatin film; C, film cast at 23°C from a solution of tropocollagen in 0.05M acetic acid; beam normal to plane of film; D, film cast as in C but X-ray beam is parallel to the plane of the film.

TABLE 3.1

CHARACTERISTICS OF THE WIDE-ANGLE X-RAY DIFFRACTION PATTERN OF COLLAGEN

(From Ramachandran and Sasisekharan, 1965)

Layer Line	Spacing, A°	Remarks
10th	2.91	true meridional reflection; spacing corresponds to the vertical height of one residue.
7th	4.2	off-meridional reflection
3rd	9.5	off-meridional reflection
d_{100}	10.4-14.6	equatorial reflection; hydration sensitive; spacing corresponds to the lateral packing distance TC molecules.
d_{200}	ca. $d_{200}/2$	equatorial reflection; spacing corresponds to one-half of d_{100}.
---	ca. 4.5	strong, diffuse reflections on and near the equator.

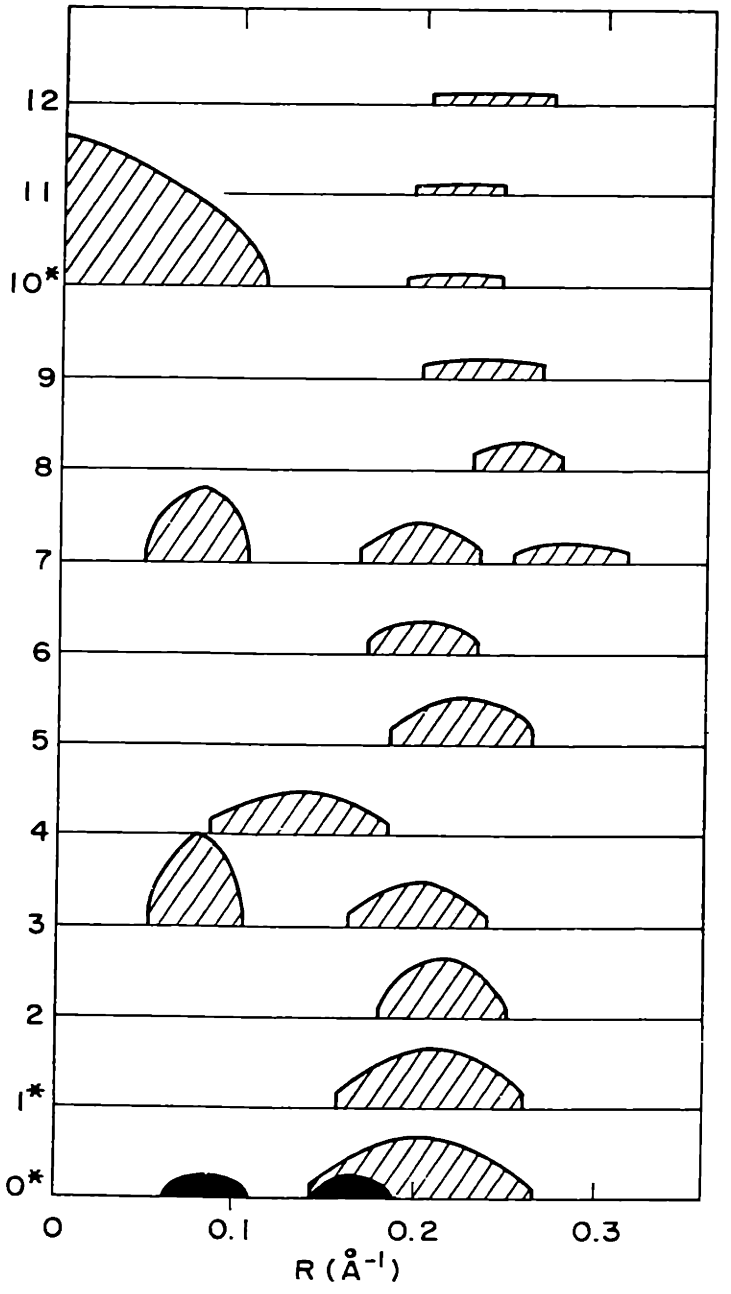


Fig. 3.19. A semi-quantitative summary of the information in the wide-angle X-ray diffraction pattern of rat tail tendon collagen. Layer lines 0 to 12 are indicated and the intensity of reflections of each is plotted vertically, Two intense equatorial reflections are represented by solid semi-circles. An asterisk denotes reflections plotted at one half scale. From Rich and Crick (1961).

cast from a tropocollagen solution is a collection of triple-helical molecules lying mostly in the plane of the film but randomly oriented otherwise. Sung (1972) has estimated the average out-of-plane orientation of the collagen molecules in the film to be about 12°.

The X-ray diffraction of denatured RTT collagen fiber and of the hot-cast gelatin film are exactly alike (Fig. 3.18B), showing a rather diffuse pattern, devoid of the 2.91A° meridional reflection characteristic of the collagen triple helix. The diffraction patterns of partially denatured gelatin film (cast at 4°C and 23°C) are indistinguishable from that of the collagen film (Fig. 3.18C). The estimation of helical content of partially denatured collagen using X-ray diffraction pattern, appears, therefore to be difficult. The estimation will involve a measurement of the intensity of the 2.91A° meridional reflection (using a X-ray diffraction densitometer) and the normalization of this intensity for the thickness and the density of the collagen sample. Furthermore, all the experimental conditions (time of exposure, radiation voltage and current, beam collimator length and cross sectional area) have to be kept precisely constant. These stringent requirements render the X-ray diffraction method rather impractical for the estimation of helical content of the collagen molecule. However, under certain circumstances, the X-ray diffraction method may be the only method capable of estimating the helical content of the collagen sample. This is so because the infrared spectroscopic method, to be described in the following section, is not able to cope with thicknesses in excess of about 0.002" while the optical rotation method (Section 3.3.5) cannot be used for samples which are birefringent.

Hence, for samples which are excessively thick and birefringent, the X-ray diffraction method (or an alternative method) must be developed to measure the helical content of the collagen sample.

3.3.4. Infrared Spectroscopy. A double-beam grating infrared spectrophotometer (Perkin Elmer Model 621) was used in the present investigation to obtain the spectra of collagen and gelain samples in the frequency range of 200 cm^{-1} to 4000 cm^{-1}. The scan speed was fixed at 16 minutes for the full spectrum. The spectra was recorded both in the transmission and absorption mode. The resolution of the relatively strong bands (i.e. those of amide I, II) was found to be better in the absorption mode, whereas relatively weak absorption bands were resolved better in the transmission mode.

Representative infrared spectra of collagen and fully denatured gelatin are shown in Fig. 3.20 and interpretation of several bands have been collected in Table 3.2. As is the case with several other proteins, the spectrum of collagen is poorly resolved. Nevertheless, Fig. 3.20 shows several changes brought about in the spectrum by the complete denaturation of collagen to gelatin. Close scrutiny of the bands shows some of these changes to be real shifts in frequency, whereas, in other cases, the shift is only apparent, arising from changes in relative intensity of several bands within a composite band (Huang, 1971). For example, the shift in frequency of the N-H stretching vibration from 3330 cm^{-1} in gelatin (Fig. 3.20) is real whereas the reported (Bradbury et al., 1958) shift in frequency of the Amide I band from 1655 cm^{-1} to 1640 cm^{-1} is apparent and arises from changes

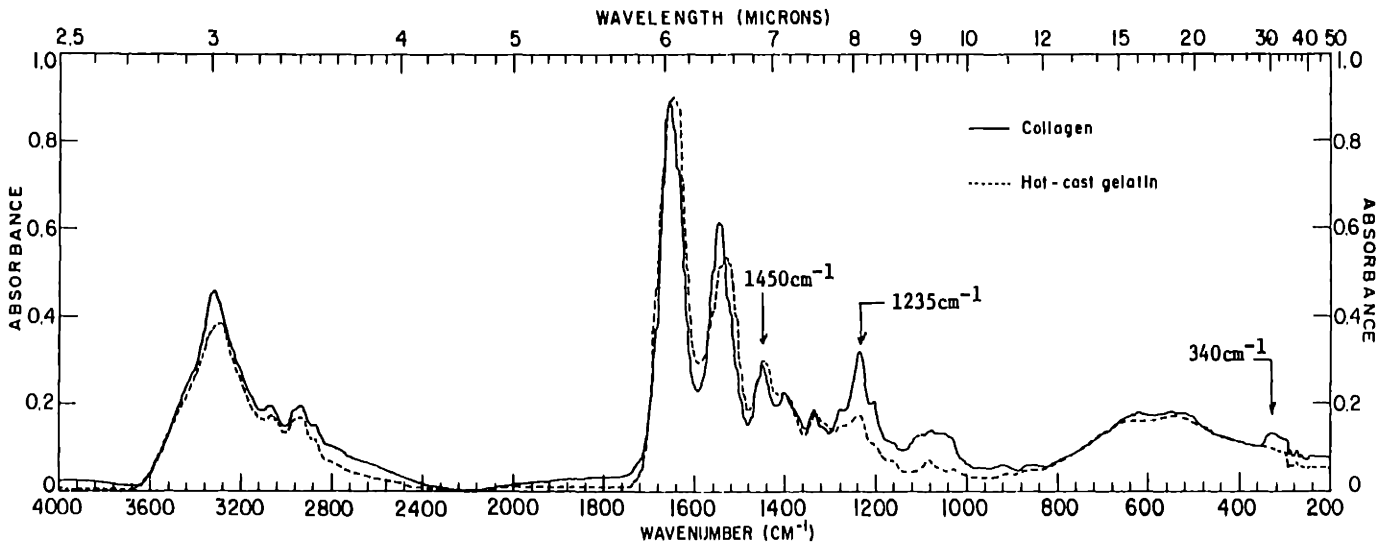


Fig. 3.20. Infrared absorption spectra of collagen and hot-cast gelatin films. The three absorption bands used for determining the helical content of partially denatured collagen are indicated.

TABLE 3.2

MAJOR BANDS IN THE IR SPECTRUM OF COLLAGEN

(From Yannas, 1972)

Frequency, cm^{-1}	Interpretation	References
4850-4890	combination of NH stretch ($3300\ cm^{-1}$) and in-plane C-N-H deformation ($1550\ cm^{-1}$)	[2,8,9]
4580-4600	combination of C=O modes	[1]
3450-3400	free water	[6,7]
3290-3330	NH stretch	[1,7,12]
3060-3100	overtone of Amide II	[10,12,13]
2930-2950	CH stretch	[6,11]
2870	CH stretch	[6]
2850	CH stretch	[6]
1710	nonionized COOH	[6]
1640-1660	C=O stretch (Amide I)	[3,4,6]
1560	COO^- band	[6]
1535-1550	NH in-plane deformation plus CN stretch (Amide II)	[3,4,6]
1445-1455	CH_2 deformation and CH_3 asym. deformation	[6,11]
1407	COO^- band	[6]

TABLE 3.2 (CONTINUED)

MAJOR BANDS IN THE IR SPECTRUM OF COLLAGEN

(From Yannas, 1972)

Frequency, cm^{-1}	Interpretation	References
1375-1391	CH_3 symmetric deformation	[6,11]
1310-1340	CH_2 wagging	[11]
1230-1270	CN stretch plus NH in-plane deformation (Amide III)	[3,4,6]
1075-1082	CO vibrations of hydroxyl groups	[7]
920-940	OH deformation of COOH (?)	[6]
650	NH deformation out of plane	[8,11]

(1) Fraser (1950)

(2) Ambrose and Elliot (1951)

(3) Fraser and Price (1952)

(4) Fraser and Price (1953)

(5) Miyazawa and Blout (1961)

(6) Huc and Sanejouand (1968)

(7) Bradbury et al. (1958)

(8) Sutherland et al. 1954)

(9) Glatt and Ellis (1948)

(10) Badger and Pullin (1954)

(11) Beer et al. (1959)

(12) Cannon (1959)

(13) Miyazawa (1960)

in relative intensity of sub-bands at 1655, 1645 and 1635 as collagen is transformed to gelatin (Huang, 1971). Similarly, the shift in the Amide II band from 1550 cm^{-1} in collagen to 1535 cm^{-1} in gelatin is apparent and arises from relative intensity changes in its satellite bands at 1550, 1533, 1525, 1520 and 1505 cm^{-1}.

In the use of the infrared spectra for the study of helical content, three absorption bands are of special interest: the 1450 cm^{-1} band, which corresponds to the CH_2 deformation and CH_3 assymetric deformation; the 1235 cm^{-1} band, which is the amide III band; and the far infrared band at 340 cm^{-1}, which has yet to be assigned. The 1450 cm^{-1} band appears to be unchanged in frequency or in intensity when the collagen is denatured whereas the intensities of the 1235 cm^{-1} and 340 cm^{-1} bands are markedly diminished upon denaturation (Figs. 3.21 and 3.22). The absorbances at 1450 cm^{-1} (A_{1450}) and at 1235 cm^{-1} (A_{1235}) were obtained by using the baselines shown in Fig. 3.21. The choice of the baseline for the 340 cm^{-1} band proved to be difficult because the change in the grating prisms of the spectrophotometer at 300 cm^{-1} gave a rather erratic and irreproducible shift in the baseline. However, it was found that using the horizontal line tangential to the trough at 370 cm^{-1} as the baseline (Fig. 3.22) yields an absorbance value (A_{340}) which gives a satisfactory estimate of the helical content (see below).

Attempts were made to use the absorbance ratios A_{1235}/A_{1450} (R_{1235}) and A_{340}/A_{1450} (R_{340}) as independent estimates of the helical content of partially denatured gelatin. The R values obtained for the various samples studied are shown in Table 3.3. By

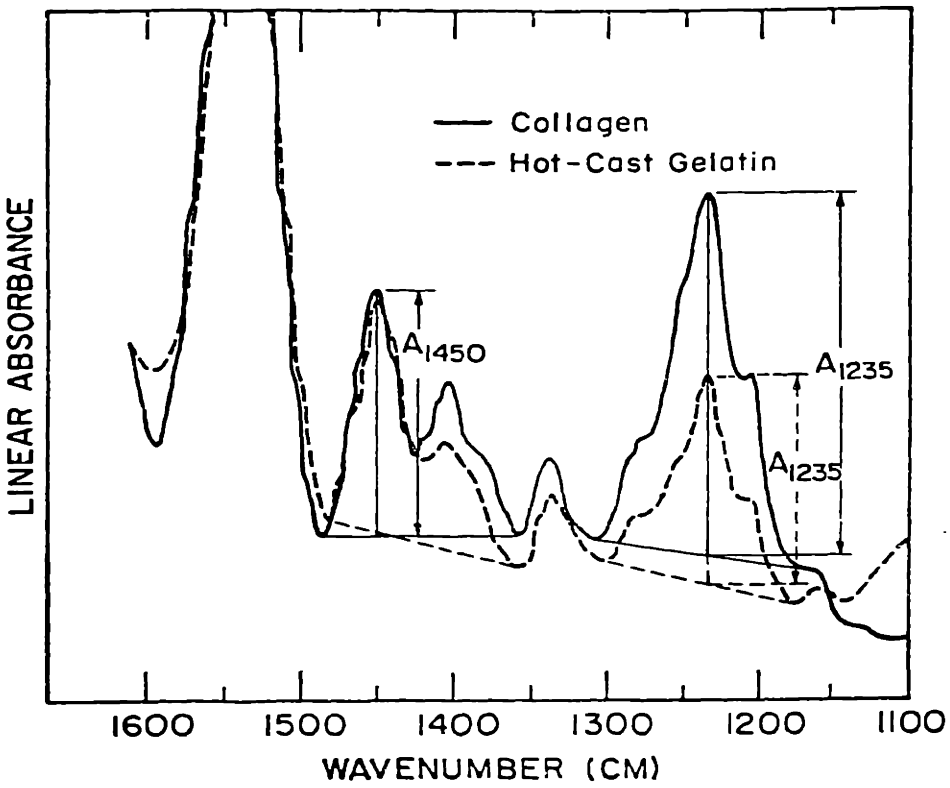


Fig. 3.21. Details of the infrared absorbance bands at 1235cm^{-1} and 1450cm^{-1} for collagen and hot-cast gelatin. Baselines used for the measurement of the absorbances are as indicated.

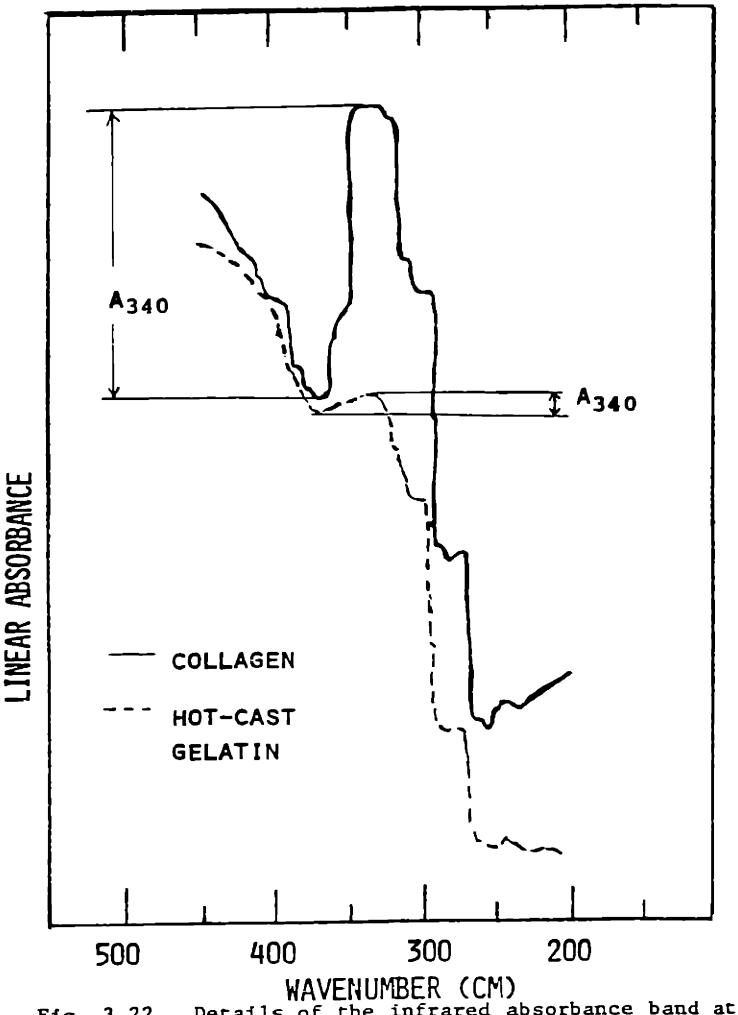


Fig. 3.22. Details of the infrared absorbance band at 340cm^{-1} for collagen and hot-cast gelatin. The baseline used for measurement of the absorbances are as indicated.

TABLE 3.3

INFRARED DATA ON THE ESTIMATION OF HELICAL CONTENT OF COLLAGEN

	A_{1235}/A_{1450} (R_{1235})	%-helicity based on R_{1235}	A_{340}/A_{1450} (R_{340})	%-helicity based on R_{340}
Collagen	1.38±0.05	100% (assumed)	0.280±0.008	100% (assumed)
Gelatin (Cast at 4°C)	1.15±0.02	71%±9%	0.227±0.010	76%±12%
Gelatin (Cast at 23°C)	0.94±0.02	44%±10%	0.158±0.005	46%±10%
Gelatin (Cast at 60°C)	0.59±0.02	0% (assumed)	0.054±0.002	0% (assumed)

assigning the R values of the collagen (cast at 23°C) and the gelatin (cast at 60°C) as the characteristic R values for 100% helix and 100% random coil respectively, an estimate of the helical contents of the two partially denatured gelatin (cast at 4°C and 23°C) can be determined if it is assumed that the helical contents are linearly related to the R values. Such estimates have been made for both the 1235 cm^{-1} and 340 cm^{-1} bands and are tabulated in Table 3.3. The agreement in the estimates of the helical content based on these two absorbance bands suggest that R_{1235} and R_{340} must be linearly related. This is confirmed in Fig. 3.23. However, the linear relationship between these two absorbance ratios do not necessarily justify the assumption that the helical content is linearly related to the absorbance ratio of the samples. An independent method for the estimation of helical content, based on optical rotation studies will be described in the next section. The agreement between the estimates of helical content based on this independent method and those obtained in the present investigation (Table 3.4) lends credence to this author's belief that the values shown in Table 3.3, though empirically obtained, represent true estimates of the helical content of the samples.

The use of R_{1235} or R_{340} for the estimation of helical content is only practical for samples which have thicknesses less than 0.0002". When the thickness of the sample is higher than this, A_{1450} becomes so large that an accurate estimation of R_{1235} or R_{340} is not possible. Another method, based on normalizing A_{340} with respect to the thickness of the sample, has been developed for the estimation of

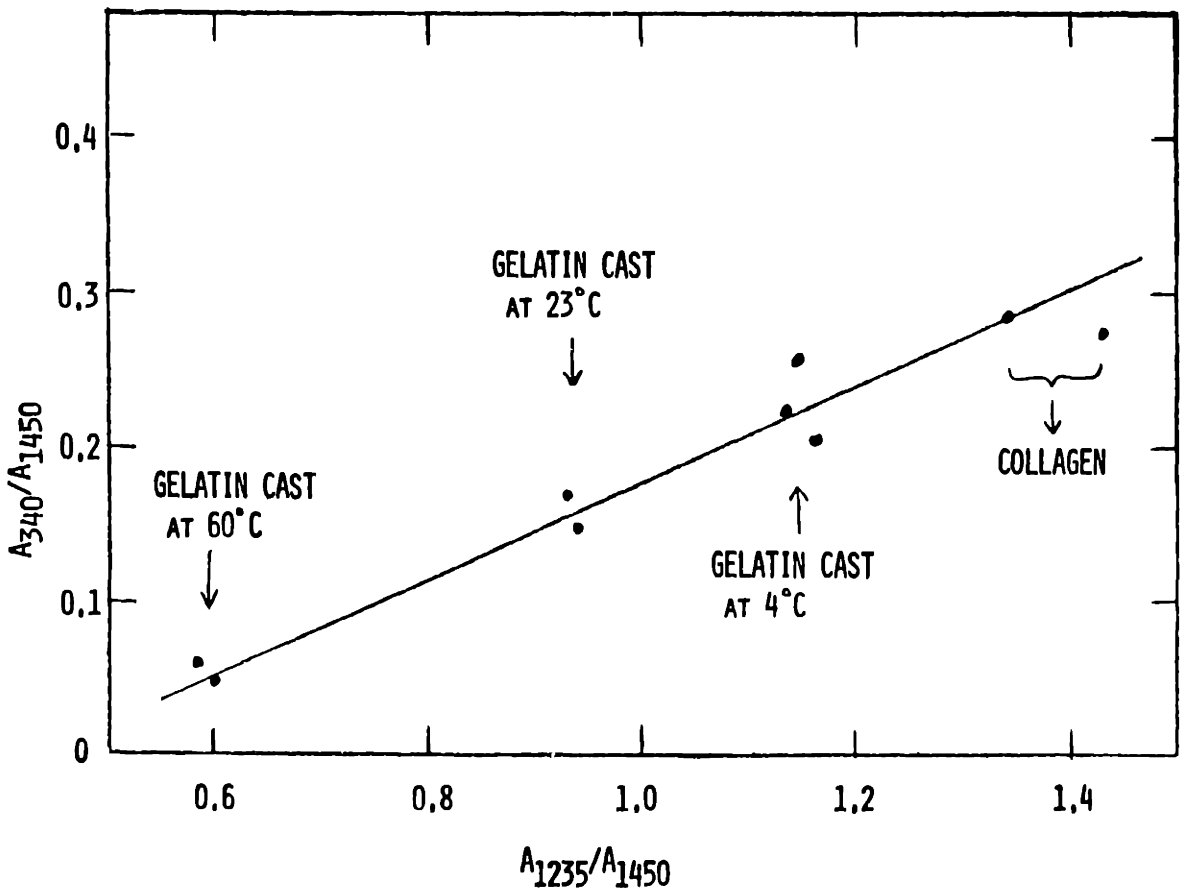


Fig. 3.23. A plot of the infrared absorbance ratio, A_{340}/A_{1450} (R_{340}) versus the absorbance ratio, A_{1235}/A_{1450} (R_{1235}) for collagen and gelatin, denatured to different extent.

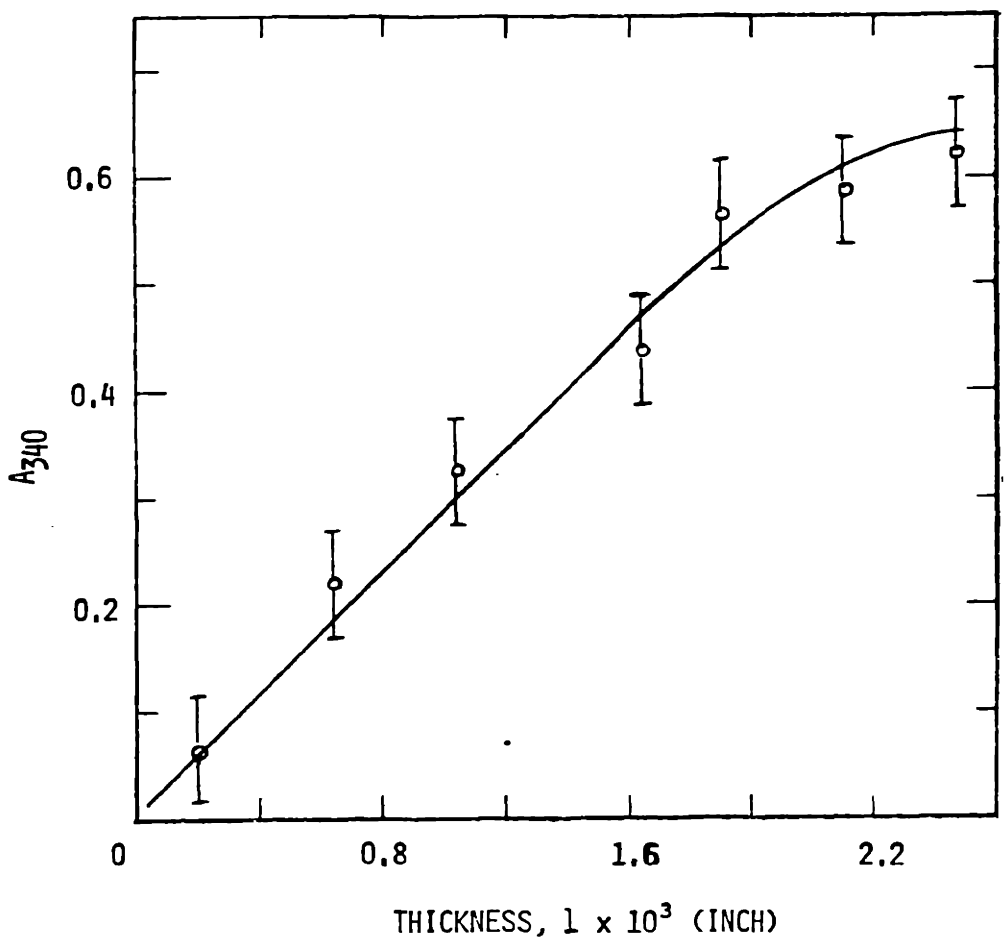


Fig. 3.24. Variation of the infrared absorbance at $340cm^{-1}$ with thickness of collagen films. Note the deviation from linearity between the absorbance and thickness at ca. 0.002 inch.

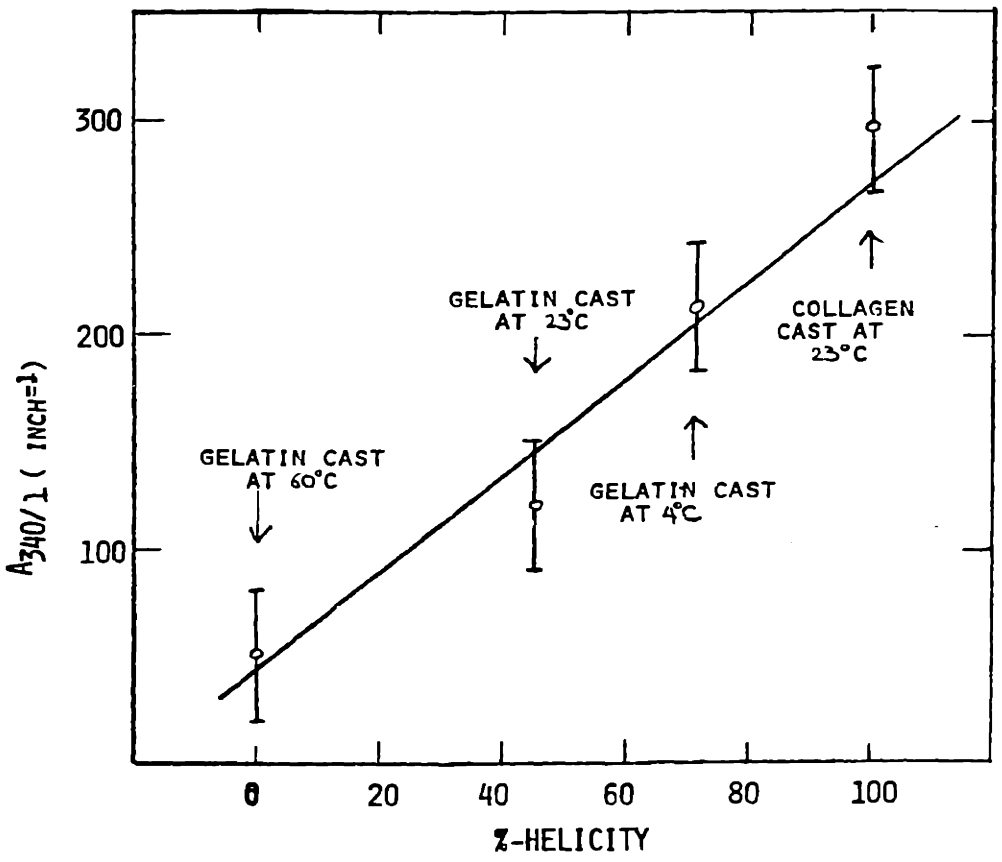


Fig. 3.25. The infrared absorbance at 340 cm^{-1} normalized for thickness of the sample, as a function of helical content of the samples. The latter was determined from the ratio of absorbances A_{1235}/A_{1450}, as shown in Table 3.3.

helical content of thicker samples. This is possible because the 340 cm^{-1} absorbance band is relatively weaker than the 1235 cm^{-1} and the 1450 cm^{-1} bands (Fig. 3.20). Fig. 3.24 shows that up to a thickness of about 0.002" , A_{340} is a linear function of the thickness, l, of the sample. By preparing samples of varying thicknesses under the various conditions shown in Table 3.3, the value of A_{340} normalized for the thickness (A_{340}/l) has been plotted against the helical contents expected (from Table 3.3) of the samples prepared under those conditions (Fig. 3.25). Although the results are subject to large errors (± 10%) a linear relationship is indicated. This correlation of A_{340}/l with the helical content was used to estimate the helical content of materials that have been implanted in experimental animals (Chapter 5).

3.3.5. Optical Rotation Studies. A Cary 60 Spectropolarimeter (Cary Instruments, Monrovia, Calif.) was used in the present investigation to obtain the optical rotation of collagen and gelatin samples at a fixed wavelength (365Mμ). A dynode voltage of 200-300 volts and a lamp current of 25 amps were employed. The measured optical rotation angle at a fixed wavelength, λ (α_λ) is normalized with respect to the thickness (l) and the density (ρ) of the film to give the specific optical rotation, $[\alpha]_\lambda$, which is defined by:

$$[\alpha]_\lambda = \frac{10\ \alpha_\lambda}{\rho l} \quad \frac{\text{degrees-cm}^2}{\text{gm}} \qquad (3.7)$$

Where:

α_λ = measured optical rotation angle in degrees.

l = thickness of the film (cms).

ρ = density of the film (gm/cc).

In cases where the specific optical rotation of samples having significant differences in refractive index are to be compared, a Lorentz correction factor (Urnes and Doty, 1961) has to be applied to the specific rotation. The corrected specific rotation, called the reduced specific rotation, is designated by $[\alpha]^r_\lambda$ and is related to the specific rotation through the relationship:

$$[\alpha]^r_\lambda = \frac{3}{n^2 + 2} [\alpha]_\lambda \qquad (3.8)$$

Where n is the refractive index of the sample. For collagen in the solid phase, containing about 15 wt-% water, n is 1.525 (Sung, 1972) and the correction factor, $3/(n^2+2)$ is 0.690. For a dilute solution of collagen (ca. 0.1 wt-%), n can be taken as 1.33 (the same value as water), and the correction factor becomes 0.795.

In considering the specific rotation of the solid phase collagen film, the question immediately arises whether the triple-helical structure of the collagen molecule undergoes a significant change in its dimensions in going from the relatively unperturbed existence in dilute solution to the highly crowded solid state. Furthermore, since the weight ratio of protein to aqueous solvent changes from about 10^{-3} to about 6 as collagen goes from dilute solution to

solid state (ca. 15 wt% water), the question of the possible effect of water content on the conformation of the helix also poses itself.

To resolve these questions, a series of experiments were conducted to obtain the specific rotation, at 365mμ, of collagen at various states of hydration. The specific rotation of collagen solutions ranging in concentrations from 0.1 to 5 wt-% were measured using solution cells of path lengths 1.0 cm, 0.1 cm and 0.01 cm. At concentrations higher than 5 wt-% collagen, the solution became very viscous and the solution cells could not be used. An aliquot of a very viscous solution (ca. 7 wt-% collagen) was therefore transferred onto the surface of a quartz plate where further dehydration was carried out in a controlled humidity chamber. Between 7 to 20 wt-% collagen, measurement of the optical rotation was done with sufficient rapidity that the flow of the viscous solution did not significantly affect the measurement. Above 20 wt-% collagen, the solution acquired a gel-like state and flow of the concentrated collagen solution become negligible. The concentration of collagen was determined gravimetrically.

Fig. 3.26 shows the variation of the specific rotation of collagen at 365mμ as it is dehydrated from a dilute solution of 0.1 wt-% collagen to a solid state film in equilibrium with laboratory conditions, containing ca. 15 wt-% water. From the dilute solution state to about 30 wt-% collagen, the specific rotation remained unchanged at a value of -1300. Above 30 wt-% collagen, the specific rotation gradually increased in its magnitude from -1300 to -3200 as the concentration increased to ca. 65 wt-% collagen. From 65 wt-%

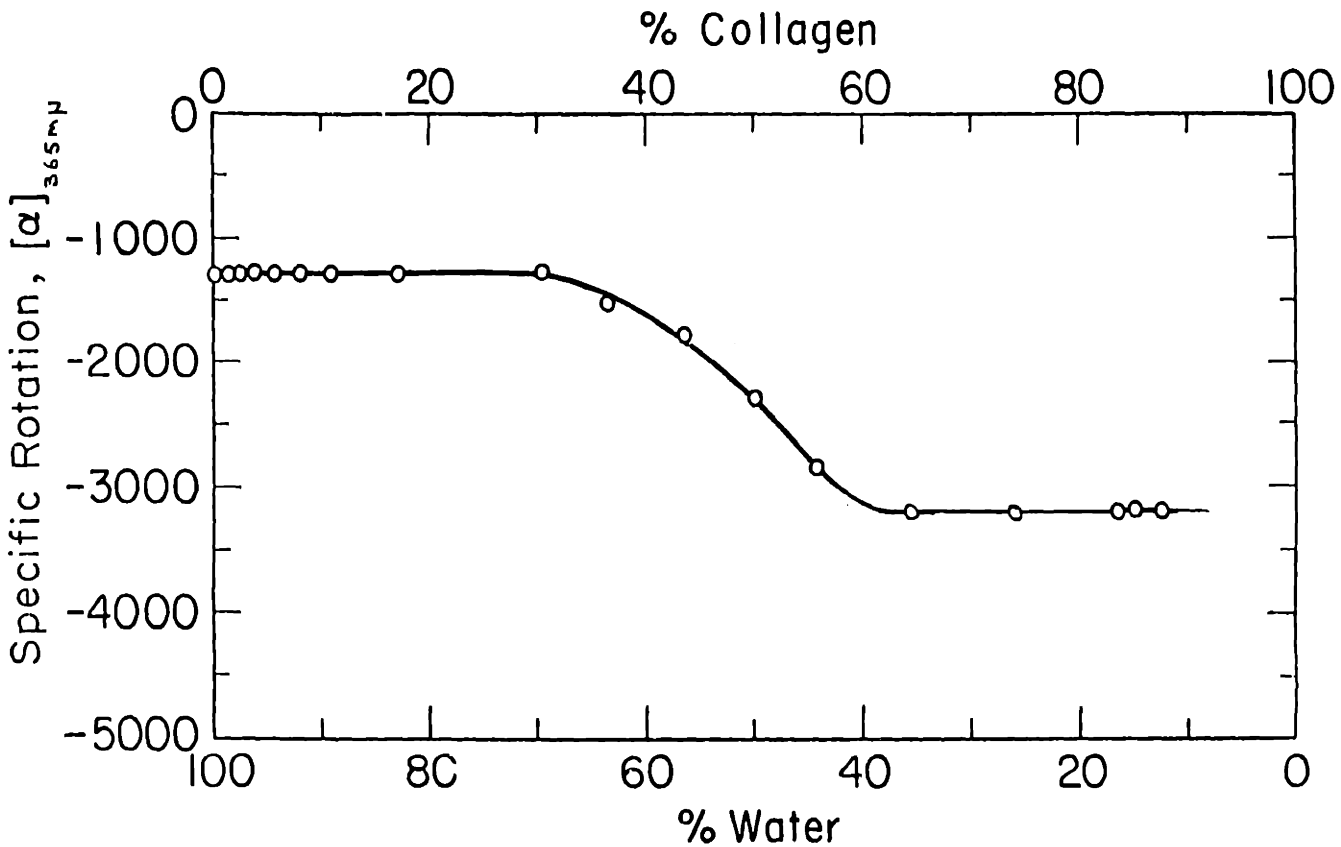


Fig. 3.26. Specific rotation of collagen at various hydration levels from dilute solution (0.1 wt-% collagen) to a solid state film (ca. 85 wt-% collagen), in equilibrium with laboratory conditions.

collagen to 85 wt-% collagen (where the collagen film maintains an equilibrium with the laboratory conditions) the specific rotation remained unchanged at a value of -3200.

The increase in specific rotation from -1300 in the dilute solution state to -3200 in the solid state appears to suggest, at first glance, that the helical content of the collagen had increased when going from the solution to the solid state. A careful analysis of the problem, however, indicates that there are significant differences between the two states that must be accounted for before a reasonable comparison of the specific rotation values can be made. As was pointed earlier, the refractive indices of the two states are significantly different and hence the Lorentz correction factor must be applied. The corrected specific rotations of -2360 for the solid state and -1035 for the dilute solution state cannot, however, eliminate the difference. An examination of the X-ray diffraction pattern of the collagen film, with the X-ray beam parallel to the plane of the film, indicated that the collagen molecules are planarly oriented along the plane of the film (Fig. 3.18D). This contrasts significantly with the random orientation of the molecules present in a dilute solution of collagen. As the optical activity of the collagen molecule has been shown to possess tensorial components of different magnitude (Tinoco, 1959), the differences in orientation of the molecules in the two states must be accounted for.

To resolve this problem, the optical activity of the collagen molecule is considered as a second-rank tensor

$$\alpha = \alpha_{ij} l_i l_j \quad (i,j = 1,2,3) \tag{3.9}$$

where α_{ij} are the components of the tensor and $l_i l_j$ are the direction cosines of the light beam with respect to the molecular axes 1, 2 and 3. Due to the cylindrical symmetry of the collagen molecule, all non-diagonal components of the optical activity tensor of collagen are zero and furthermore, $\alpha_{11} = \alpha_{22}$. In this notation, $\alpha_{11} = \alpha_{22}$ is the optical activity measured with the light beam perpendicular to the helical axis, while α_{33} is the optical activity measured parallel to that axis. In dilute solution, where the molecules are randomly oriented with respect to the light beam,

$$\alpha_{solution} = \frac{\alpha_{11}+\alpha_{22}+\alpha_{33}}{3} = \frac{2\alpha_{11}+\alpha_{33}}{3} \tag{3.10}$$

For planar orientation of the molecules in the film, the average values of the direction cosines are $\overline{l_1^2} = \overline{l_2^2} = 1/2$ and $\overline{l_3^2} = 0$, so that the optical activity measured with the beam normal to the film surface is

$$\alpha_{film} = \frac{\alpha_{11}+\alpha_{22}}{2} = \alpha_{11} = \alpha_{22} \tag{3.11}$$

In this way, the reduced specific rotation normal to the helical axis, $[\alpha^r_{11}]_{solid}$ was found equal to -2360 ± 140, as reported above.

To answer the question as to whether the collagen triple helical structure is changed when going from solution to the solid state, the value of $[\alpha_{33}]^r$ in the solid state must be determined. With this value of $[\alpha_{33}]^r_{solid}$ and the value of $[\alpha_{11}]^r_{solid}$ which has already been

found, the "solution" specific rotation for the solid state $[\alpha]^r_{solid}$ can be determined through Eq. (3.10). This can then be compared with the value of $[\alpha]^r_{solution} = -1035$ already found for the dilute solution.

In attempting to obtain $[\alpha_{33}]^r_{solid}$, a method was developed whereby the optical activity of the collagen film, cast on a fused quartz disk, was measured for several levels of angle between the beam direction and the normal to the plane of the film (tilt angle). Purely on trigonometric grounds, it can be shown (Sung, 1972) that the optical activity with tilt angle Θ is given by

$$[\alpha_\Theta] = [\alpha_{11}] + \frac{([\alpha_{33}] + [\alpha_{11}])}{2} \sin^2\Theta \qquad (3.12)$$

However, corrections have to be made for the change in angle of the polarized light as it is refracted by the specimen and supporting disk and for the change in optical path length which accompanies tilting. Specifically, the apparent tilt angle, β is different from the actual tilt angle, Θ and is related to it through the relationship, $\sin\beta = n \sin\Theta$. After introducing the corrections and assuming $[\alpha]_{\Theta=0}=[\alpha_{11}]$, Eq.^{n} (3.12) becomes

$$\frac{\alpha_\beta}{\alpha_0}\cos\Theta = 1 + \frac{\sin^2\beta}{2n^2} \frac{[\alpha_{33}] - [\alpha_{11}]}{[\alpha_{11}]} \qquad (3.13)$$

Where α_β is the rotation angle of the specimen at tilt angle, β.

Fig. 3.27 shows the variation of the optical rotation with the tilt angle, plotted with the appropriate functions. The experimental points fall on a straight line, as predicted by Eq^n. 3.13.

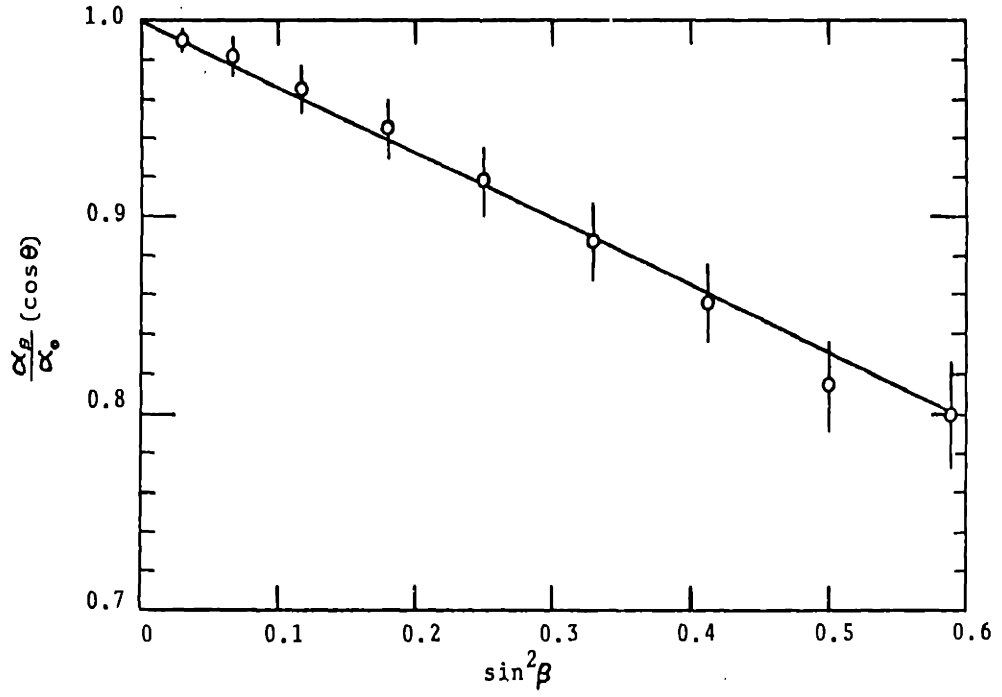


Fig. 3.27. Specific rotation of collagen film at various tilt positions. (Tilt angle, β) The experimental points fall on a straight line, as predicted by the theoretical treatment presented in the text (see Equation 3.13).

From the slope of the Line, $[\alpha_{33}]$ for the collagen film can be determined if the value of $[\alpha_{11}] = -3200$, obtained previously, is assumed. After correction with the Lorentz factor, it was found that $[\alpha_{33}]^r_{solid} = +1301 \pm 190$. This value agrees quite well with the value of $+1100 \pm 140$ determined by Sung (1972) who used a direct method of measurement. Using the value of $[\alpha_{11}]^r_{solid} = -2360 \pm 140$ and the presently determined value of $[\alpha_{33}]^r_{solid}$, a value of $[\alpha]^r_{solid} = -1140 \pm 220$ is found through Eq.[n] 3.10 for the solid state collagen. This compares with the value of $[\alpha]^r_{solution} = -1035 \pm 40$ obtained for the dilute solution. It is clear that the optical activity of collagen in the solid state is almost indistinguishable from the activity in dilute solution.

It is therefore concluded that the collagen helix does not undergo any appreciable change as it goes from the dilute solution to the solid state (ca. 85 wt % protein). It appears instead that the large apparent difference between the optical activity of collagen in dilute solution and in the form of a solid film can be largely explained in terms of the transition from a random molecular orientation (in solution) to a planar orientation (in the film). Nevertheless, a small but significant difference, undetected in this work may exist between the helical structure of collagen in these two states.

In an attempt to explore the possibility of using the optical rotation measurement as a method for estimating the helical content of partially denatured gelatin, the specific rotations at 365mμ of collagen, hot cast gelatin and partially denatured gelatin (cast at 4°C and 23°C) were obtained. The results are tabulated in Table 3.4. By assuming the specific rotations of collagen and hot cast gelatin to be

TABLE 3.4

COMPARISON OF OR AND IR METHODS FOR THE ESTIMATION OF HELICAL CONTENT OF COLLAGEN

	$[\alpha]_{365m\mu}$	%-helicity based on $[\alpha]_{365m\mu}$	%-helicity based on R_{1235} (Table 3.3)	%-helicity based on R_{340} (Table 3.3)
Collagen	-3200±200	100% (assumed)	100% (assumed)	100% (assumed)
Gelatin (Cast at 4°C)	-2440±130	74%±4%	71%±9%	76%±12%
Gelatin (Cast at 23°C)	-1700±120	49%±5%	44%±10%	46%±10%
Gelatin (Cast at 60°C)	-260±30	0% (assumed)	0% (assumed)	0% (assumed)

the characteristic values of specific rotations of 100% native helix and 100% random coil, respectively, an estimate of the helical content for the two partially denatured gelatin can be obtained if a linear relationship between the specific rotation and helical content is assumed. The results, so calculated, are shown in Table 3.4 where they are compared with the estimates of helical content of identical samples based on the infrared absorbances at 1235 cm^{-1} and 340 cm^{-1}, described in the previous section. The excellent agreement in the estimates of helical content using these three independent methods strongly justify the assumption of linearity between the helical content and the parameters used in each of the three methods.

The error in the optical rotation (OR) method (±5%) is, however, less than that with the infrared (IR) methods (±10%) indicating that the OR method is more sensitive than the IR method. However, the OR method is only applicalbe to specimens which are non-birefringent and whose thicknesses are no greater than 0.001". If the OR measurements are conducted at a higher wavelength, say 600 mμ, this limiting thickness can be extended by about three fold. A linear relationship between the specific rotation and helical content, similar to the one described in this section, has been found to hold also for OR measured at these higher wavelengths. Hence, for specimens which are birefringent or are thicker than 0.003", the X-ray diffraction method (described in Section 3.3.4) or an alternative method must be developed to measure the helical content of collagen.

3.4. Enzymatic Degradation of Insoluble Collagen.

3.4.1. Introduction. One of the problems of using collagen as a biomaterial is its susceptibility to attack by collagenolytic enzymes (Chvapil, 1973). The degradation of the collagen by these enzymes (collagenases) induces a loss in the mechanical strength of the material (Salthouse et al., 1969) and seriously impairs its usefulness as a material for skin replacement. It is therefore necessary to find a method for determining the extent of degradation by collagenase of collagen and composite materials containing collagen which may be used for skin replacement.

Previous studies on the enzymatic degradation of collagen have been made on collagen in solution or in the form of reconstituted fibrils. The disadvantage of using collagen solution is, as discussed in Section 2.4.1, the necessity to use temperatures which are lower than the physiological temperature of 37°C since otherwise denaturation of the collagen, followed by non-specific attack of the molecule by the collagenase, occurs. Furthermore, since the collagen used for skin replacement must necessarily be in the solid phase to provide any structural integrity the methods used for studying the degradation of collagen in solution are not applicable for our present investigation.

The methods that have been used for studying the degradation of reconstituted collagen fibrils generally involve measuring the amount of fibrils which is solubilized by the enzyme by determining either the amount of the residual undigested collagen or the amount of solubilized protein. The amount of residual undigested collagen was

determined gravimetrically by Kono (1968) while the amount of solubilized protein has been determined by colorimetric assay of the amino acids released (Mandl, 1953), by the amount of radioactivity released from ^{14}C-labelled reconstituted collagen fibrils (Nagai et al., 1966; Lapiere and Gross, 1963), and by the analysis of chain fragments released using acrylamide gel electrophoresis (Lazarus, 1973). Many of the studies have been done on reconstituted fibrils which are not crosslinked and which can, therefore, be redissolved by appropriate alteration of the pH and ionic strength of the medium in which the fibrils are incubated.

The study of enzymatic degradation of insoluble (i.e., crosslinked) collagen fibrils has been rather limited. The colorimetric assay of amino acids released by the degradation (Mandl et al., 1953) has been used for studying the enzymatic degradation of insoluble bovine tendon collagen and presently serves as a standard assay for collagenases of commercial sources. However, since the release of free amino acids represents the final stages of the enzymatic degradation, the assay is necessarily very time consuming (involving the incubation of the insoluble collagen at 37°C for 18 hours) and does not yield any information on the earlier stages of the degradation. The gravimetric method for determining the amount of residual undigested collagen has also been used (Kono, 1968) to study the degradation of insoluble bovine tendon collagen. This method has been found useful in our investigation for studying the extent of degradation in materials that have been implanted in experimental

animals for a long time period - about 10 days (see Chapter 5). However, as a method for determining the extent of *in vitro* degradation in collagen that has been highly crosslinked, it has been found to be time consuming and not as sensitive as the machano-chemical method which we have developed and which will be described below. This observation is consistent with the observation of Loeven (1967) who found that the gravimetric method for determining the extent of degradation of elastin is not as sensitive as a mechanochemical method which is somewhat similar to the one in our present investigation.

Recently, an attempt was made (Harris and Farrell, 1972) to study the degradation of reconstituted collagen gels that have been crosslinked by formaldehyde by using ^{14}C-labelled collagen fibrils. A measure of the radioactivity in the collagenase solution in which the fibrils were incubated was taken as the extent of enzymatic degradation. This method was found to be suitable for detecting the degradation of lightly crosslinked collagen gels but was found to be lacking in sensitivity to detect degradation of moderately or highly crosslinked gels. No attempt was made in this study to measure quantitatively the extent of crosslinking and to correlate this with the extent of degradation of the collagen. In the present investigation, such a correlation was made using a mechanochemical method for determining the extent of degradation and the method outlined in Section 3.2 for determining the extent of crosslinking.

It must be pointed out that none of the methods described so far for determining the extent of degradation of insoluble collagen convey any information on the effect of the degradation of the mechanical behaviour of the collagen. In order to study such an effect, we have developed a mechanochemical method whereby the force induced on a collagen fiber (held at a fixed extension) in the presence of a solution of collagenase is recorded as a function of time. The rate at which the force relaxes (after correction for the mechanical force relaxation that would occur when a fiber is stretched in an enzyme-free solution) is then taken as a measure of the rate at which the fiber is degraded by the collagenase. In this section, the effect on this rate of degradation by a variety of factors that have been shown to affect the degradation of collagen in solution (Seifter and Harper, 1970) will be investigated. These factors include the temperature, pH and concentration of the collagenase solution and the presence of inhibitors in the solution. Other factors that will be shown to affect greatly the extent of degradation of insoluble collagen in this investigation are the strain on the collagen fiber, its helical content and its extent of crosslinking. The effect of these factors on the degradation of the insoluble collagen are carefully assessed with a view of attempting to retard as much as possible the extent of degradation.

3.4.2. Materials. The collagen fibers used in this investigation are either the bovine Achilles tendon (BAT) collagen tape supplied to us by Ethicon, Inc., N.J. (described in Section 3.2.3) or the rat tail

tendon (RTT) collagen teased out of tails of 3-month-old Sprague-Dawley rats by the method of Dumitru and Garrett (1947) and described in Section 3.1.2. In the case of the RTT, the reticular membrane surrounding the tendon fibers (Fig. 3.1) have been removed by immersion in 0.5M NaH_2PO_4 at 23°C over a two-hour period. Further cross-linking of the collagen fibers have been achieved by immersing them in solutions of aldehydes with the procedure described in Section 3.2.3. Characterization of the crosslink density was done using the method described also in that Section. Denatured collagen fiber was produced by immersing the fiber for 1 minute in a Tris-HCl buffer solution, pH 7.4 kept at 95°C.

Collagenase from Clostridium histolyticum, CLSPA grade, was purchased from Worthington Biochemical Corporation, Freehold, New Jersey. The collagenolytic activity of this bacterial enzyme has been carefully documented in several studies (Mandl, 1961; Seifter and Gallop, 1966; Kono, 1968; Harper and Kang, 1970; Harper, 1972). Several recent studies by independent investigators have confirmed the earlier discovery by Grant and Alburn (1959) that a crude preparation of the enzyme contains more than one collagenolytic enzyme (Mandl et al., 1964; Yoshida and Nodo, 1965; Harper et al., 1965; Kono, 1968; Kesselring et al., 1972). The Worthington CLSPA grade collagenase used in this investigation had been purified by precipitation with ammonium sulfate and by chromatographic fractionation. The preparation is substantially free of peptidase or trypsin-like activity. Never-

theless, Harper and Kang (1970) have shown that the preparation contains two collagenases (A and B), each possessing a distinct specificity which they were able to characterize, using as substrates collagen peptides of known sequence that had been cleaved from collagen with cyanogen bromide.

Characterization of collagenases A and B from Clostridium histolyticum has been previously made by Harper et al. (1965). Sedimentation equilibrium studies of solutions of the enzymes showed molecular weights of 105,000 for collagenase A and 57,400 for collagenase B. This observation, coupled with the observation that the amino acid compositions of the two collagenases are almost identical led to the postulation (Harper et al., 1965) that collagenase A is a dimer of collagenase B. Furthermore, Levidkova et al. (1963, 1964) have demonstrated that the abstraction of calcium ions from the collagenase with EDTA at ph 11 leads to the dissociation of the enzyme into four inactive subunits with molecular weights of ca. 25,000. All these observations would lead one to infer that collagenase A and B are made up of 4 and 2 subunits respectively, each subunit being of molecular weight ca. 25,000 and is held to each other through calcium ions. The identification of a collagenase with molecular weight 79,000 by Yoshida and Moda (1965) suggests that a trimer may possibly exist while the observation of heat precipitation of the collagenase, to be reported later in this investigation, suggests that larger aggregates are also possible. So far, the evidence points to the dimer

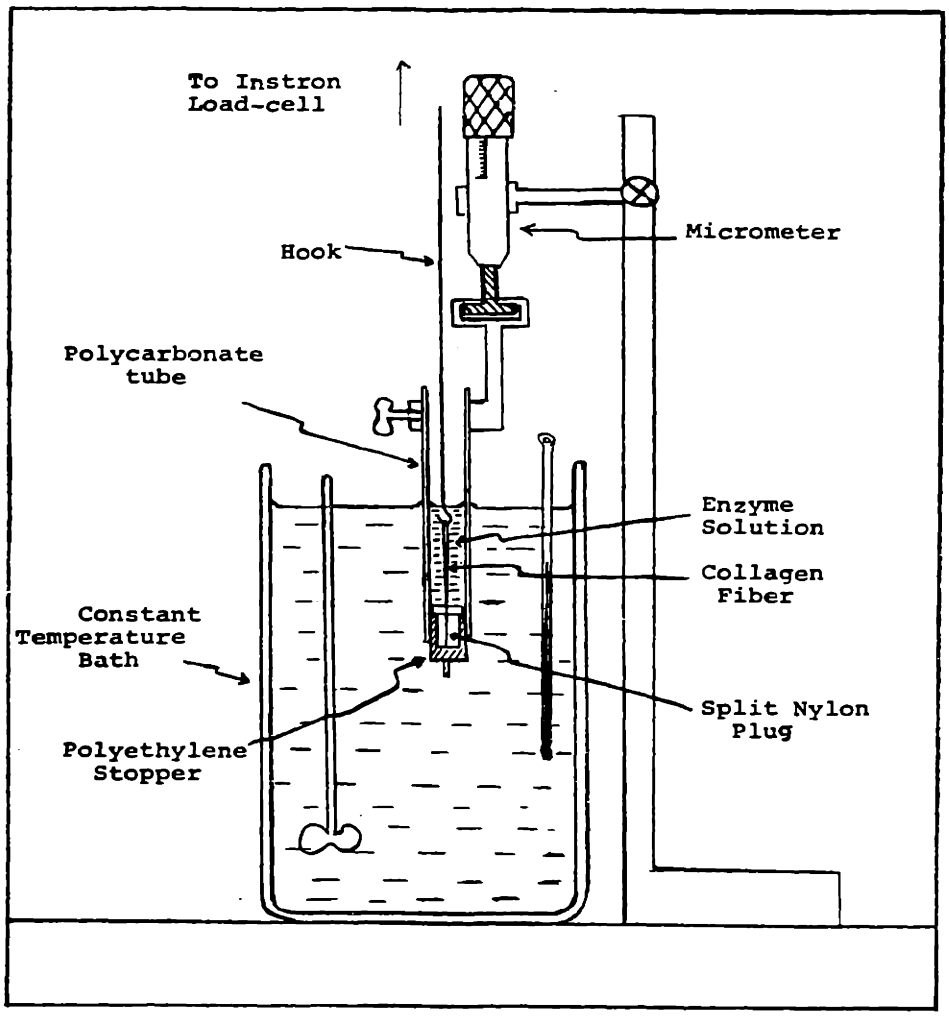


Fig. 3.28. Apparatus for determining the rate of enzymatic degradation of insoluble collagen.

(collagenase B), trimer and tetramer (collagenase A) being active against collagen in solution while the monomer and the larger aggregates are inactive.

Harper et al. (1965) have compared the activities of collagenases A and B on insoluble bovine collagen using the colorimetric method of Mandl et al. (1953) for determining the extent of degradation. They found that while collagenase A degraded the insoluble collagen, collagenase B did not. Since, in our present investigation, only insoluble collagen is used, the Worthington CLSPA grade collagenase was used as received without further fractionation into the two components.

The buffer solutions used in the present investigation were either 0.05M Tris-HCl buffer (for pH ranges of 7.0 to 9.5) or 0.05M Tris-Maleate buffer (for pH ranges of 5.0 to 7.5). Unless otherwise stated, the buffer solutions contain 0.005M $CaCl_2$. The inhibitors of collagenase that have been used in this investigation are EDTA (0.005M), o-phenanthroline (0.005M), 2, 3 dimercaptopropanol (5×10^{-5}M), and D, L-cysteine (0.005M), all purchased from Sigma Chemical Co., St. Louis, Missouri.

The activity of enzyme used in this investigation is expressed in terms of a unit which corresponds to the number of micromoles of leucine liberated from bovine tendon when incubated for 18 hours at 37°C in a solution of the enzyme (Mandl et al., 1953). In studying the effect of collagenase concentration on the enzymatic

degradation, the solutions of varying concentration of collagenase were prepared by diluting, with buffer solutions, a stock solution containing ca. 40 units/ml collagenase.

3.4.3. Experimental. The operating procedure consisted in stretching a length of the collagen fiber to a fixed, known extension and then monitoring continuously the relaxation of the force exerted by the fiber. The apparatus used for stretching the collagen fiber is shown in Fig. 3.28. This apparatus is basically the same as that used in Section 3.2.3 for studying the stress-strain behaviour of the fiber (Fig. 3.10) except that the present set-up enables one to use only a small volume (ca. 7cc) of the enzyme solution. The free ends of a looped collagen fiber, originally about 12 cm long, were clamped between the two halves of a split nylon plug which fits tightly into a hollow T 19/22 polyethylene stopper. The stopper was then inserted into the open end of a polycarbonate tube (1/2" ID, 5/8" OD, 6" long) and ca. 7cc of the enzyme solution was added into the tube. A thin rod with hooks at both ends was used to connect the looped collagen fiber to one of the load cells of the Instron Tester (Instron Engineering Corporation, Canton, Mass.) whose output in grams was calibrated before every measurement and was displayed on a Perkin-Elmer strip chart recorder Model 165.

The polycarbonate tube, containing the collagen fiber immersed in the enzyme solution was partially submerged into a constant temperature bath such that the level of the enzyme solution was just

above the water level of the bath. After equilibration, the bath maintains a constant temperature (within ± 0.2°C) in the enzyme solution. Stretching of the collagen fiber was achieved by moving the polycarbonate tube vertically downwards using a clamping system attached to the moving rod of a micrometer head (Fig. 3.28). The strain on the fiber was determined with the help of a cathetometer.

Prior to its introduction into the collagen fiber, the collagenase solution was incubated for ca. 30 mins at the temperature of measurement. Over that same period of time, the collagen fiber was immersed in a Tris buffer solution, kept at the temperature of measurement. This was found necessary in order to eliminate the considerable amount of stress relaxation that takes place when the relative dry fiber is swollen in an aqueous medium. When conditioned in this manner and then stretched in the presence of Tris buffer, the fiber undergoes a small amount of stress relaxation over the first 40 min (Fig. 3.29), but showed no relaxation beyond that point over the duration of the experiments (2 1/2 hours). By contrast, when the preconditioned fiber was immersed in a bath containing collagenase, the force relaxed continuously until the specimen failed. Force data obtained in the presence of collagenase were corrected for the "baseline" relaxation which occurs in Tris buffer (Fig. 3.29).

Relaxation data obtained at 37°C (Fig. 3.29) showed that up to the point before failure of the fiber occurs the force f can be represented by a single negative exponential term:

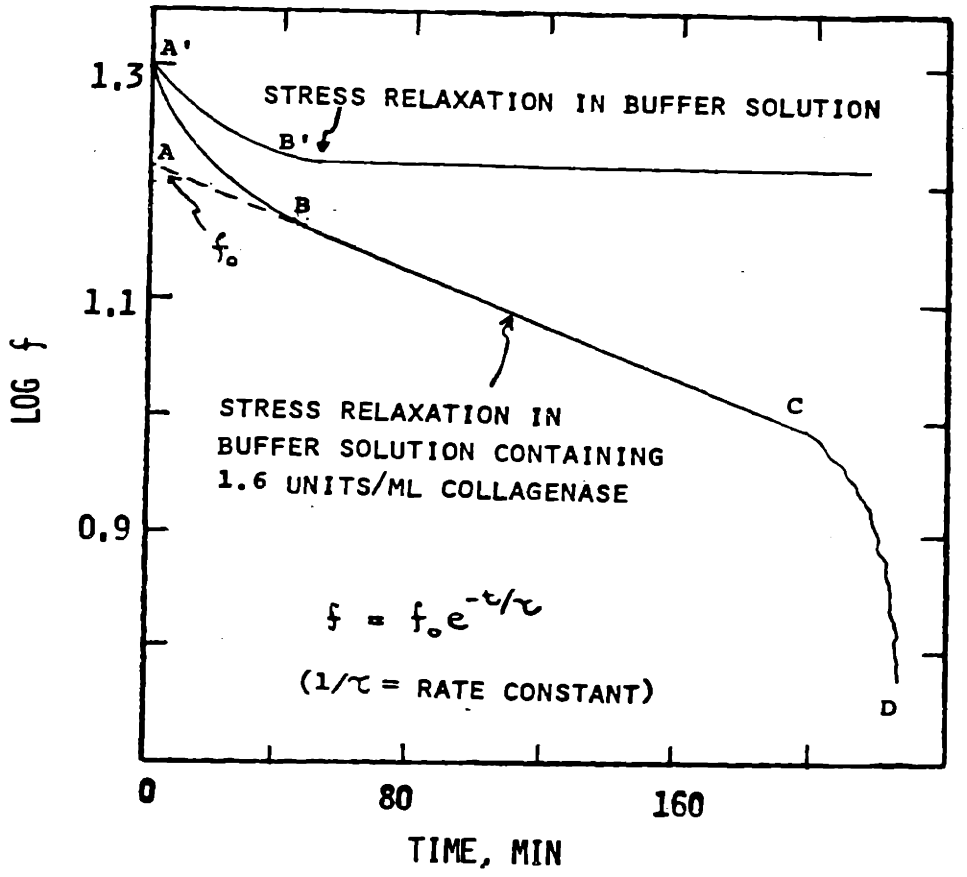


Fig. 3.29. Typical Stress relaxation curves of collagen fibers, stretched to fixed extensions, in the presence and absence of collagenase in buffer solution (0.05M Tris-HCl buffer, pH 7.4).

$$f = f_o e^{-t/\tau} \tag{3.14}$$

where τ is the characteristic relaxation time and f_o is the initial force on the fiber after correction for mechanical relaxation in the buffer solution. The slope of the semilogarithmic plot in Fig. 3.29 yields the reciprocal relaxation time ($1/\tau$) - a quantity identical to the rate constant of a first order chemical reaction. This quantity is taken as a measure of the rate of enzymatic degradation of the collagen fiber by the collagenase solution.

The effect on $1/\tau$ of stretching the collagen fibers to different strain levels was initially determined in order to establish an appropriate level of strain which can be used for subsequent investigations. It was found that for both crosslinked BAT and RTT collagen fibers, $1/\tau$ is almost invariant with strain in the strain region of $4.0 \pm 0.5\%$ (Fig. 3.35). All subsequent studies were done in this strain region in order to avoid the large errors involved in operating in strain regions where $1/\tau$ is very sensitive to the small variation in strain levels that invariably occurs when collagen fibers are stretched from one experiment to another.

By varying the concentration, pH and temperature of the collagenase solution, the effect of these factors on $1/\tau$ was determined. In the studies involving inhibitors of collagenase, the inhibitors were incubated with the enzyme for 30 min at the temperature of measurement (37°C) before the mixture was introduced into the collagen fibers. In all cases, reversibility of the inhibition was

tested by the addition of $CaCl_2$ solution (in excess of the concentration of the inhibitors present) to the inhibited collagenase solution after $1/\tau$ in latter solution was determined. A new collagen fiber was used to determine $1/\tau$ in the resultant solution.

The effect of denaturation of the collagen fibers on $1/\tau$ was also determined. It was found that, in contrast to the undenatured collagen fiber, $1/\tau$ for the denatured fiber decreases monotonously with strain on the fiber (Fig. 3.36). An arbitrary strain level of 45 ± 2% was used to study the effect of collagenase concentration on the rate of degradation of the denatured collagen fiber. The helical content of the denatured collagen fiber was determined by the IR absorbance at frequency $340cm^{-1}$, normalized for thickness (described in Section 3.3.4). Finally, the effect of crosslinking of the collagen on $1/\tau$ was determined by using collagen fibers of different M_c values prepared and characterized as outlined in Section 3.2.

3.4.4. Results and Discussion. Most of the results reported in this section will be based on studies done on BAT collagen tapes. The uniformity in cross sectional area and the homogeneity in property of these tapes make them ideally suited for the present studies since very reproducible results can be obtained for collagen tapes cut from different parts of the same batch of preparation. However, batch-to-batch variations are greater and, hence, for each series of experiment involving the variation of an influencing factor, tapes from the same batch were used. In contrast, the variation in behaviour of the RTT is

considerably greater even for tendons removed from the same tail. A limited amount of studies on the RTT have, therefore, been made. These studies, however, indicate that the various influencing factors on the degradation of the BAT collagen, to be discussed below, have identical influence on the degradation of the RTT collagen.

Fig. 3.29 shows a typical curve of force (plotted on a logarithmic scale) versus time for a collagen fiber (held at fixed extension) in the presence of a known concentration of collagenase solution. Both BAT and RTT collagen fibers yield curves with identical features shown in the figure. The initial portion of the curve (A'-B) exhibits a rapid relaxation of the force followed by a more gradual relaxation (B-C) which appears linear when plotted semilogarithmically as shown in Fig. 3.29 . Correction for the baseline relaxation which occurs in the Tris buffer (A'-B') results in a linear plot (A-B) for the initial portion of relaxation of the fiber in the collagenase solution. Hence, up to point C (Fig. 3.29), relaxation of the force can be represented by Eq. 3.14, presented in the last section. At point C, the relaxation plot becomes non-linear and, at the same time, extensive fibrillation of the collagen fibers can be observed visually. Shortly afterwards (at point D), failure of the collagen fiber occurs. The rate at which point C occurs in an experiment is dependent on the rate of degradation of the collagen fiber which in turn is dependent on the various factors to be described later in this Section. In all the experiments done in this investigation, the conditions were chosen such that the linear portions (A-C) of the

relaxation plots (Fig. 3.29) were of sufficiently long duration (>100. min) to enable an accurate determination of $1/\tau$ from the slopes of the plots.

The effect of altering the concentration of the collagenase on the rate of enzymatic degradation at 37°C is shown in Fig. 3.30. The strain level used (ε) was in the region of 4.0 ± 0.5% where the rate is almost invariant with strain (see Fig. 3.35). The crosslink density of the collagen (indicated by M_c) was determined using the method outlined in Section 3.2.3 while its helical content (%-H), as measured by the IR absorbance at 340cm^{-1} (Section 3.34) shows that it was fully native. It can be seen from Fig. 3.30 that the rate of degradation, indicated by the slope of the semilogarithmic plots, increases with the concentration of the enzyme. A plot of the rate of degradation ($1/\tau$) versus concentration of enzyme (C) is shown in Fig. 3.31. For the concentration range used in the experiment, the rate of enzymatic degradation ($1/\tau$) is a linear function of the collagenase concentration. This observation is consistent with that made by Gallop et al. (1957) who showed that a similar relationship exists (for low concentrations of enzyme) in the degradation of collagen in solution. Analtyically, this result can be represent as

$$1/\tau = Kc \qquad (3.15)$$

where c is the enzyme concentration in units/cc and K is a constant equal to 2.18 x 10^{-3} cc unit^{-1} min^{-1} for conditions shown in Fig. 3.31.

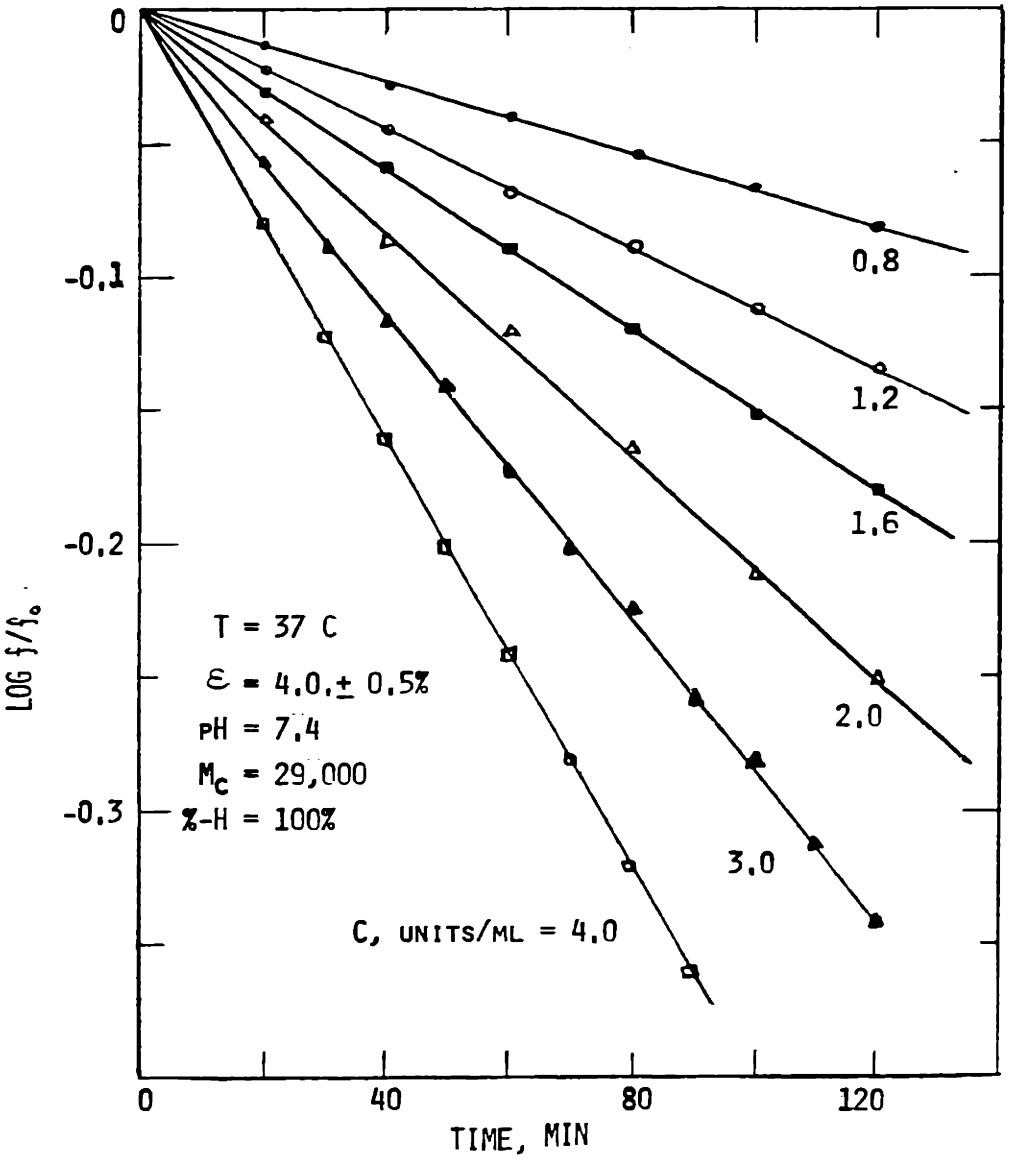


Fig. 3.30. Stress relaxation of collagen fibers, stretched to a fixed strain level (ε), in the presence of collagenase solutions of varying concentrations (c). The temperature (T) and pH of the collagenase solutions are as indicated. The extent of crosslinking (M_c) and helical content (%-H) of the collagen fibers used are as shown.

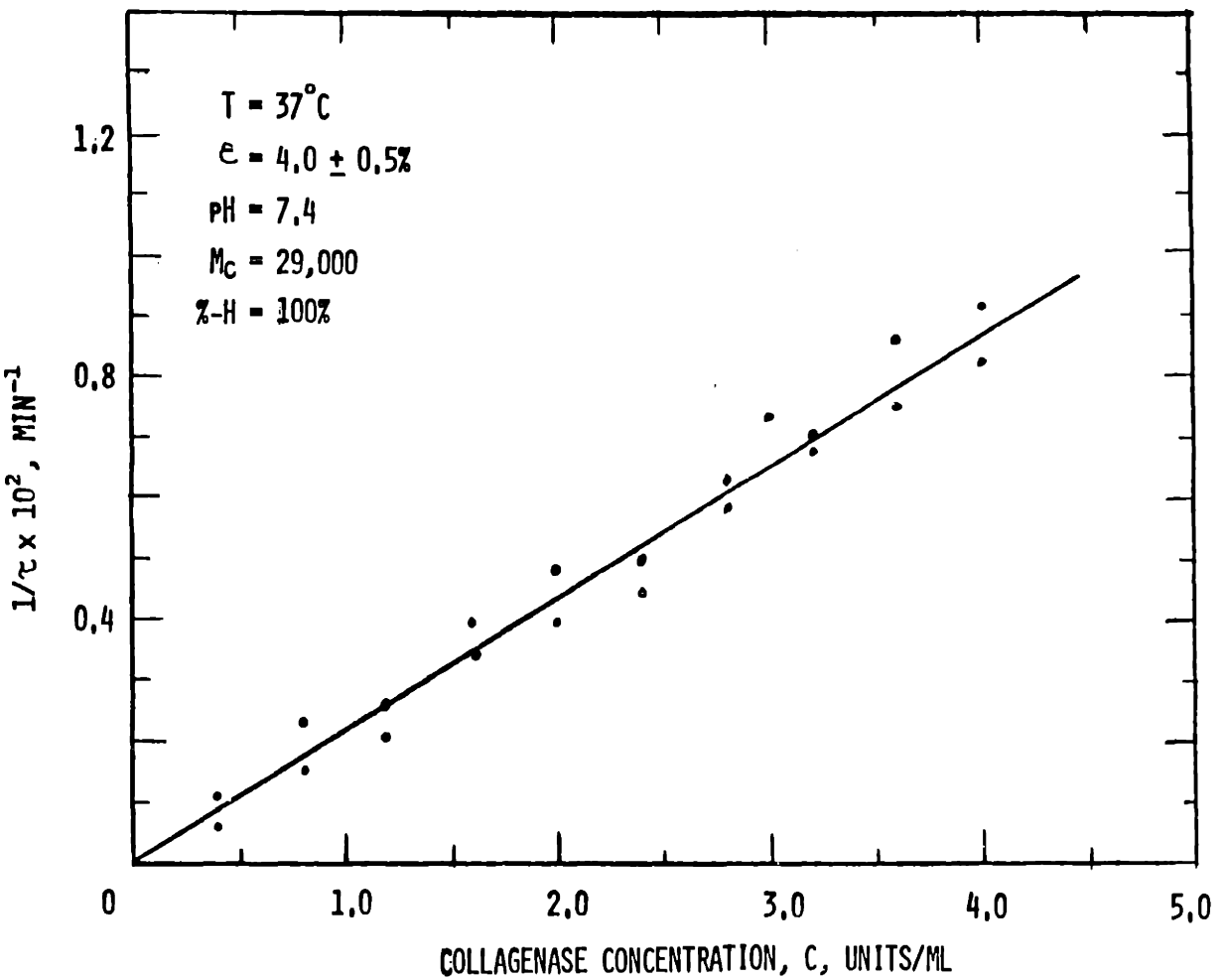


Fig. 3.31. The rate of enzymatic degradation of collagen fibers ($1/\tau$) as a function of the collagenase concentration. See legends to Fig. 3.30 for explanation of symbols.

The effect of pH of the collagenase solution on $1/\tau$ is shown in Fig. 3.32. The bell-shaped curve, exhibiting an optimum enzymatic activity at ca. pH 7-8 is similar to those obtained by other investigators who used collagenases from both bacterial (Mandl et al., 1953; Takahaski, 1967; Rippon, 1968) and animal (Kang et al., 1966; Perez-Tamayo, 1970) sources. The discovery of these collagenases with maximum activity at physiological pH had weakened the previous postulations that the enzymes are lysosomal in nature (see Section 2.4.1) and had led to postulations (Gross, 1964; Nagai et al., 1966; Kang et al., 1966) that the in vivo degradation of collagen occurs extracellularly. The inactivation of the collagenase solution by altering the pH to either extremes from the optimum was found to be irreversible. The possible mechanisms by which the optimum pH of activity of an enzyme can be explained are reviewed exhaustively in a book by Webb (1963) while the techniques involved in establishing the exact mechanism is treated excellently in a book by Volkenstein (1969). These two books can serve as useful guides to further studies in explaining the bell-shaped curve obtained in Fig. 3.32 for the collagen-collagenase system.

The effect of temperature (T) on the enzymatic activity of the collagenase is shown in Fig. 3.33. Below ca.56°C, the rate of enzymatic degradation ($1/\tau$) appears to follow the Arrhenius relationship:

$$\frac{1}{\tau} = Ae^{-E/RT} \tag{3.16}$$

where A is the frequency factor and E is the activation energy. For

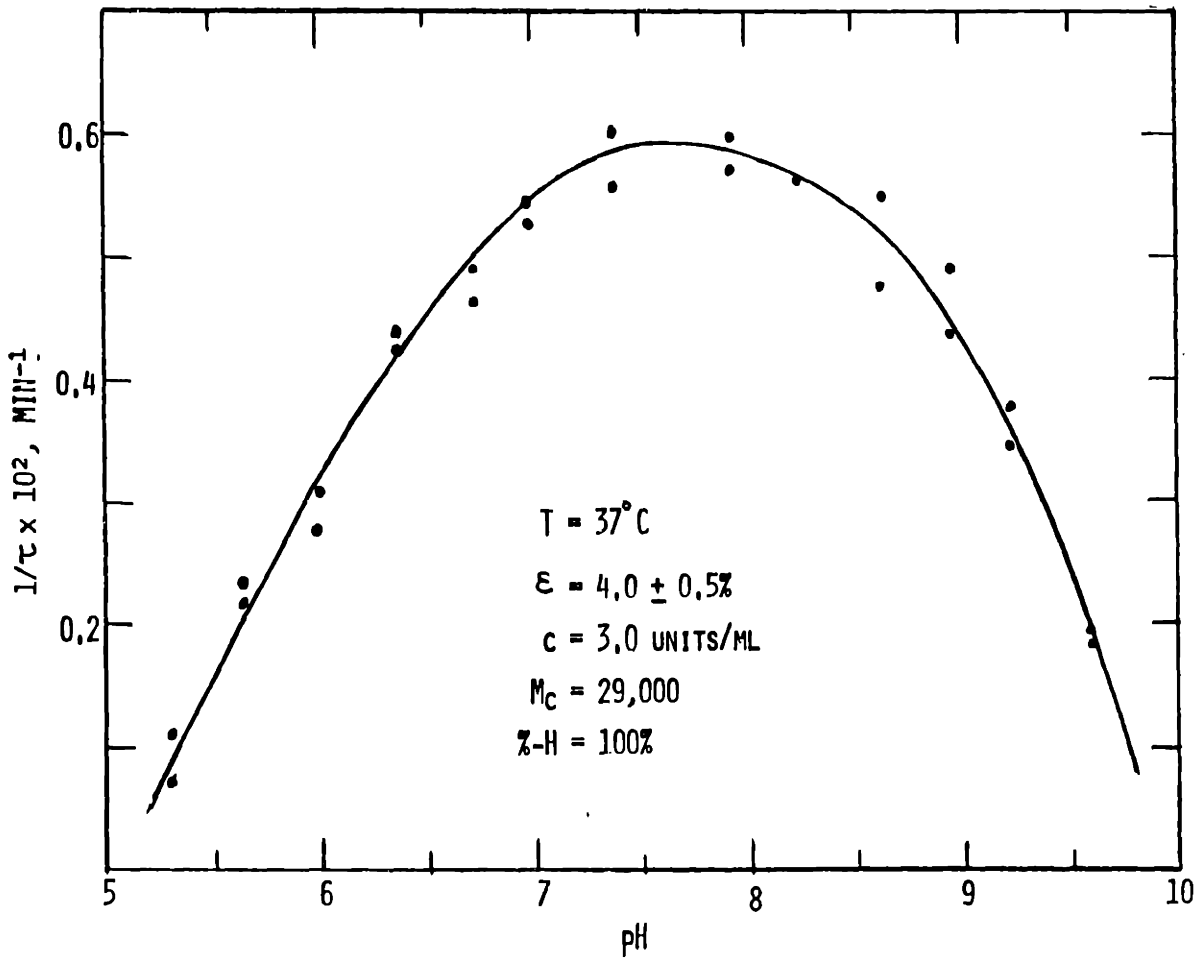


Fig. 3.32. The rate of enzymatic degradation of collagen fibers as a function of pH of the collagenase solution. See legends to Fig. 3.30 for explanation of symbols.

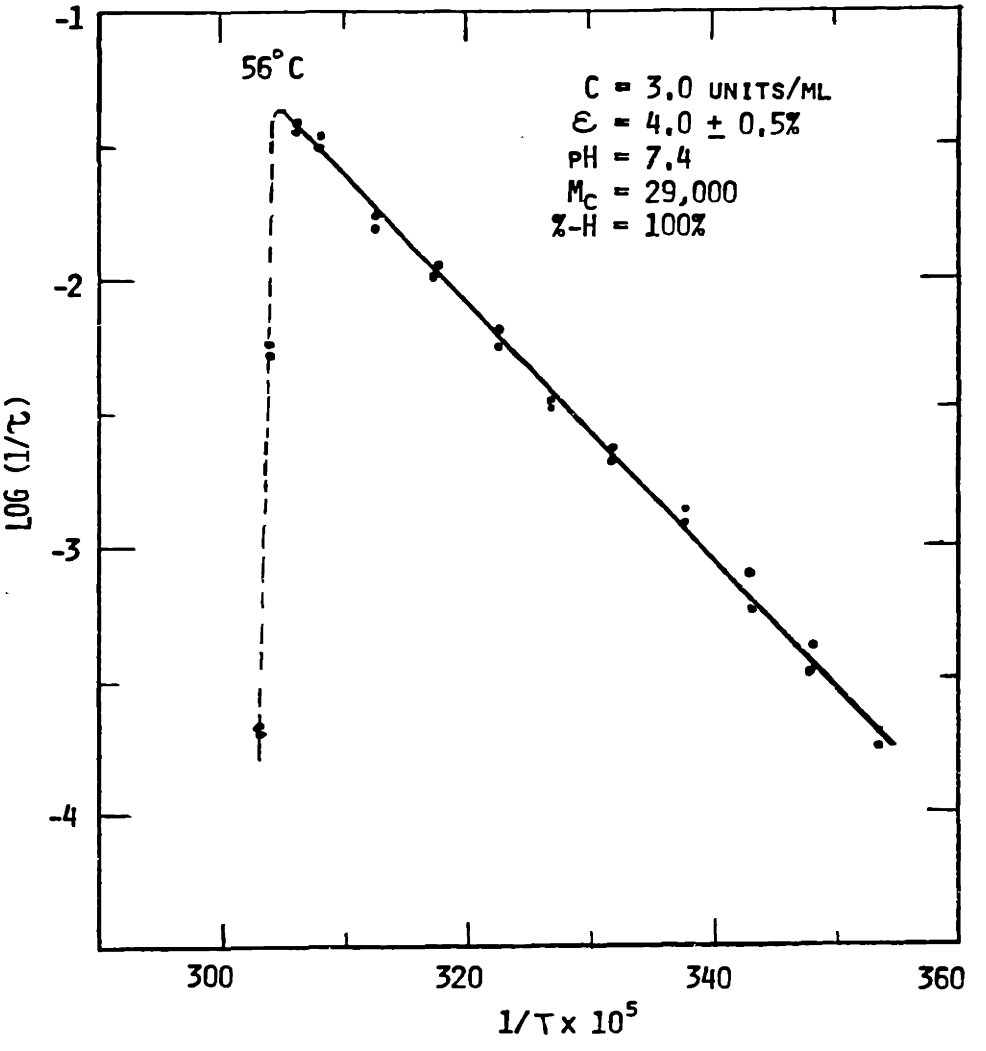


Fig. 3.33. The rate of enzymatic degradation of collagen fibers as a function of temperature of the collagenase solution, plotted in a manner which tests the applicability of the Arrhenius relationship (Equation 3.16). See legends to Fig. 3.30 for explanation of symbols.

the conditions listed in Fig. 3.33, $A = 3.16 \times 10^{11}$ min^{-1} and $E = 19{,}700$ cal/mol. The existence of a single activation energy over this fairly wide range of temperature (10° to 56°C) suggests that only one species of collagenase in the enzyme solution is capable of attacking the insoluble collagen. This is consistent with the findings of Harper *et al.* (1965) who showed that the Worthington CLSPA grade collagenase contains two collagenolytic enzymes, only one of which (collagenase A) is capable of degrading insoluble collagen. The complete and irreversible inactivation of the enzyme at ca. 56°C (Fig. 3.33) is due to the precipitation of the collagenase at this temperature. This can be observed clearly if a concentrated solution of the enzyme (ca. 1000 units/ml) is heated above this temperature. A turbid solution develops almost instantly when the solution reaches ca. 56°C.

The effect of a number of inhibitors on $1/\tau$ is shown in Fig. 3.34. Complete inhibition was achieved by the addition of 0.005M EDTA or 0.005M of o-phenanthroline. The addition of 0.01M $CaCl_2$ to the resultant inhibited collagenase solutions shows that while the inhibition of the EDTA can be reversed (Fig. 3.34), that of the o-phenanthroline cannot be reversed by the addition of excess Ca^{++}. This is consistent with the findings of Seifter *et al.* (1959) who showed that the EDTA chelates calcium ions which are necessary for the binding of the collagenase to collagen in solution while the o-phenanthroline chelates zinc ions which are necessary for the active

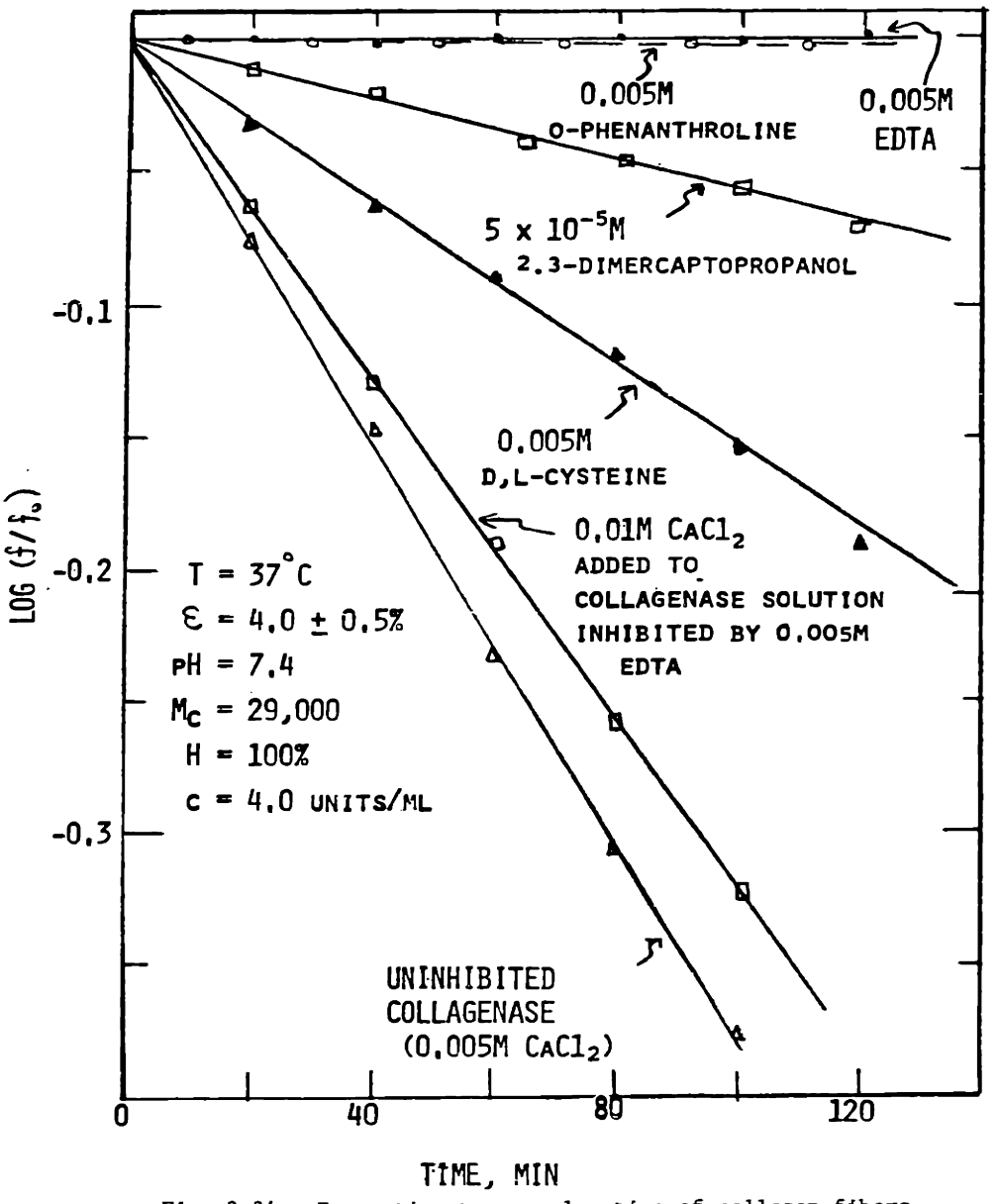


Fig. 3.34. Enzymatic stress relaxation of collagen fibers conduced in the presence of a number of inhibitors, as indicated. Note the reversibility of the inhibition of the 0.005M EDTA by the addition of 0.01M Ca Cl_2 to the inhibited solution. See legends to Fig. 3.30 for explanation of symbols.

centers of the enzyme. The present study shows that the ion requirements for the binding and active centers of the enzyme are the same for both soluble and insoluble collagen substrates. The inhibition by 5×10^{-5}M 2,3 dimercaptopropanol and 0.005M cysteine (due also to the chelation of zinc ions) have also been found for degradation of soluble collagen by collagenase (Seifter et al., 1959). The difference in mode of reaction of these two inhibitors has been demonstrated by Seifter and Harper (1971). Cysteine apparently combines with the zinc ion and is fixed to the enzyme through this interaction, whereas the 2,3 dimercaptopropanol abstracts the zinc ion from the enzyme.

The effect of varying the strain level applied to the collagen on its degradation by collagenase is shown in Fig. 3.35. The rate of degradation of the collagen ($1/\tau$) apparently goes through a minimum at ca. 4.0 ± 5% strain level as the strain on the collagen is varied from ca. 1.0% to 7.5%. The rate increases sharply for strain levels below this minimum activity strain region while for strain levels above this region, the rate increases moderately. A possible explanation for this behaviour is provided by the stress-strain curve of the collagen tape which is also included in Fig. 3.35. The initial, low-modulus portion of this curve probably corresponds to the uncrimping of the collagen fibers which usually occur as crimped fibers with crimp frequency of ca. $0.01\mu^{-1}$ (See Fig. 3.1) (This uncrimping behaviour has been confirmed by Rigby et al. (1959) for RTT which we have found to possess a similar strain dependence of $1/\tau$ as the

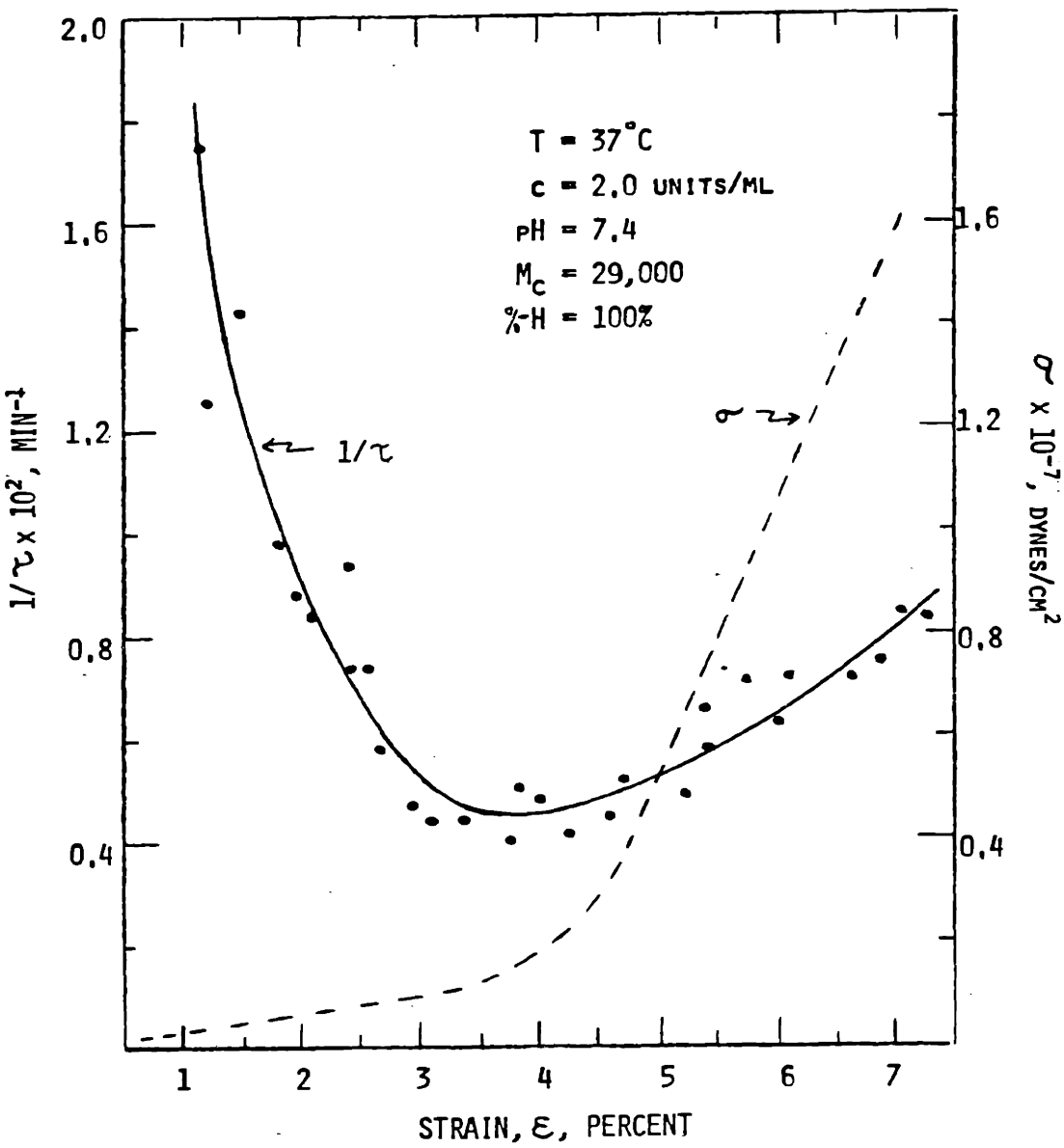


Fig. 3.35. The rate of enzymatic degradation of collagen fibers as a function of the strain levels imposed on them. Included in the graph is the stress-strain curve of the collagen fibers. See legends to Fig. 3.30 for explanation of symbols.

collagen tape). At strain levels higher than ca. 4.0%, the fibers are straightened out and thus exhibit their characteristic high modulus. The concomitant occurrence of a minimum enzymatic degradation rate at the strain level where uncrimping of the fiber is completed suggests that the initial sharp decrease in enzymatic degradation with strain (between 1% to 4%) is due to uncrimping of the fibers. The result of the latter is a closer packing of the fibers so that the "pores" between them are reduced in size and in number. The reduction of these "pores" effectively retard the diffusion of the collagenase molecules into the fibers and, hence, reduce their rate of degradation of the collagen.

The increase in rate of enzymatic degradation of the collagen with increasing strain at strain levels exceeding 4% (Fig. 3.35) is probably due to the opening of new sites of attack by collagenase on the highly strained collagen molecules. This increase in enzymatic degradation due to the development of high stresses on the collagen fibers may be important in explaining the development of osteoarthritis. This condition, which is marked by extensive degradation of the collagenous structure of cartilage (Bollet, 1969) can be induced in experimental animals by the application of impact load on the cartilage (Radin and Paul, 1971; Simon et al., 1972). The relase of collagenolytic enzymes in the synovium of a number of degenerative joint diseases has also been demonstrated (Harris et al. 1969).

The effect of strain alone may not be sufficient to explain the normal development of osteoarthritis since other factors may be involved. It has been shown that the mucopolysaccharide (MPS) content of osteoarthritic cartilage is significantly lower than that of normal cartilage (Hjertquist and Lemperg, 1972). As will be shown in Chapter 4 of this thesis, the presence of MPS bound to collagen can effectively inhibit its enzymatic degradation by collagenase. The loss of MPS from osteoarthritic cartilage may thus induce an accelerated enzymatic degradation of the collagen fibers of the cartilage. Furthermore, Nimni and Deshmukh (1973) has shown that the collagen produced in osteoarthritic cartilage differ in structural features from cartilage produced in normal cartilage. It should be interesting to compare the susceptibility of these two types of collagen to collagenase degradation, and to compare their ability to interact with MPS. In this context, it is interesting to note that normal cartilage collagen is not attacked by collagenases which are active against skin and bone collagens (Robertson and Miller, 1973).

The effect of denaturation of the collagen on its susceptibility to attack by collagenase is shown in Figs. 3.36 and 3.37. In Fig. 3.36, the effect of strain on the degradation of both denatured (45% helicity) and native collagens are compared. It can be seen that the denatured collagen fiber is considerably more susceptible to collagenase attack than the native fiber. This is in agreement with the studies of Harrington and von Hippel (1961) who showed that denatured collagen in solution is considerably more

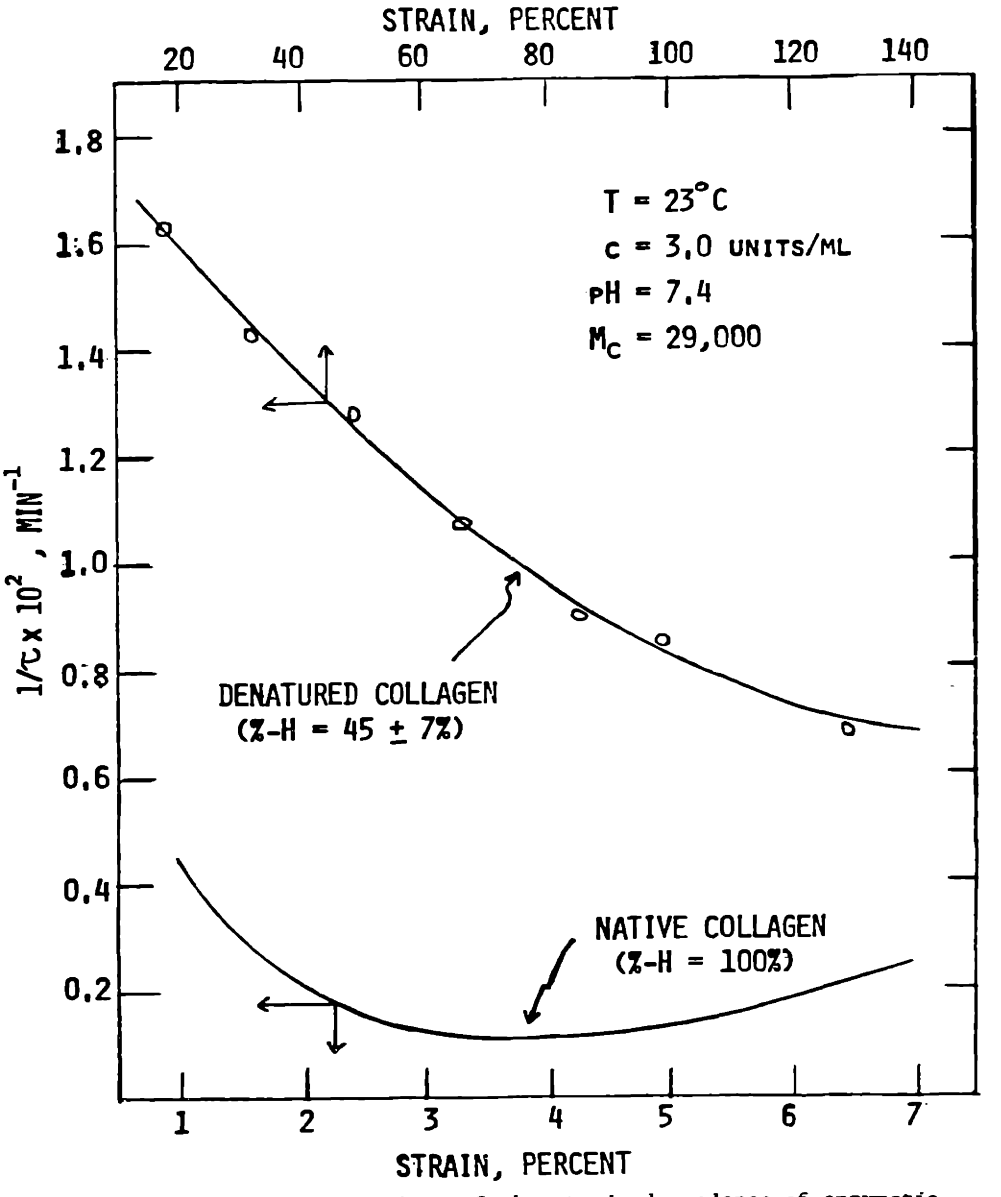


Fig. 3.36. Comparison of the strain dependence of enzymatic degradation rates for native and denatured collagen. Note the substantially higher rate of enzymatic degradation of the denatured collagen. See legends to Fig. 3.30 for explanation of symbols.

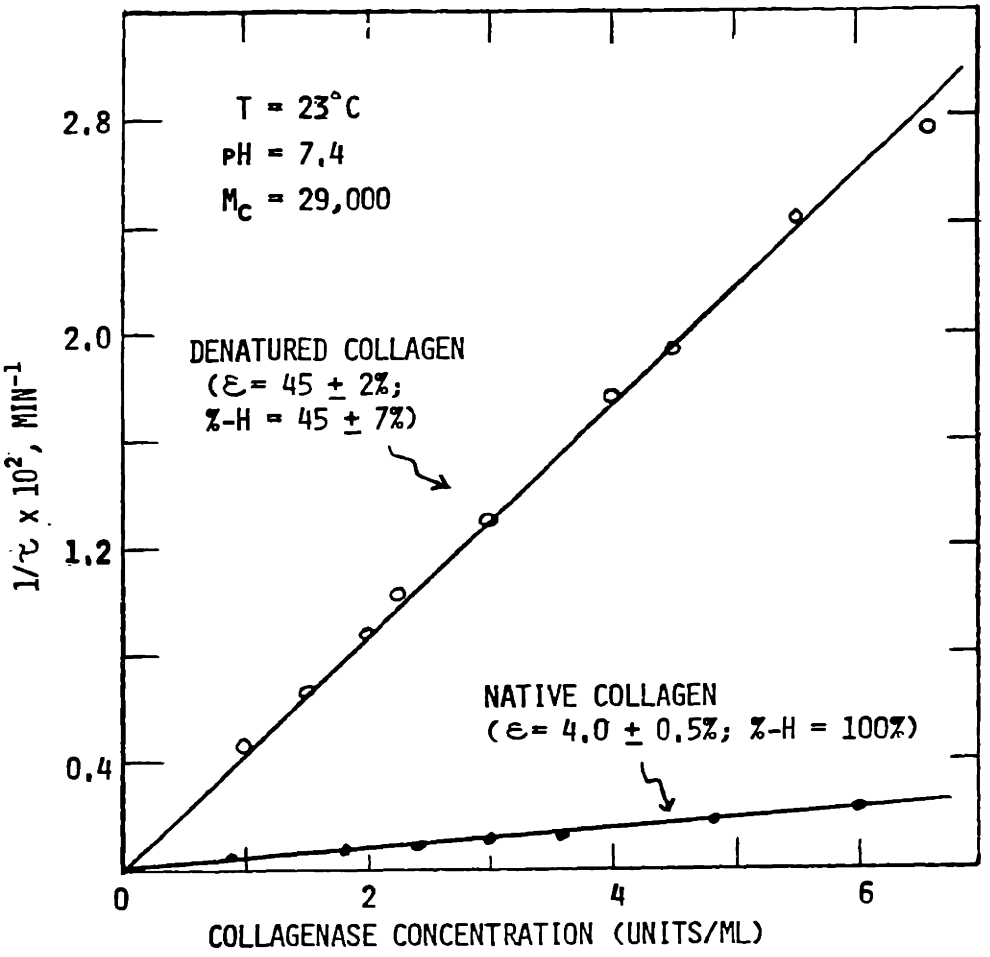


Fig. 3.37. Comparison of the collagenase concentration dependence of enzymatic degradation rates for native and denatured collagen. Note the substantially higher rate of enzymatic degradation of the denatured collagen. See legends to Fig. 3.30 for explanation of symbols.

susceptible to attack by bacterial collagenase than the native collagen in solution.

Studies on semipurified preparations of mammalian collagenases (Sakai and Gross, 1967; Jeffrey and Gross, 1967) showed that these collagenases attack native collagen at physiological temperature to yield two fragments which spontaneously denature into non-helical gelatin polypeptides. The latter were shown to be rapidly degraded by the same preparation of mammalian collagenase to yield much smaller polypeptides, although it remains unclear whether this action was caused by the same collagenase that attacked the native collagen or by other proteases which might have been present in the preparation. A recent study by McCroskery et al (1973), who used a highly purified form of mammalian collagenase (from rabbit tumor), showed that while the collagenase attacked collagen in a way similar to other mammalian collagenases (see Section 2.4.1), it was not capable of attacking denatured collagen. Whether this is a unique case or is general for all the mammalian collagenases remains to be answered. Nevertheless, the study suggests that the complete degradation of collagen in vivo may involve proteases other than collagenase. Such a protease has been isolated in cultures of rheumatoid synovial tissues and shown to be capable of degrading gelatin to small fragments at a rate much faster than synovial collagenase (Harris and Krane, 1972).

In view of these studies with mammalian collagenases,

the question as to whether the bacterial collagenase preparation used in the present study contained proteases other than collagenase poses itself. Although the preparation is known to be free of peptidase or trypsin-like activity, the presence of other proteases capable of degrading denatured collagen may explain the accelerated attack of the latter by the collagenase preparation (Figs. 3.36 and 3.37). The identity of such proteases has yet to be demonstrated. However, the observation that denatured collagen, when implanted in experimental animals are also more readily resorbed than native collagen (Woessner, 1968) suggests that the in vitro degradation of collagen (whether native or denatured) by bacterial collagenase may be utilized with advantage for estimating the extent of in vivo degradation of collagenous materials implanted in experimental animals. A correlation of this kind for native collagen crosslinked to different extent has been obtained (see Chapter 5).

The helical content of the denatured collagen shown in Fig. 3.36 has been measured (using the IR absorbance at $340cm^{-1}$) with the fiber in an unstrained position. Stretching the denatured collagen fiber can increase the helical content of the collagen, as deduced by x-ray studies (Ramachandran, 1967). This probably explains the monotonous decrease in the susceptibility of the denatured collagen fiber to collagenase degradation as the strain of it is increased. The increase in helical content of the denatured collagen fiber as it is stretched shows that it is adopting conformations which approach that of the collagen. Consequently, the enzymatic

degradation of the denatured collagen decreases and tend to approach that of the native collagen fiber. However, the latter never actually occurs because failure of the denatured collagen fiber occur before this point. Fig. 3.37 shows that the linear relationship between $1/\tau$ and collagenase concentration (c) found for native collagen (Fig. 3.31) holds true also for the denatured collagen fiber although the slope of the $1/\tau$ vs c plot is much greater for the latter case.

Finally, the effect of crosslinking the collagen fiber on the rate of enzymatic degradation is shown in Fig. 3.38. The rapid decrease in the rate of $(1/\tau)$ with increase in crosslinking density (decreasing M_c) is consistent with the qualitative observations of Harris and Farrell (1972) and justifies quantitatively the age-old practice of crosslinking collagen sutures to reduce their rate of resorption by the body. In attempting to produce collagenous materials which are resistant to degradation it is clear that maximum crosslinking of the collagen should be achieved. Of the crosslinking agents studied, glutaraldehyde was found to be the most efficient crosslinking agent (see Section 3.2.4). A correlation curve of the sort shown in Fig. 3.38 for the *in vitro* degradation of the collagen can be invaluable for predicting the extent of *in vivo* degradation of collagen if the latter can be correlated with crosslink density of the collagen implanted. Such a correlation has been obtained and will be described in Chapter 5 of this thesis.

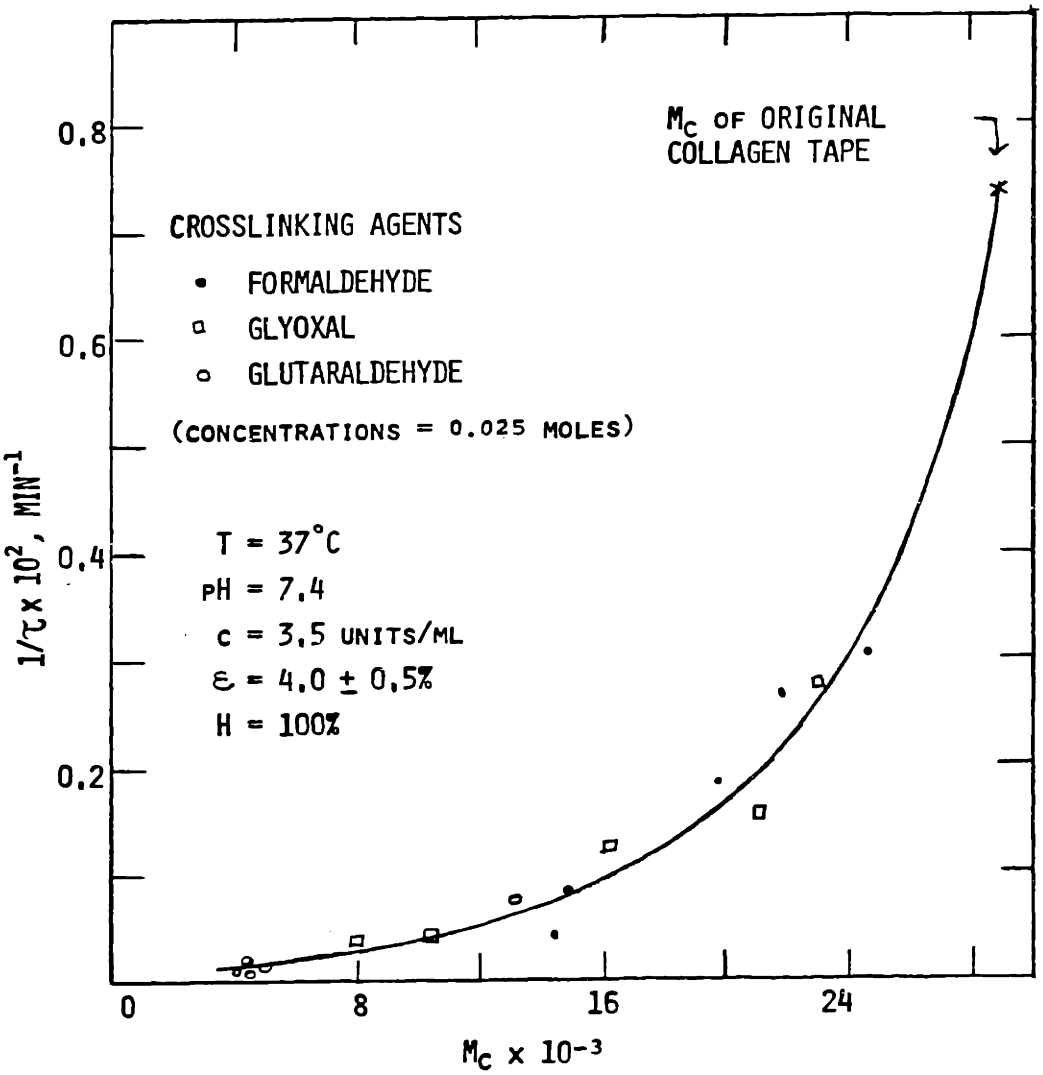


Fig. 3.38. The rate of enzymatic degradation of collagen fibers as a function of crosslinking density of the collagen. The various extent of crosslinking of the collagen fibers were obtained by utilizing different crosslinking agents, as indicated, and different duration for crosslinking. See legends to Fig. 3.30 for explanation of symbols.

Chapter 4

COLLAGEN-MUCOPOLYSACCHARIDE COMPOSITE MATERIALS

While the importance of collagen as a structural protein in the connective tissue is well recognized, the functional significance of the mucopolysaccharides (MPS) and protein-polysaccharides (PPS) that are generally found associated with the collagen fibers is still unclear. Much of the studies aimed at elucidating the functions of MPS/PPS have been focussed on the interaction of collagen with the MPS/PPS and on the physical properties of the MPS/PPS. A review of the interaction of collagen with MPS/PPS will be made in Section 4.1 while the physical properties of the MPS/PPS have been discussed in Sections 2.2.4 and 2.6.2.

From these studies, a number of functions of the MPS/PPS have been postulated. These include the ability of the MPS/PPS (1) to regulate the growth of collagen fibrils and, hence, control their size; (2) to direct the location of growth of the collagen fibrils in the extracellular space; (3) to stabilize the fibrils, making them more resistant to denaturation and dissolution; (4) to strengthen the collagen fibrils by amalgamating them; (5) to lubricate the collagen fibers and thus enable them to be more deformable; (6) to act as a molecular sieve in the connective tissue, allowing the diffusion of selective molecules in and out of the tissue; and

(7) to form a high osmotic pressure within the tissue and thus prevent the loss of fluid caused by deformation.

The main objection to the postulations of functions of MPS/PPS based on *in vitro* studies of their interaction with collagen is that the conditions *in vivo* are never exactly simulated in the *in vitro* studies. Postulations on the functions of MPS/PPS based on their physical properties *per se* tend to overlook the probable alteration of these properties by the interaction of the MPS/PPS with collagen. It is for these reasons that this author feels that a study of composite materials made up of collagen and MPS/PPS may give a better indication of the functions of the MPS/PPS in connective tissues. In this Chapter, special emphasis will be given to the mechanical behaviour of these composite materials (Section 4.3) and their susceptibility to enzymatic degradation (Section 4.4).

4.1. Interaction of Collagen with Mucopolysaccharides and Protein-Polysaccharides (Review).

4.1.1. *Introduction.* The concept of an interaction between mucopolysaccharides and protein-polysaccharides (MPS/PPS) on one hand, and collagen fibers on the other, dates back to almost a century ago when Flemming (1876) noted that a mucinous substance ("Kittsubstanz") could be extracted from tendon with lime water and that this resulted in the tendon fibers splitting into very thin fibrils. He suggested that these so called "ground substances" acted as a con-

nective tissue cement, holding the collagen fibrils together in a network swollen with water. This suggestion, however, went largely unnoticed for over half a century, with researches on collagen and MPS/PPS going on in divergent ways. Studies on collagen, both histologically and biochemically, surged ahead of studies on MPS/PPS because the collagen fibrils were easier to identify, both structurally and chemically, and were assumed to play the major role in the properties of connective tissues (see Section 2.2.1).

In the mid 1930's, Karl Meyer, in search of a substrate for the enzyme lysozyme, became interested in the "mucoid" ground substances and has since contributed significantly to the understanding of the chemistry and structure of the MPS/PPS (see Section 2.2.4). It was Meyer (1946) who first proposed that the regularly spaced acid groups of the MPS/PPS might act as a template upon which insoluble collagen fibrils are put together.

Since Meyer's proposal, a considerable amount of data has accumulated which suggests that the MPS/PPS may play an important role in both collagen fibril formation and in fibril stabilization (i.e. resistance to dissolution). However, evidence to the contrary has been brought forward. Some of these evidence are convincing enough to make the whole question of an interaction between collagen and MPS/PPS a controversial one. Two recent reviews on this subject (Jackson and Bently, 1968; Balazs, 1970, Vol. 2) appear to assume a direct interaction between collagen and MPS/PPS. In this Section the author will attempt, however, to survey the situation without such an assumption and will review the various pieces of

evidence that have been presented for or against the importance of in vivo interaction of MPS/PPS with collagen.

The studies that have been done in this area can be broadly divided into three groups:

(i) Physicochemical studies of in vitro interactions: These are in vitro experiments in which complexes of collagen and individual MPS/PPS (formed under physiological pH and ionic strength) are detected and studied by physiochemical methods. The observed effects of such complex formation on collagen fibril formation and collagen fibril stability are then used to deduce the nature of in vivo interactions.

(ii) Physicochemical studies of in vivo interactions: These involve treatment of a specimen of connective tissue with reagents or enzymes which apparently destroy the interactions between the collagen fibrils and the MPS/PPS, followed by study of the structural integrity of treated tissue which includes determination of such physicochemical properties as tensile strength, shrinkage temperature, swelling capacity and resistance to dissolution by various solvents.

(iii) Studies based on biosynthetic events: These generally involve histological studies of fixed or unfixed tissues that have been stained preferentially to show the presence of the MPS/PPS or the collagen fibrils. By use of drugs, the rate of biosynthesis of collagen or individual MPS/PPS (in incubated tissues) can be varied

and the influence of the rate of biosynthesis of one on that of the other can be studied, either by chemical assay or by histological techniques. Synchronous studies of collagen and MPS/PPS synthesis in wound healing have also been used to study the interaction between collagen and individual MPS/PPS.

4.1.2. Physicochemical Studies of in vitro Interactions. Faure-Femiet (1933) first demonstrated that crude chondroitin sulfate (Ch-S) extracted from cartilage could precipitate collagen from its solution in dilute acetic acid and that it is co-precipitated with the collagen. Thereafter, a considerable number of reports, reviewed by Jackson and Bentley (1968), illustrated similar phenomena involving solutions of collagen and gelatin and a wide variety of polyanions (including Ch-S, H, and HA). However, as pointed out by Jackson and Bentley (1968), these studies were done at the non-physiological pH range of 3-4 and were probably a mere demonstration of the non-specific co-precipitation of oppositely charged colloids. Furthermore, most of the fibrils formed in the studies were structureless and did not show the ca. 640 A° periodicity characteristic of native collagen fibrils.

When Gross et al (1955) demonstrated that native-type fibrils could be precipitated out of polyanion-free neutral salt solutions (at physiological pH and ionic strength) by simply warming the solution to 37°C, the whole question of the role of MPS/PPS in the in vivo formation of collagen fibrils became an open one. Gross and

Kirk (1958) further demonstrated that with two doubtful exceptions, a large number of preparations of mucopolysaccharides (Ch-S, HA, KS, HS) had little or no effect on the rate of fibril formation of collagen under the experimental conditions that they used.

Wood (1960) made a more extensive study of the problem, using a wide range of pH and ionic strength and came to the conclusion that the rate of fibril formation could be usefully treated in terms of two phases - a nucleation and a growth process. He demonstrated the ability of small amounts of MPS to influence independently these two processes by showing that the rate of fibril formation and the thickness of the fibrils formed (Wood and Keech, 1960) could be deliberately altered. For example, chondroitin sulfate-1 (a mixture of Ch4-S and Ch6-S) was shown to accelerate the nucleation phase and retard the growth phase and as a result, the collagen fibrils formed were thin. On the other hand, heparin was shown to inhibit nucleation and, hence, resulted in thick fibrils. Wood (1960) suggested that these studies may indicate that the role of MPS in vivo is one of regulating nucleation and growth, thereby determining the rate of formation of fibrils and their ultimate size. Unfortunately, very little of the subsequent studies done in this area has been aimed at testing this hypothesis. Objections to the extrapolation of these studies to in vivo conditions have been raised based on the fact that they were conducted at the non-physiological temperature of 25°C. In a subsequent study, Wood (1962) was able to confirm the

two-step mechanism of fibril formation by isolating the nuclei produced during the nucleation phase (by ultracentrifugation) and showing that inoculation of these fibrils into a fresh solution of collagen induced rapid fibril formation.

Largely from the observation that small changes in the ionic strength of the solution affects greatly the influence of MPS and PPS on fibril formation Mathews (1965) concluded that the interaction must be of an electrostatic nature, involving the anionic charged groups of the MPS and the cationic groups of the collagen. Using the method of free electrophoresis (which essentially separates components of different mobilities under an electric field), Mathews (1965) was able to identify a complex of the MPS with the collagen. The formation of the complex was reversible and dependent on the ionic strength and the molecular weight of the MPS. The use of a PPS of Ch4-S enhanced fibril formation compared to the use of Ch4-S, suggesting that the protein moiety of the PPS may also play a role in the fibril formation. On the basis of these studies, Mathews (1965) proposed a model for the interaction of the PPS with the collagen fibrils. Jackson and Bentley (1968) have extended this model to include the involvement ofglycoproteins (see Fig. 2.6 and Section 2.2.4 for details of the model). Subsequent ultrastructural studies of connective tissues (Smith and Frame, 1969; Serafini-Fracassini et al., 1970; Pease and Bouteille, 1971; Bouteille and Pease, 1971) have confirmed substantially the correctness of such a model.

Nemeth-Coska and Kaiser (1965) have studied extensively the effect of temperature on the formation of collagen fibrils in the presence of MPS/PPS. Unlike many other forms of precipitation, the precipitation of collagen fibrils exhibit a positive temperature coefficient, i.e. the higher the temperature, the easier the precipitation. The temperature dependence of the collagen fibril formation was found (Nemeth-Csoka and Kaiser, 1965) to follow an Arrhenius relationship (as in Equation 3.16) and, hence, an activation energy for the fibril formation can be determined. Nemeth-Csoka and Kaiser (1965) reported a decrease in the activation energy of fibril formation for collagen due to the addition of Ch4-S. They further showed that the activation energy of fibril formation was influenced by the age of the tissue from which the collagen was extracted. The presence of an increased fraction of β component in the older rat was postulated to explain this effect. In a subsequent study, Nemeth-Csoka (1971) classified the MPS into three classes according to their ability to influence the rate of fibril formation: (1) CH 4-S, CH 6-S; (2) HA; and (3) H. Members of first class enhanced, of the second did not and of the third delayed the rate of fibril formation. The activation energy of fibril formation was decreased, unchanged, and increased respectively. Nemeth-Csoka (1971) believed that it is possible to explain the various effects on a thermodynamic basis.

The possibility that the MPS and the PPS are physically entangled with the collagen fibrils was first stated by DiSalvo and

Schubert (1966) who showed that precipitation of collagen fibrils in the presence of PPS resulted in a precipitate which contained a considerable fraction of the PPS. This latter fraction increased with the amount of PPS in the original solution. An increased fraction of PPS in the precipitate resulted, in turn, in greater swelling ability and water retention of the precipitate. Disalvo and Schubert (1966) concluded that long range entanglements and excluded volume effects may play an important role in collagen fibril formation in vivo. Hoffman and Mashburn (1970), however, raised the objection that if this were so, it was difficult to see how the collagen fibrils that were extruded form the fibroblast could meander through the PPS and lay down in an orderly fashion, as they obviously do in certain connective tissues.

Obrink and Wasteson (1971) also do not believe that the excluded volume effect play any important role in the formation of collagen fibrils. Using the method of chromatography of PPS and MPS over gels made from collagen, they confirmed the ability of PPS and MPS to form complexes with collagen. They also confirmed the earlier observations of Mathews (1965) that increased ionic strength of solution lowered the interaction. They considered the possibility of the effect of ionic strength on the degree of coiling of the PPS and the contribution of the associated excluded volume effect to the change in elution characteristics. They pointed out that their studies on chromatography of polysaccharides on Sephadex G-200 showed that the Stokes radius of the macromolecule over the range of

ionic strength used (0.1-0.4) decreased only from 45.4 A° to 42.5 A°. Such a change in radius was found to be too small to account for the substantial differences in elution characteristics due to comparable changes in ionic strength. Accordingly, it was concluded that the effect of excluded volume on collagen fibril formation is insignficant.

Prodrazky et al. (1971),using the method of isoelectric focussing technique (which essentially enables separation of components with different isoelectric potentials), have identified the groups that are responsible for the interaction of the PPS with tropocollagen. They showed that when PPS was desulphated, no complex was formed between the PPS and the tropocollagen and thereby deduced that the sulfate group was necessary for the interaction of the PPS with the collagen. Furthermore, they showed that pepsin-treated tropocollagen interacted with the PPS and deduced from this that the randomly coiled telopeptide region of the tropocollagen was not necessary for its interaction with the PPS. They also showed that acetoacetylated tropocollagen failed to complex with the PPS and thus concluded that the positively charged amino groups of lysine and arginine were the sites of interaction on the tropocollagen molecule. Finally, they found that the corresponding MPS can complex with the tropocollagen as well as the PPS and thus concluded that the protein moiety of the proteoglycan has little effect on the primary interaction of the tropocollagen with the MPS. However, we recall the conclusion previously reached that the protein moiety has a strong effect on collagen fibril formation (Mathews, 1965; Obrink and

Wasteson, 1971).

Toole and Lowther (1968) have presented another interesting aspect of the collagen-MPS/PPS interaction by showing that there are at least two fractions of collagen within a solution of collagen which differ in their ability to interact with PPS. By titrating solutions of PPS into the collagen solution, they showed that a fraction of the collagen was instantaneously precipitated out of the solution. Electron micrographs of the precipitate showed that the collagen fibrils were of the native type. This instantaneous precipitation of collagen fibrils by the PPS occurred at both 4°C and 37°C. If performed at 37°C, the nucleation and growth phase, as described by Wood (1960) were observed. If performed at 4°C, no further precipitation occurred. Toole and Lowther (1968) showed that removal of the precipitate formed instantaneously at either 4°C or 37°C and subsequent incubation of the supernatant at 37°C resulted in a nucleation period four or five times longer than that observed on a portion of the untreated solution (not containing PPS) diluted to comparable concentrations. These authors suggested that the original solution contained two fractions differing both in ability to react with the PPS and to form fibrils at 37°C. The possibility that the fraction which was precipitated instantaneously might have consisted of fibrils that were crosslinked intramolecularly to a higher extent was eliminated by the observation (Lowther et al., 1970) that the proportions of α and β components in both precipitated and unprecipitated fractions were alike.

Based on the above observations, Toole and Lowther (1968) proposed the following sequence of events for the formation of collagen fibers *in vivo*:

(i) The connective tissue cell, which is mobile and sensitive to stresses in the tissue (Porter 1964) align itself in the appropriate direction.

(ii) The cell secretes tropocollagen (Porter 1964) and PPS (Godman and Lane, 1964) into the grooves or folds on the cell surface in a pattern of order and in a concentration suited to the specific requirements of the tissue.

(iii) A series of interaction takes place at or near the cell surface resulting in the formation of a gel comprising a network of fibrils, nuclei of tropocollagen and PPS and further occluded PPS and tropocollagen. This network would act as a restrictive framework for accretion of tropocollagen around the nuclei; the diameter and orientation of the fibrils within the framework are controlled by the shape and the number of nuclei which, in turn, are dependent on the type and concentration of the PPS in the aggregates.

(iv) The PPS may then interact with the fibrils formed in the ways suggested by Mathew (1965) and Disalvo and Schubert (1966).

All of the studies described in this Section have demonstrated that the *in vitro* interaction of collagen with MPS/PPS is very sensitive to a large number of factors. These include: (1) the pH, ionic strength and temperature of the solutions involved; (2) the extent of crosslinking (i.e. age), the helical content, and the purity of the collagen; (3) the concentration, molecular weight

and nature of the MPS; and (4) the presence of the protein moiety in the PPS. The sensitivity of the collagen-MPS/PPS interaction to such a wide variety of factors makes the extrapolation of *in vitro* experiments to *in vivo* conditions rather questionable since the latter conditions are never exactly simulated in *in vitro* experiments.

4.1.3. Physicochemical studies of in vivo interactions. As mentioned above, Flemming (1876) first demonstrated the possible role of the mucopolysaccharides as a cement substance when he showed that treatment of tendon with lime water caused it to split into very fine fibrils. McClean (1931) made similar observations on tendon injected with hyaluronidase. Later, Jackson (1953) demonstrated the ability of PPS to contribute to tendon stability by reporting that:

(i) pretreatment of the tendon with a crude hyaluronidase (which does not affect H-bond linkages between polypeptide chains) resulted in 85% of the tendon becoming soluble in dilute acetic acid as compared to 25% of the untreated tendon.

(ii) similar enzyme treatment lowered the hydrothermal shrinkage temperature to 53°C (native being 67°C).

(iii) removal of chondrotin sulfate from chondroitin sulfate-precipitated fibers reduced the shrinkage temperature from 55°C to 47°C

(iv) the enzyme-treated tendon swelled much more than the native tendon.

Subsequently, neither Banga and Balo (1960), nor Partington and Wood (1963), nor Coulson & Linker (1968) were able to

confirm the stabilizing role of chondroitin sulfate as reported by Jackson (1953). However, the work of Kuhnke (1962) in which the electron microscope appearance of hyaluronidase-treated collagen fibrils was studied and that of Elden (1964) on the effect of hydration on tendon collagen fiber elasticity lend support to the hypothesis. Recently, Toole (1969), has proved quite convincingly that fibrils precipitated in the presence of PPS are much more stable (i.e., more resistant towards dissolution) than those precipitated in the absence of the PPS.

Milch (1966) investigated the effect of a number of MPS on the physical properties of collagen and came to the conclusion that within physiological pH limits, they act as "plasticizers" to soften (or lower the elastic modulus of) the collagen matrix. The apparent contradiction that the MPS can both strengthen (Jackson's hypothesis) and soften (Milch's hypothesis) the collagen matrix was apparently resolved by Bryant and Weeks (1967), who studied the tensile strength of secondary wound tissues. They noted that while Jackson's hypothesis appear to be true for normal tissues, under certain pathological conditions the structural stability of collagen bundles is reduced in the presence of excessive MPS. For example, gestational changes in human and rat cervices are characterized by: (i) an accelerated production of MPS; (ii) marked tissue edema; and (iii) histological evidence of collagen bundle disorganization associated with increasing distensibility of the cervix. After parturition, the excessive MPS is resorbed, the collagen bundles become reorganized and more compact and the normal rigidity of the

cervical tissues is established (Ullery et al., 1966). Bryant and Weeks (1967) suggested that the alteration of the interfilamentous cohesive forces of the collagen macromolecules may be dependent on a quantitiative relationship with the surrounding MPS; that is, the ratio of collagen to MPS may be directly related to the tissue tensile strength. By measuring the tensile strength of secondary wounds inflicted on rats and mice and analyzing the wounded tissue for collagen and MPS, they were able to show a direct correlation between the tensile strength of the tissue and the ratio of the collagen to MPS content.

Using the above observation, Bryant and Weeks (1967) concluded that the initial rapid gain in tensile strength normally observed in secondary wounds is due to a direct interaction between collagen and MPS. As demonstrated by Fessler (1960), a mixture of water, MPS and fibers provide two types of frictional interactions: (1) between water and MPS and (2) between the resulting solution and the fibers. If a favorable collagen to MPS ratio (e.g. 9.1:1 for rats) exists in the wound, the MPS contributes to tensile strength gain by amalgamating larger collagen units (filaments, fibrils, fibers and larger bundles). If an unfavorable collagen to MPS ratio (e.g. 3.8:1 for mice) exists, the excess MPS interferes with aggregation and development of cohesiveness between larger collagen units. However, as the excess MPS is resorbed, a more favorable ratio is established permitting tensile strength gain. The observation of Prudden (1964) seems to support this reasoning. He found that the

addition of mucopolysaccharides from cartilage powder significantly accelerated wound tensile strength gain when applied at the time of wounding. On the other hand, powdered cartilage added after the third postoperative day produced a negative effect on wound tensile strength.

4.1.4. Studies based on biosynthetic events. The question of interactions between collagen and MPS/PPS can also be pursued by direct observation of connective tissue. Using histochemical techniques, it is possible to stain the MPS/PPS with, for example, toluidine blue and collodial iron and thereby differentiate them from collagen (which can be stained with phosphotungstic acid and uranyl acetate). A large number of studies of this kind have been performed; most of these have been concerned with the healing of wounds (Wolbach and Howe, 1926; Stearns 1940; Gersh and Catchpole, 1949; Buck, 1953; Taylor and Saunders, 1957; De Vito, 1965). From these studies, there appeared a pattern of events which seem to implicate the MPS/PPS with the formation of collagen fibrils:

(i) Following the appearance of fibroblasts, an amorphous matrix appeared which had the staining characteristics of the MPS/PPS (e.g. toluidine blue metachromasia);

(ii) On the fifth or sixth day after wounding, this metachromasia reached a peak, and then declined rapidly thereafter.

(iii) This decline is followed by a rapid appearance of collagen fibrils.

The belief in the validity of this pattern was further strengthened by the chemical assay which was made on the wound contents. The tissue hexosamine rose to a maximum at about the fourth to sixth day after wounding. This was followed by a rapid increase in the tissue hydroxyproline (used as a measure of the collagen content) and with a simultaneous decrease in the hexosamine concentration (Ahmad, 1961). From this pattern of events, there rose the concept that early production of the MPS/PPS was necessary for the subsequent formation of collagen fibrils. In fact, Meyer (1946) suggested that the fibroblasts secrete into the surrounding space a mixture of HA and Ch-S and soluble collagen and under the influence of these MPS the soluble collagen is then presumably precipitated in the form of insoluble fibers.

As pointed out by Jackson and Bently (1968), however, the use of hexosamine as a measure of the total MPS can lead to erroneous results because the other constituents of the connective tissue, glycoproteins and mucoproteins also contain hexosamine. These authors also pointed out the disadvantage of using toluidine blue metachromasia for detecting the MPS since the degree of the metachromasia was also affected by other factors such as the degree of sulfation of, for example chondroitin sulfate (Larack-Stone, 1965), by the presence of certain lipids (Schubert and Hamerman, 1956) and by the binding of proteins to the dye (Kelly, 1955). These and other considerations led Jackson and Bentley (1968) to conclude that no really strong evidence had been presented to show that the presence of MPS/PPS is necessary for the formation of collagen fibrils.

The use of autoradiographic studies to correlate the synthesis of collagen and MPS have become increasingly popular in the past decade. Bhatnagar and Prockop's (1966) reasoning was as follows: if the pathways for the synthesis of collagen and MPS are interrelated, and if the formation of a complex between collagen and MPS is a prerequisite for the extrusion of these macromolecules, the synthesis of one type should require a concomitant synthesis of the other. Therefore, the inhibition of the synthesis of one macromolecule should result in the inhibition of the synthesis of the other. They proceeded to test this hypothesis by removing the tibiae of chick embryo and incubating them under physiological conditions in solutions containing labelled proline, glucosamine and sodium sulfate. The conversion of the proline to hydorxyproline was taken as a measure of the biosynthesis of the collagen while the incorporation of the glucosamine and the sodium sulfate into the tissue was taken as a measure of the biosynthesis of the MPS. By alternately inhibiting the synthesis of collagen and mucopolysaccharides (with α-α' dipyridyl and 6-diazo-5-oxonorleucine respectively) and observing the effect of inhibition of synthesis of one component on the synthesis of the other, Bhatnagar and Prockops (1966) concluded that the biosynthesis of collagen and MPS were not interrelated.

However, the studies of Rokosova-Cmuchalova and Bentley (1968), who followed the same line of approach as Bhatnagar and Prockop (1966) but using puppy epiphyseal cartilage instead, showed

that the inhibition of biosynthesis of collagen did cause a reduction in the biosynthesis of MPS. The explanation offerred (Rokosova-Cmuchalova and Bentley, 1968) was that the effect of inhibition of synthesis of one component on the synthesis of the other is apparent only after a lag time (6 hours, in the case of puppy epiphyseal cartilage). The experiment of Bhatnagar and Prockop (1966), which lasted only 4 hours, was thought to be of too short a duration to detect such an effect.

The recent studies of Nemeth-Csoka (1970) on the growth of granulomata on rats provide additional evidence that the presence of some MPS can accelerate the formation of collagen fibrils. She experimented with rats of different age groups, inducing granulomata by injecting them with carrageenan. The amount of sulfated MPS in each of the granulomata was found (by chemical assay) and so were the weight of the granulomata. It was found that while the granulomata of young and adult rats consisted of 68% sulfated MPS, that of the weanlings have sulfated MPS of less than 18%. The greater granuloma growth in young and adult age groups (2-3g/100g body weight) was thought to be in close relation to and/or a result of the high content of sulphated MPS in those groups; at the same time the low granuloma weight in the weanlings (0.6-1.5g/100g body weight) was related to the low content of sulfated MPS.

Munro et al. (1970) followed the uptake of tritiated proline and $Na_2S^{35}O_4$ by the healing of injuries inflicted on the flexor tendon of chickens. The uptake of the proline and $Na_2S^{35}O_4$ were

taken as the incorporation of collagen and MPS respectively by the healing wound. The variation of the collagen (proportional to H^3) and the MPS (proportional to S^{35}) showed distinct similarities. From this observation, it was concluded (Munro et al., 1970) that MPS and collagen "are not only interrelated but are interdependent". It must be kept in mind, however, that since both the collagen and the MPS are produced by the same cell (see Section 2.3.1), the fact that the amounts of each component produced mimic each other may be simply a reflection of the metabolic activity of the cell. On the other hand there may be physiological reasons for maintaining the ratio of concentration of collagen and MPS within a narrow range as suggested by the studies of Bryant and Weeks (1967) which were discussed above.

These above studies uniformly suggest that MPS are involved in the biosynthesis of collagen in a complicated way. A recent finding by Katzman and Jeanloz (1971) has weakened the view that MPS are essential for collagen fibril formation in vivo or for fibril stabilization. They found that the body wall connective tissue of a sea anemona, Metridium dianthus did not contain any MPS (within the limit of detectability, which was 0.05%). Yet, physicochemical examination of the collagen fibrils found in that tissue showed that they closely resemble those of mammalian collagen. For example, the amino acid composition, wide- and low-angle x-ray diffraction pattern, electron microscopic band patterns, melting behaviour, susceptibility to collagenase, electrophoretic

pattern, molecular weight and optical rotation all show that these fibers have a strong structural resemblance to mammalian collagen. In view of this finding, it would seem that MPS which are produced in the vicinity of collagen fibers may influence fibril formation and fibril stabilization, but that they would not seem to play an indispensable role in these processes. Of course, the rather primitive nature of the organism chosen for this study (Katzaman and Jeanloz, 1971) may detract from the generality of such a conclusion.

4.1.5. Summary. The above studies point to at least three types of collagen-MPS/PPS interactions:

(1) Interaction between the tropocollagen and the MPS/PPS. This involves the positively charged amino groups of lysine and arginine in the tropocollagen and the negatively charged sulphate group of the PPS/MPS (Podrazky et al., 1971). This interaction probably controls the instantaneous precipitation of collagen from solution by the MPS/PPS. (Toole and Lowther, 1968). It probably also has a strong influence on formation of the nucleus during a two step precipitation as described by Wood (1960).

(2) Interaction between collagen fibrils and the MPS/PPS. This is the level that can usually be observed by electron microscopy (Smith and Frame, 1969; Serafini-Fracassini, 1970; Pease and Bouteille, 1971; and Bouteille and Pease, 1971). The interaction is reversible, depending strongly on the ionic strength of the solution. It probably controls the growth phase in a two step

precipitation of the collagen from solution (Wood, 1960). It helps to regulate the final width of the fibril in the *in vitro* precipitation (Wood and Keech, 1960), but the extent to which it contributes to regulating the size of the fibril *in vivo* is not clear.

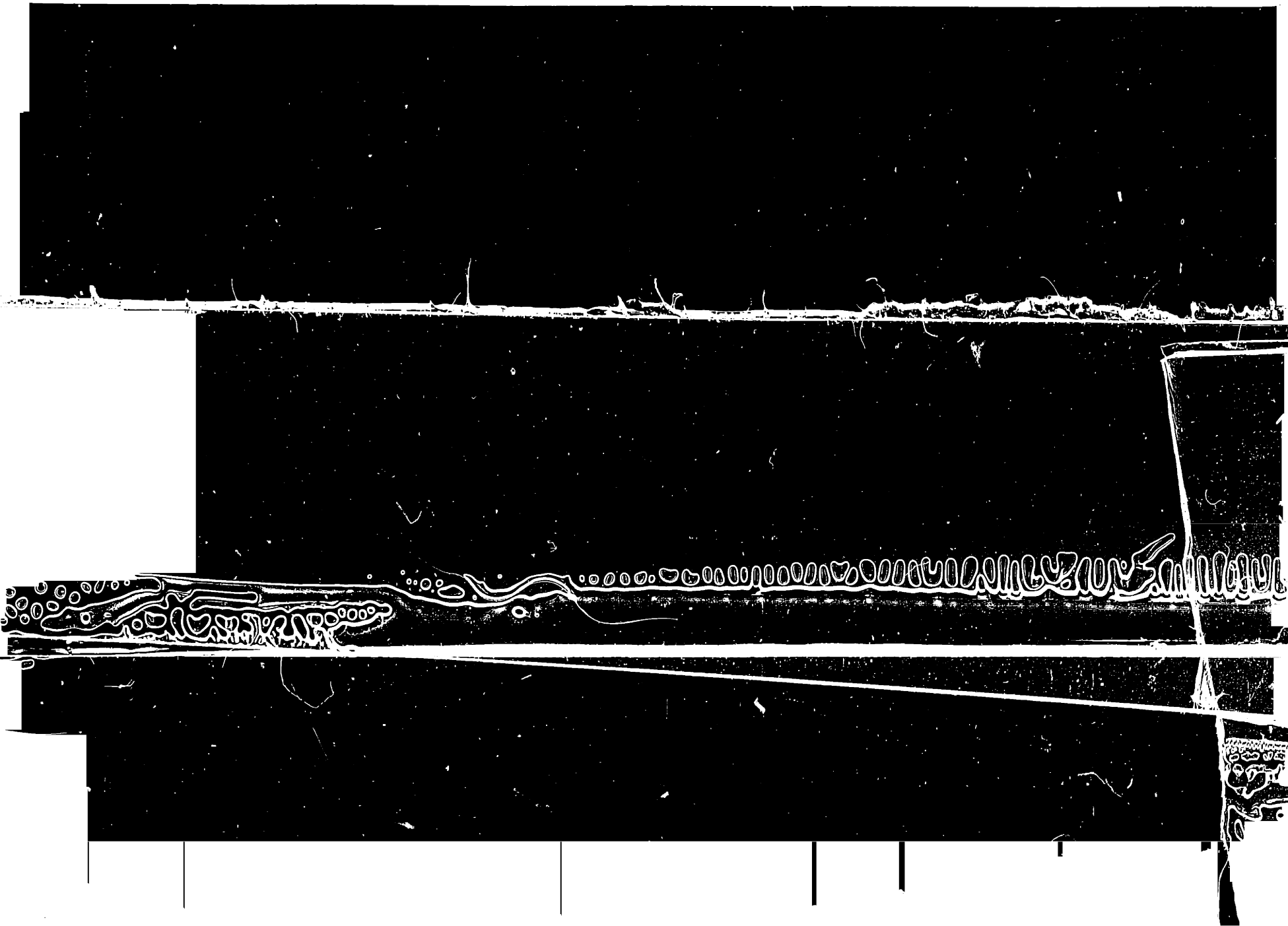
(3) Entanglement and excluded volume interaction between the fibrils and the MPS/PPS. The effect of this interaction on collagen fibril formation is considered to be minimal (Obrink and Wasteson, 1971). However, the interaction may be important in controlling such properties as its swelling ability, ability to retain water, permeability, shrinkage temperature and possibly its tensile strength. At low concentrations, the MPS/PPS contribute to the tensile strength by binding the collagen fibrils together (Jackson, 1953). At high concentrations of MPS/PPS, a plasticizing effect results, making the collagen matrix soft and more extensible (Milch, 1966).

The interrelationship between the biosynthesis of MPS/PPS, on one hand, and the biosynthesis of collagen, on the other, has been clearly demonstrated (Rokosova-Cmuchalova and Bentley, 1968; Nemeth-Csoka, 1970; Munro *et al.*, 1971). The physiological significance of such an interrelationship remain, however, unclear. The necessity for the body to maintain the collagen/MPS ratio to a fixed range in order to produce a tissue with good mechanical strength has been demonstrated (Bryan and Weeks, 1967). The ability of the MPS/PPS to stabilize the collagen fibril has also been shown (Jackson, 1953; Kuhnke, 1962; Toole, 1969), although other studies (Banga and Balo, 1960; Partington and Wood, 1963; Coulson and Linker, 1968) disclaim

such an effect. The ability of MPS/PPS to influence the *in vitro* formation of collagen fibrils has been demonstrated but extrapolation of such results to *in vivo* situations is difficult. Finally, the the existence of a connective tissue containing no detectable MPS/PPS has been found (Katzman and Jeanloz, 1971); the apparently normal form of collagen fibrils formed in this tissue has weakened the hypothesis that the MPS/PPS are indispensable in the formation of collagen fibrils.

4.2. Sample Preparation and Characterization.

4.2.1. Introduction. Most of the studies of the *in vitro* interaction of collagen with MPS/PPS, described in the last Section involve solutions of collagen and MPS/PPS. With only a few exceptions (Einbinder and Schubert, 1951; Obrink and Wasteson, 1971: Seraffini-Fracassini, *et al.*, 1970 a) very little studies on the *in vitro* interaction of insoluble collagen with MPS/PPS have been made. We have initiated a study of the interaction of insoluble collagen with a variety of MPS with the objective of preparing collagen-MPS composite materials which may be suitable for use as skin replacement. The insoluble collagen used was either in the form of a condensed solid phase or in the form of a dispersion of fibrils in solution. The interaction of the former with MPS resulted in a material consisting of a collagenous core coated with MPS on the surface (This material will be called MPS-coated collagen). On the other hand, the interaction of a dispersion of collagen fibris in solution with MPS



resulted in a material consisting of collagen fibrils dispersed within a MPS matrix; this material is analogous to the fiber reinforced plastics that have been used in materials applications with increasing popularity in the last decade. (This material will be called collagen-MPS composite).

In this Section, a physicochemical characterization of these two materials (MPS-coated collagen and collagen-MPS composite) will be made using some of the techniques described in Chapter 3. In order to determine the amount of each component in a composite material containing both collagen and mucopolysaccharide, it is necessary to use methods by which each component can be analyzed without interference from the other. In our present investigation, the analysis for hydroxyproline content, by the method of Woessner (1961) was used as a determination of the collagen content while the analysis for hexosamine content, by the method of Elson and Morgan (1933) was used as a measure of the MPS content. Since the analyses of both substances (hydroxyproline and hexosamine) are based on their initial conversion to derivatives of pyrrole (see Figs. 4.1 and 4.3) and the subsequent reactions of these derivatives with p-dimethylaminobenzaldehyde (DAB) to form chromagens which can be analyzed spectrophotometrically, the possibility of one substance interfering with the analysis of the other is a real one. A careful study was, therefore, made to investigate this possibility. The feasibility of using either analysis for determining the concentration of the components in a collagen-MPS composite was then assessed.

4.2.2. Materials. The collagen used in this investigation was from bovine Archilles tendon (BAT) and was generously donated to us by Dr. R. K. Kronenthal, Ethicon, In., Somerville, New Jersey. Detailed descriptions of the extraction and purification steps have been previously reported in detail by Oneson et al. (1970). Briefly, insoluble bovine tendon was treated sequentially with ficin, sodium borohydride and ammonium nitrate; it was subsequently washed thoroughly, swollen with acetic acid, centrifuged and filtered. The collagen fibrils were subsequently dispersed in acetic acid and used as such to interactwith the MPS to form collagen-MPS composite materials.

The MPS-coated collagen was prepared from collagen tapes described in Section 3.2.3. These tapes were prepared from the collagen fiber dispersions (described above) by deswelling the fibers with ammonium nitrate. The deswollen fibers were subsequently extruded and crosslinked by irradiation (to a M_c value of 28,900 - see Section 3.2.3). In both the collagen fiber dispersions and tapes, the total amount of impurities (protein-polysaccharides, glycoproteins, various tissue and serum proteins) was less than 0.2%-wt. (Oneson et al., 1970). The collagen fibers present in both preparations showed no degradative change when examined with the electron microscope (Oneson et al., 1970) or by wide-angle X-ray diffraction and infrared spectroscopy in our laboratory (see Section 3.3).

The MPS used in this work have been generously donated to us by a number of researchers prominent in the field of connective tissue research. Hyaluronic acid (HA), from human umbilical cords, chondroitin 4-sulfate (Ch 4-S), from bovine nasal cartilage and chondroitin 6-sulfate (Ch 6-S), from rock sturgeon (Acipenser fulvescens) cartilage, all prepared by the methods described by Roden et al. (1972) were supplied to us by Dr. M. B. Mathews, University of Chicago, Chicago, Illinois; heparan sulfate (HS) and dermatan sulfate (DS), both extracted from hog mucosal tissues and purified by the methods described by Cifonelli and Roden (1968) was supplied by Dr. J. A. Cifonelli, University of Chicago, Chicago, Illinois; and heparin (H), extracted from pig intestinal mucosa and purified by the method described by Lindahl et al. (1965) was supplied by Dr. U. Lindahl, University of Uppsala, Uppsala, Sweden. A complete analytical and physical characterization of these materials is given in Table 4.1.

4.2.3. Hydroxyproline analysis. The basic reactions involved in the analysis is shown in Fig. 4.1. The first step in the analysis is the oxidation of the hydroxyproline to the pyrrole-2 carboxylic acid. Various oxidants that have previously been used include sodium peroxide (Neuman and Logan, 1950), hypochlorite (Lang, 1935), and hypobromite (Waldsmith-Leitz and Akabori, 1934). The oxidant used in the present analysis is sodium p-toluene-sulfonchloramide (Chloramine T) which was found by Woessner (1961) to give the most reproducible result compared to the other oxidants. The next step in the analysis involve

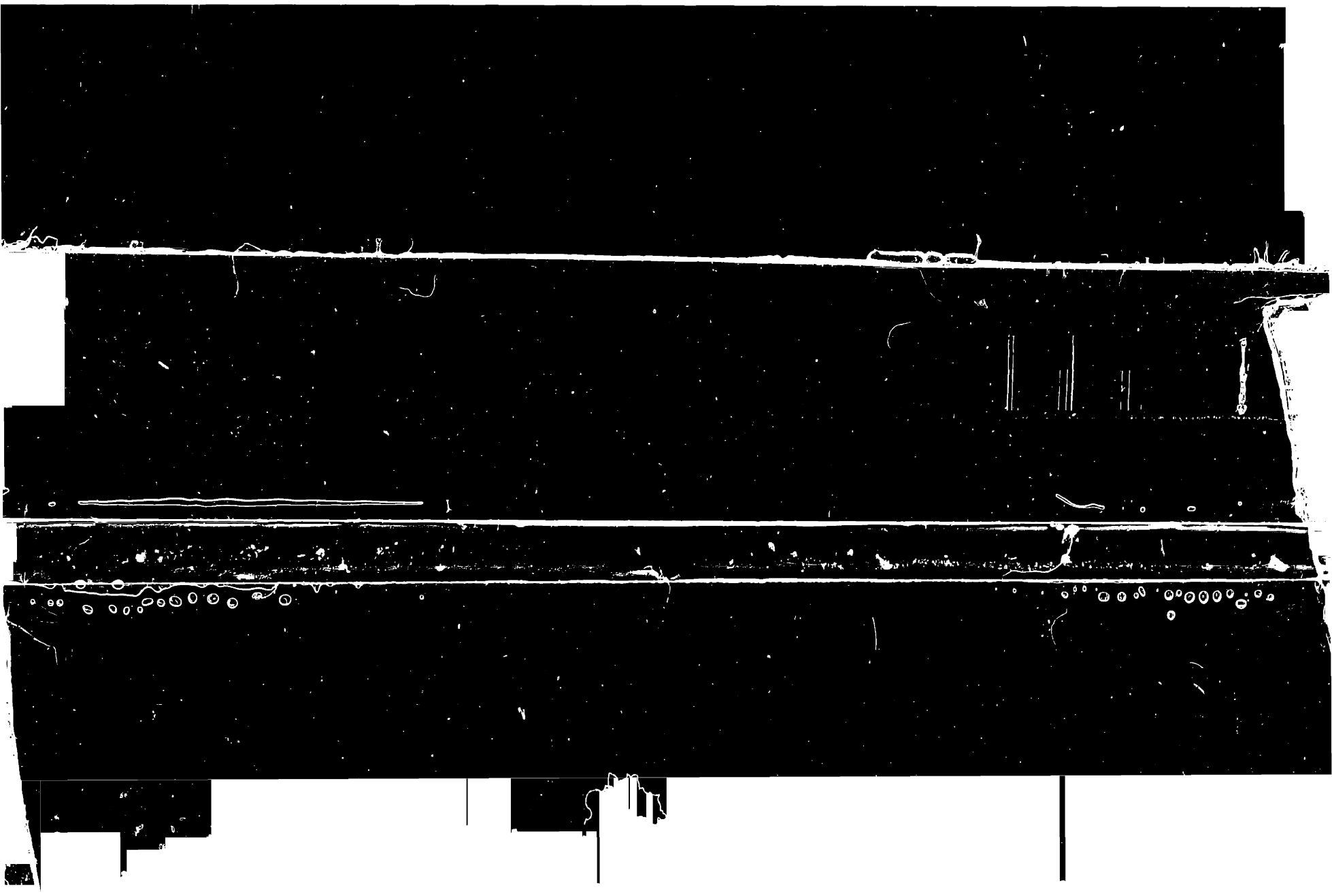


TABLE 4.1

ANALYTICAL AND PHYSICAL DATA FOR SODIUM SALT OF ACID MUCOPOLYSACCHARIDES

	HA	Ch6-S	Ch4-S	DS	H	HS[10]
Nitrogen[1,2]	3.0	2.7	2.6	2.3	2.6	2.5
Hexuronic Acid[1,3]	47.2	34.6	34.1	12.8	38.7	44.1
Hexosamine[1,4]	38.3	26.2	27.2	23.0	23.8	24.6
Sulfate[5,6]	0.00	0.98	0.97	1.29	2.33	0.99
Galactosamine[7]	0.001	1.000	1.000	1.000	0.001	0.002
Glucosamine[7]	1.000	0.007	0.001	0.029	1.000	1.000
Asp[7]	0.001	0.006	0.030	0.010	tr	tr
Ser[7]	0.001	0.017	0.067	0.015	0.028	tr
Thr[7]	tr	0.008	0.012	tr	tr	tr
Glu[7]	0.001	0.015	0.023	tr	tr	tr
Gly[7]	0.001	0.015	0.052	tr	tr	tr
$[\alpha]_D^{24}$	-69°	-11°	-25°	-70°	+52°	+73°
$[\eta]$[8]	5.4	0.95	0.55	0.59	0.16	0.39
Mol. Wt.[9]	23,000	30,000	23,000	27,000	11,000	...

1. Per cent by weight.
2. By Kjeldahl - nesslerization method.
3. Carbazole method (Dische, 1947).
4. By modified Elson-Morgan method (Elson and Morgan, 1953) with correction for loss on hydrolysis for HA, CSC and CSA only.
5. Molar ratio of ester sulfate to hexosamine.
6. By method of Muir (1957).
7. By amino acid analyser; molar ratio to major hexosamine.
8. Intrinsic viscosity measured in 0.38 M NaCl with 0.01 M phosphate buffer pH 7.0. (Mathews and Dorfman 1953); based upon anhydrous weight of sample.
9. Estimated from relationships between $[\eta]$ and molecular weight (see Table 2 of Mathews 1967).
10. Data for calcium salt.

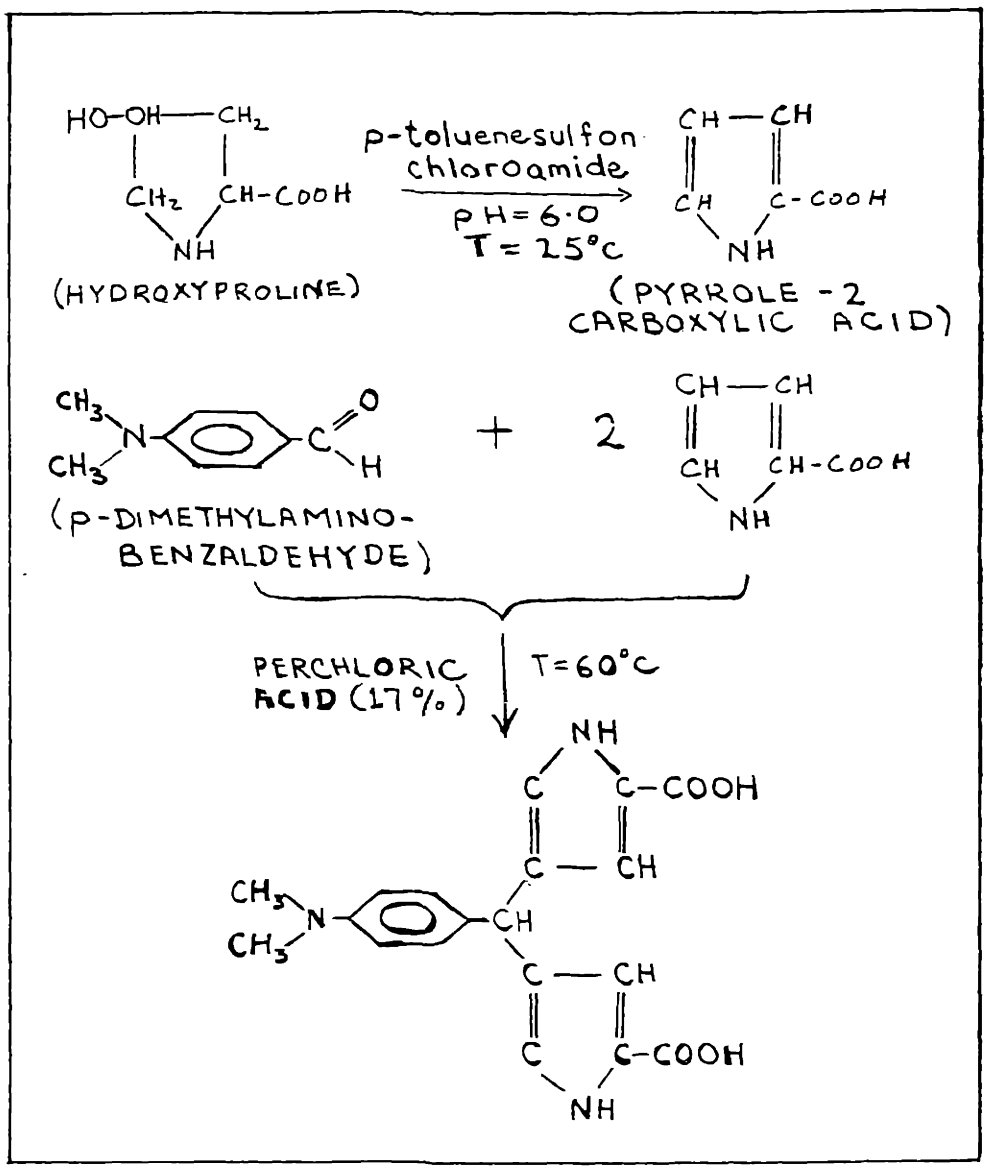


Fig. 4.1. Reactions involved in the analysis for hydroxyproline.

stopping the oxidation with perchloric acid, followed by reaction of the oxidation product (pyrrole-2-carboxylic acid) with DAB under the same acid medium. The chromagen produced by the latter reaction (Fig. 4.1) yields an intense red color, the absorbance of which can be measured with a spectrophotometer. For low concentrations of hydroxyproline (< 6μg/ml), the absorbance is proportional to the concentration (See Fig. 4.2a) and thus the concentration of hydroxyproline in an unknown solution can be determined by this method if the constant of proportionality is known (via a calibration curve).

The following reagents are used in the analysis:

(i) Hydroxyproline standard. A stock solution was prepared by dissolving 25mg of vacuum-dried L-hydroxyproline (Matheson Coleman and Bell, East Rutherford, N.J.) in 250 ml 0.001N HCl. Standards were prepared daily by diluting the stock with water to obtain concentrations of 1-5μg/ml.

(ii) Buffer solution. 50g citric acid monohydrate (J. T. Baker Chemical Co., Phillipsburg, N. J.), 12ml of glacial acetic acid (J.T. Baker Chemical Co., Phillipsburg, N. J.), 120g of sodium acetate trihydrate (Matheson Coleman and Bell, East Rutherford, N. J.) and 34g of sodium hydroxide (Allied Chemicals, Orristown, N. J.) were made up to a final volume of one liter in distilled water. The pH was carefully adjusted to 6.0 and the buffer was stored in the refrigerator under toluene.

(iii) Chloramine T. A 0.05M solution was prepared fresh daily by dissolving 1.4g of chloramine-T (J. T. Baker Chemical Co.,

Phillipsburg, N. J.) in 20ml of water. 30 ml of methyl cellosolve (see below) and 50 ml of buffer were added. The solution was kept in a glass-stoppered flask.

(iv) Methyl cellosolve (ethylene glycol monoethylether). A preparation, free of interfering substances, was obtained from Union Carbide Chemicals Company, South Charleston, W. Virginia.

(v) Perchloric acid. A 3.15M solution was prepared by diluting 27.0ml of a 70% perchloric acid (Frederick Smith Chemical Co., Columbus, Ohio) to 100ml with water. Care should be taken in handling the perchloric acid because of its explosive nature (Everett and Graf, 1971).

(vi) p-dimethylaminobenzaldehyde (DAB). A 20% solution was prepared shortly before use by adding methyl cellosolve to 20g of DAB (Fischer Scientific Co., Fair Lawn, N. J.) to get a final volume of 100ml.

The procedure for the analysis is as follows: 2ml portions containing 1-5µg/ml hydroxyproline were placed in 16 x 150 mm test tubes. 1 ml chloramine-T was added to each tube and the contents were shaken a few times to ensure adequate mixing. Oxidation was allowed to continue for 20 min at room temperature, and the excess chloramine-T was destroyed by adding 1 ml of perchloric acid, shaking and allowing to stand for 5 min. When analyzing a number of samples, the chloramine-T and perchloric acid should be added in exactly the same sequence to ensure consistent oxidation time for each sample. Woessner (1961) found that the oxidation time may be varied between 15 and 25 min without causing much error in the

analysis. Using oxidation times beyond this range resulted in increasing errors. The time allowed for destruction of chloramine-T with perchloric acid (5 min) was found (Woessner, 1961) to be quite critical and should not be extended by more than 2-3 min.

Finally, 1ml DAB solution was added, and the mixture was shaken to ensure complete mixing. The tubes were placed in a water bath at 60°C for 20 min, then cooled in tap water for 5 min. The absorbance of the solution was then determined with a spectrophotomer (Coleman Junior II A Spectrophotometer, Coleman Instruments, Maywood, Illinois) using a wavelength of 557 mμ. This wavelength corresponds to the maximum absorbance of the chromogen produced (Swann and Balazs, 1966). Heating of the mixture at 60°C can be delayed up to 30 min after addition of DAB solution. However, after 25 min of heating, the color development begins to decrease. After removal from the 60°C water bath and cooling, this color is quite stable and may be left as long as 1 h without appreciable fading.

The result of such an analysis using standard hydroxyproline solution is shown in Fig. 4.2(a). It can be seen that, up to a concentration of ca. 5 μg/ml, the absorbance is linearly proportional to the concentration. In order to determine whether the presence of hexosamine would interfere with the analysis, a series of analyses were repeated using hydroxyproline containing 102 μg/ml of either glucosamine or galactosamine. The results of these experiments are also included in Fig. 4.2 (a) where it can be seen that the analysis is not interfered by the presence of hexosamine. The

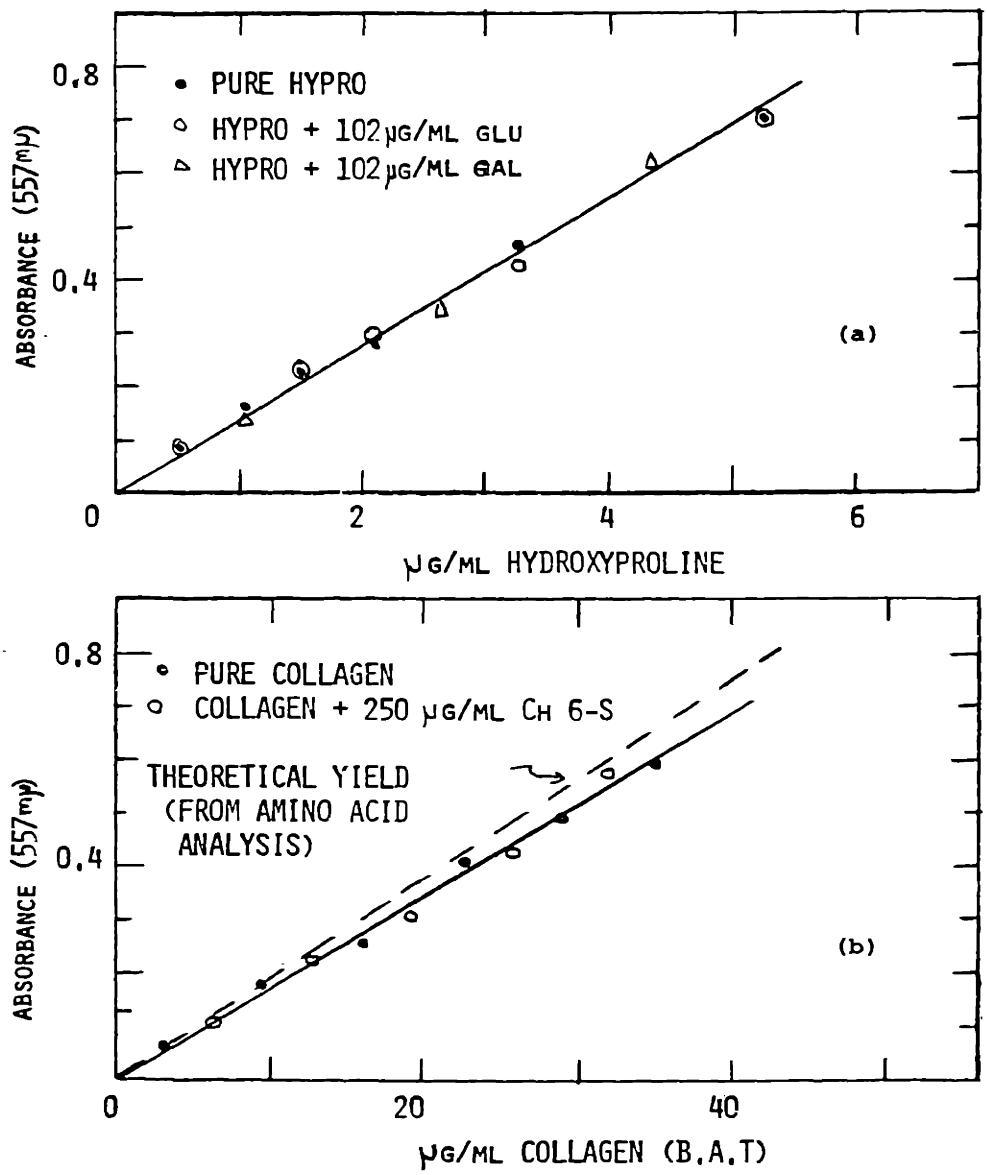


Fig. 4.2. Relationship between absorbance and the concentration of (a) hydroxyproline and (b) collagen used in the hydroxyproline analysis. In the analysis involving standard solutions of hydroxyproline (a), the presence of glucosamine (Glu) and galactosamine (Gal) did not affect the absorbance measured. In the analysis involving known concentrations of collagen (b), the presence of chondroitin 6-sulfate also did not interfere with the analysis. The dotted line in (b) was calculated based on the expected yield of hydroxyproline from the collagen as determined by amino acid analysis and on the calibration line in (a).

probable explanation is that the reaction involved in converting the hydroxyproline to the pyrrole (Fig. 4.1) is quite different from the reaction involved in converting the hexosamine to the pyrrole (Fig. 4.3). Presumably, the former reaction (which is an oxidation reaction) is not capable of converting the hexosamine to pyrrole and since the hexosamine itself does not react with the DAB in the subsequent reaction, no interference results.

In order to apply the hydroxyproline analysis to the determination of collagen concentration, an additional step involving the hydrolysis of the collagen to yield its hydroxyproline is necessary. The hydrolysis condition used in the present study is as follows: A known weight of vacuum-dried (105°C at 10^{-3} mm Hg pressure for 48 h), collagen was placed in a sealing tube (NS-33 Ampules, Wheaton Glass Co., Millville, N. J.) and 1 ml of 8M HCl was added. The tube was then evacuated and and flushed three times with dry nitrogen gas before it was sealed under vacuum (10^{-3} mm Hg). The tube was then placed in a bath at 95°C for 4 h and then allowed to cool in tap water. The seal was then broken and the tube and its contents were cooled to 2°C in order to prevent boiling of the contents in the next step which involved evacuation (Ogston, 1964). The contents of the tube were then evaporated to dryness by placing the tube in a vacuum desiccator containing dry silica gel and sodium hydroxide and kept at 40°C.

Finally, the last traces of HCl which may be present in the dried content of the tube was removed by placing the desiccator in a forced-air circulating oven maintained at 40°C and connecting the

desiccator to a vacuum line (producing a vacuum < 5mmHg) through two plexiglass tubes (60cm x 4.5cm) coupled in series. The first tube was filled with silica gel for the absorption of water and the second was filled with NaOH pellets (technical grade) for the absorption of HCl. After evacuation for about 24h, the contents of the tube are dissolved in distilled water to give a concentration equivalent to ca. 7 to 35µg/ml of collagen (based on the original weight of collagen used). This concentration of collagen corresponds to a theoretical yield of ca. 1 to 5µg/ml of hydroxyproline since the hydroxyproline content of the BAT collagen is ca. 14g/100g of collagen (Oneson et al., 1970). The solution of hydroxyproline, obtained from the hydrolysis product of collagen, is then analyzed as above.

The results of such an analysis are shown in Fig. 4.2(b) where the reported concentration of collagen is based on the original weight of collagen used in the analysis. The dotted line in the Figure is the theoretical line based on the expected yield of hydroxyproline from the BAT collagen (ca. 14g hydroxyproline/100g collagen) as determined by amino acid analysis (Oneson et al., 1970) and on the calibration line shown in Fig. 4.2(a). The discrepancy between the theoretical and experimental results may be due to loses during hydrolysis. This discrepancy points to the necessity of using, whenever possible, a calibration line based on the use of a known weight of collagen in the analysis, as in Fig. 4.2(b) as opposed to a calibration line based on the use of a known weight of hydroxyproline in the analysis, as in Fig. 4.2(a). The former

calibration line should be obtained each time an unknown sample is to be determined since it was found that the slope of the line changes from one set of experiment to the next, due to the sensitivity of the method to small variations in experimental conditions.

In order to determine whether the presence of MPS would interfere with the determination of collagen content, a series of analyses was repeated using collagen containing 250 μg/ml Ch6-S. The results, as shown in Fig. 4.2(b) show that the presence of Ch 6-S does not interfere with the analysis. The use of this analysis for determining the collagen content of a collagen-MPS composite material is thus established.

4.2.4. Hexosamine analysis. The basic reactions involved in the analysis is as shown in Fig. 4.3. The first step in the analysis is the condensation of the hexosamine with acetylacetone in alkaline solution to form derivatives of pyrrole. Cornforth and Firth (1958) have shown that, of the derivatives of pyrrole that are formed, 2-methyl pyrrole (shown in Fig. 4.3) is responsible for about 2/3rds of the color developed in the subsequent reaction with DAB. The latter reaction results in a chromagen which yields an intense red color, the absorbance of which can be measured with a spectrophotometer. For low concentrations of hexosamine (< 120 μg/ml), the absorbance is proportional to the concentration (see Fig. 4.4a) and hence the concentration of hexosamine in an unknown material can be determined if a calibration line is established.

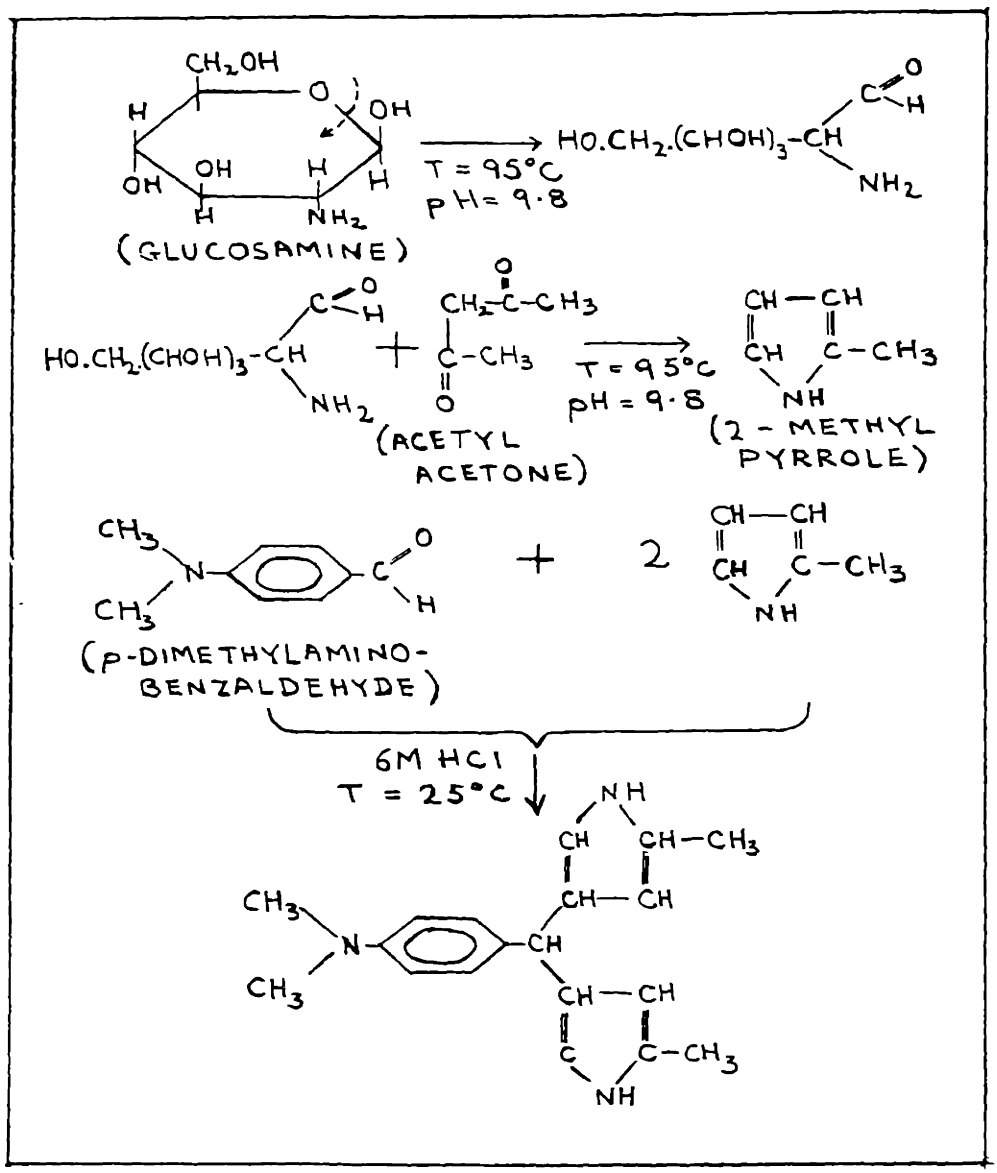


Fig. 4.3. Reactions involved in the analysis for hexosamine.

The following reagents are used in the analysis:

(i) Hexosamine standard. Stock solutions were prepared by dissolving 100 mg of vacuum-dried glucosamine or galactosamine (both purchased from Sigma Chemical Co., St. Louis, Mo.) in 100 ml of distilled water. Standards were prepared daily by diluting the stock with water to obtain concentrations of 10-100 μg/ml.

(ii) Acetylacetone reagents A 8% (v/v) solution of acetylacetone (Fischer Scientific Co., Fair Lawn, N. J.) in 1 M Na_2CO_3 (Fischer Scientific Co., N. J.) was prepared. The reagent was prepared daily immediately before use.

(iii) Erhlich reagent. This was prepared by dissolving 1.33g of DAB (Fischer Scientific Co., Fair Lawn, N. J.) in 50 ml of 6M HCl, to which was then added 50ml of 95% ethanol. The reagent was also prepared daily immediately before use although it is fairly stable when stored at 4°C (Swann and Balazs, 1966).

The procedure for the analysis is as follows: 1ml portions of the sample containing 10-100μg of hexosamine were placed in 16 x 150 mm test tubes and 1ml of the acetylacetone reagent was added in each. The tubes were then covered with screw caps and heated for 1h in a covered water bath at 95°C. After removal from the water bath, the tubes were cooled immediately in running tap water and 5 ml of 95% ethanol added to each tube. Ehrlich reagent (1ml) was then added and the contents of the tubes were mixed thoroughly. The tubes were allowed to stand at room temperature for 2h and the absorbances were then measured at 527mμ against a reagent blank within

30 min using a spectrophotometer (Coleman Junior IIA, Coleman Instruments, Maywood, Illinois).

The result of such an analysis, using standard hexosamine solutions, is shown in Fig. 4.4(a). It can be seen that up to a concentration of ca. 110 μg/ml, the absorbance is a linear function of the concentration of hexosamine. The absorbances for both galactosamine and glucosamine coincide for all concentrations of hexosamine. In order to determine whether the presence of hydroxyproline would interfere with the analysis, a series of analyses were repeated using hexosamine solutions containing 50 μg/ml hydroxyproline. The results are shown in Fig. 4.4(a) where it can be seen that the analysis is not interfered by the presence of hydroxyproline. The condensation reaction involved in converting the hexosamine to pyrrole presumably did not convert any of the hydroxyproline present to pyrrole and the hydroxproline itself apparently does not react with DAB to cause any interference.

In order to apply the hexosamine analysis to the determination of MPS content, the additional step involved is the hydrolysis of the MPS to yield its hexosamine. The procedure involved in the hydrolysis has already been described in Section 4.2.3. The result of an analysis of the hydrolysis product of Ch 6-S is shown in Fig. 4.4(b), where the reported concentration of Ch 6-S is based on the original weight of the MPS used in the analysis. The dotted line in the Figure is the theoretical line based on the expected yield of hexosamine from Ch 6-S as determined by its known

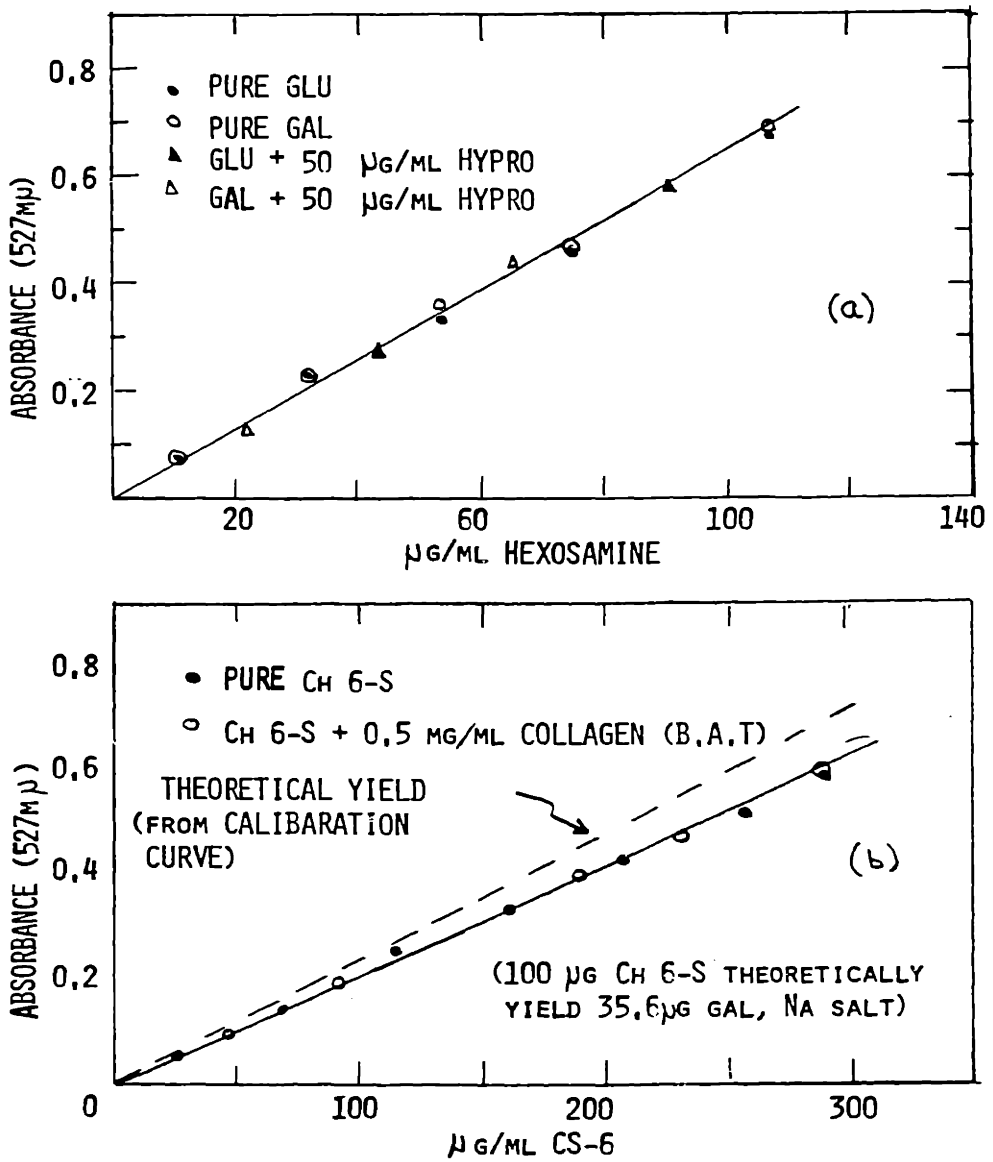


Fig. 4.4. Relationship between absorbance and the concentration of (a) hexosamine and (b) chondroitin 6-sulfate used in the hexosamine analysis. In the analysis involving standard solutions of hexosamines (a), the absorbances for both glucosamine (Glu) and galactosamine (Gal) were the same. The presence of hydroxyproline did not interfere with the analysis. In the analysis involving known concentrations of Ch 6-S, the presence of collagen did not interfere with the analysis.

chemical structure (see Section 2.2.4) and on the calibration line shown in Fig. 4.4(a). The discrepancy between the theoretical and experimental results is probably due to loses during hydrolysis. This discrepancy again points to the necessity (as in the case of collagen) of using a calibration line based on the use of a known weight of MPS (as in Fig. 4.4 b) as opposed to the use of a known weight of hexosamine (Fig. 4.2 a) in the analysis.

In order to determine whether the presence of collagen would interfere with the determination of MPS content, a series of analyses was repeated using Ch 6-S containing 500 μg/ml of collagen. The results, as shown in Fig. 4.4(b) indicate that the presence of collagen does not interfere with the analysis. It is, therefore, feasible to use this analysis for determining the MPS content of a collagen-MPS composite material. If the composite material does not contain extraneous substances, the determination of MPS content using the analysis described in this Section can be used to supplement the determination of collagen content by the analysis described in the previous Section.

4.2.5. Mucopolysaccharide-coated Collagen. The preparation of collagen fibers coated with MPS was initiated because previous studies (Einbinder and Schubert, 1951; Obrink and Wasteson, 1969; Serafini-Fracassini, 1970a) showed that insoluble collagen can interact with MPS under appropriate conditions and the ease with which these materials can be prepared makes them rather attractive from the

practical point of view. Hence a study was made on the interaction of a number of MPS with insoluble collagen fibers and on the characterization of materials resulting from the interaction.

One distinct difference between the interaction of MPS with collagen in solution and collagen in the insoluble form is that whereas the former can occur at neutral pH (see Section 4.1) the latter occurs at acidic pH (Einbinder and Schubert, 1951; Serafini-Fracassini, 1970a). Studies in our laboratory show that MPS-coated collagen specimens that have been prepared at acidic pH tend to lose their MPS when placed in solutions maintained at physiological pH (7.4) and ionic strength (0.14). Since these materials were made with the objective of their being used as skin replacement, and hence will be in constant contact with physiological fluid, it is necessary to immobilize the MPS with an appropriate cross-linking agent. Our studies showed that the use of glutaraldehyde crosslinking can successfully immobilize the MPS although a definitive proof of crosslinking between the MPS and collagen has yet to be demonstrated.

The collagen used in the studies to be presented in this Section is the Ethicon bovine Archilles tendon collagen tape, described in Section 4.2.2. The MPS used (HA, Ch 4-S, Ch 6-S, DS, H, HS) have also been described in that Section. The buffer solution used for studying the interaction of the collagen with the MPS and the subsequent crosslinking reaction was a citric acid-phosphate buffer made up of a 2.2M disodium phosphate (Na_2HPO_4) and a

0.1M citric acid solution. By varying the proportion of these reagents, a range of pH (2.2 to 8.0) can be obtained (e.g. 9.15 ml citric acid and 90.85 ml Na_2HPO_4 yields a pH of 7.4). The crosslinking agent used was glutaraldehyde (J. T. Baker Chemical Co., Phillipsburg, N. J.) and the concentration used (by dilution with the citric acid-phosphate buffer) was 0.025M.

The procedure for preparing the MPS-coated collagen fibers is as follows. The MPS solutions were prepared by dissolving 40mg of the MPS in 20ml of citric-acid phosphate buffer (pH range used was 2.2 to 7.4). A length of insoluble collagen tape (ca. 200 mg) was then added to the MPS solution, maintained at a constant temperature (23°C or 37°C) and allowed to incubate for ca. 24 hours. Glutaraldehyde was then added to the solution to give a resultant concentration of 0.025M of aldehyde. The collagen was kept in this solution for another 24 hours and was subsequently transferred to a 0.025M solution of glutaraldehyde maintained at a pH of 7.4. The latter step was done in order to ensure efficient crosslinking of collagen, since it is known (Theis and Schaffer, 1936) that crosslinking of collagen at low pH (< 5.5) is very ineffective. After 24 hours in the glutaraldehyde solution, the collagen fibers were rinsed three times with distilled water and tranferred to a 0.2 wt-% of dimedone (Eastman Kodak Co., N. Y., N. Y.) in order to remove excess, unreacted aldehydes (MacFayden, 1945). After another 24 hours in the dimedone solution, the fibers were rinsed five times with distilled water and kept in a citric-acid phosphate

buffer solution pH 7.4 at 4°C until they were required for subsequent characterization.

The wt-% of MPS (dry weight) attached to the collagen was determined by both the hexosamine (Section 4.2.4) and hydroxyproline (Section 4.2.3) analyses. The latter analysis yields a value for the collagen content of the sample from which the wt-% of MPS can be determined by difference. The values obtained from the two analyses agree quite well and an average of the two values was taken as the value representing the wt-% of MPS attached to the collagen fibers. Fig. 4.5 shows the variation of this quantity with pH and temperature for the MPS that have been studied in this investigation. It can be seen that at both 23°C and 37°C, for all the MPS, the amount of MPS attached to the collagen is small at pH > 5. At low pHs, the amount of MPS attached progressively increases, but at pH 3.2 this amount appears to level off for some of the MPS. The amount of MPS attached is seen to be ca. 2 to 3 times greater for interaction at the higher temperature (37°C). However, materials that were prepared at 37°C and at pHs lower than 3.2 were found to be partially denatured as determined by shrinkage temperature measurements (Section 3.2), by infrared spectroscopic studies (Section 3.3.4) and by enzymatic degradation studies (Section 3.4). Hence, for the collagen fibers used in the present investigation, the condition for maximum amount of attachment of MPS to the collagen without partially denaturing the collagen is pH 3.2 and temperature of 37°C.

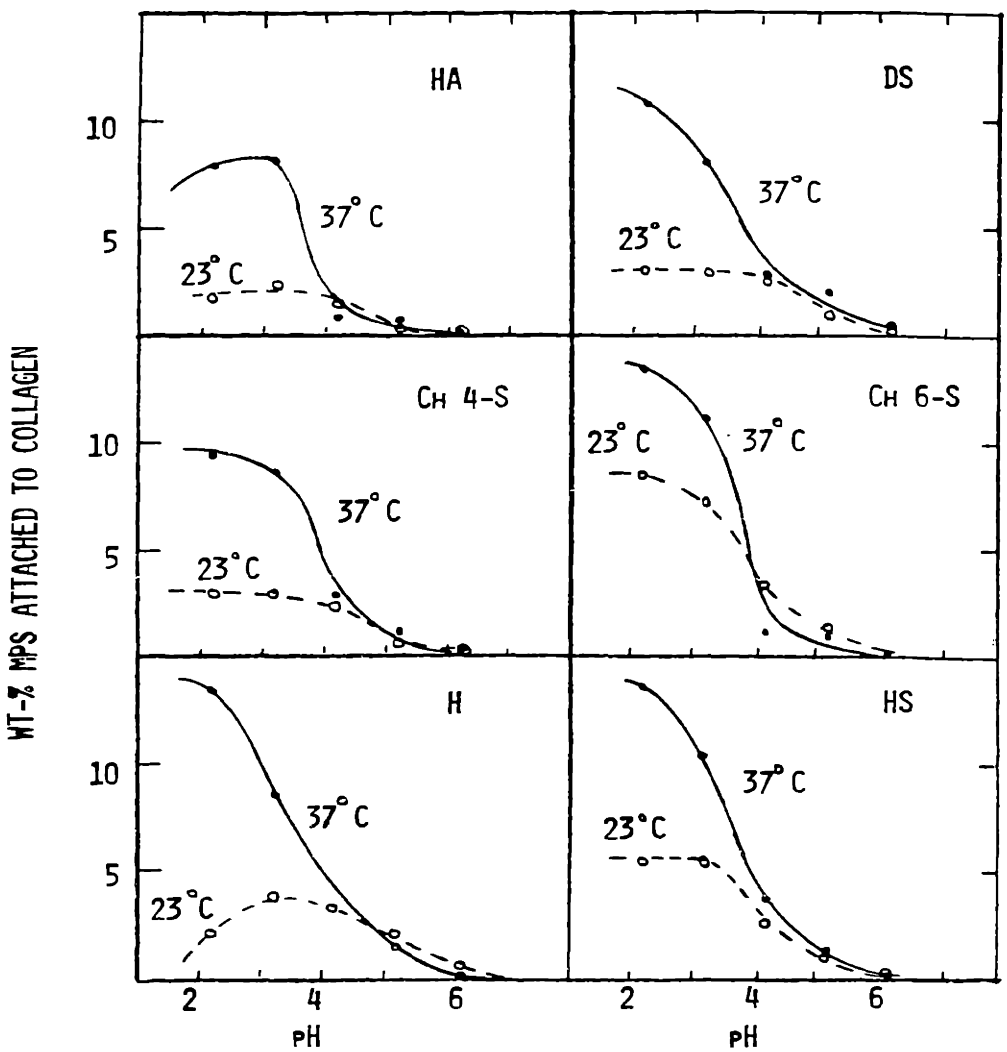


Fig. 4.5. The wt-% of mucopolysaccharides attached to collagen when interaction followed by crosslinking of the two polymers were achieved at the indicated pH and temperature. See text for details of interaction and crosslinking.

Materials prepared under these conditions were then characterized in order that they may be used for the subsequent implantation studies to be reported in Chapter 5.

Table 4.2 gives the results of characterization of MPS-coated collagen fibers prepared under the conditions just noted. The polymer-solvent interaction parameter, χ and the molecular weight between crosslinks, M_c were obtained from denatured collagen fibers using the methods described in Section 3.2.3. The presence of a coating of MPS apparently does not alter significantly the extent of crosslinking or the polymer-solvent interaction parameter of the inner core of collagen fibers.

The modulus of the collagen fiber, E was measured as outlined in Section 3.2.3, using the apparatus shown in Fig. 3.10. The modulus was taken as the slope of the later portion of the stress-strain curve after the initial uncrimping of collagen fibers (see Fig. 3.35). The swelling ratio, R, of the collagen fiber was taken as the ratio of the swollen weight of the fiber (when swollen to equilibrium in a citric acid-phosphate buffer, pH 7.4) to the dry weight of the fiber (after dehydration at 105°C at a pressure of 10^{-3} mm Hg for 48 hours). It can be seen in Table 4.2 that the moduli of the uncoated collagen and of the HA-coated collagen are slightly lower than the other MPS-coated collagen fibers. This may be due to the destruction of secondary forces or labile covalent links (see Bailey, 1968) caused by immersion of the collagen

TABLE 4.2

PHYSICOCHEMICAL PROPERTIES OF MUCOPOLYSACCHARIDE-COATED COLLAGEN

	wt-% MPS	χ	$M_c \times 10^{-3}$	$E \times 10^{-9}$ dynes cm^{-2}	%-H	swelling Ratio (R)	$1/\tau \times 10^{4}$ (min^{-1})
Collagen Control	0	0.52±0.04	3.8±0.5	3.3±0.3	100±7	3.5±0.5	8.48±0.07
Collagen -CH6-S	11.3±0.5	0.53±0.04	4.1±0.5	4.0±0.3	97±7	2.7±0.5	1.46±0.07
Collagen -CH4-S	8.7±0.5	0.52±0.04	4.0±0.5	4.2±0.3	99±7	3.1±0.5	0.88±0.07
Collagen -HA	8.2±0.5	0.58±0.04	4.2±0.5	3.5±0.3	100±7	3.6±0.5	5.38±0.07
Collagen -DS	8.2±0.5	0.53±0.04	3.9±0.5	3.9±0.3	95±7	2.7±0.5	0.90±0.07
Collagen -H	8.7±0.5	0.53±0.04	3.8±0.5	4.2±0.3	100±7	2.5±0.5	0.98±0.07
Collagen -HS	10.5±0.5	0.55±0.04	3.8±0.5	4.0±0.3	96±7	3.2±0.5	1.10±0.07

fibers in the acid medium (pH 3.2) during preparation of the samples. The presence of sulfated MPS (Ch 6-S, Ch 4-S, DS, H, HS) apparently prevents or inhibits such a destruction whereas the absence of MPS or the presence of a non-sulfated MPS (HA) does not. This is further reflected in the higher swelling ratio of the uncoated and HA-coated collagen when swollen to equilibrium in a citric acid-phosphate buffer solution, pH 7.4 (Table 4.2).

The helical content, %-H, of the collagen in the MPS-coated collagen samples was determined by the IR absorbance at 340cm^{-1}, corrected for both the thickness of the sample and the concentration of collagen in the sample (see Section 3.3.4 for details). The use of this method for determining %-H was necessary because orientation of the collagen fibers precludes use of the optical rotation method (Section 3.3.5) while the thickness of the samples (0.0012") preclude the use of relative intensitites of IR absorbances, A_{1235}/A_{1450} (see Section 3.3.4). Furthermore, the IR spectra of the MPS (Brogna, 1973; Bernardi et al., 1957; Orr, 1954; Mathews, 1958) show that the absorbances at 1235cm^{-1} and 1450 cm^{-1} of collagen are interfered by absorbances of the MPS at or near these frequencies whereas the absorbance at 340cm^{-1} is relatively free of interference by the MPS IR spectra.

The susceptibility of the MPS-coated collagen fibers to collagenase attack was studied using the method described in Section 3.4. The use of a considerably higher collagenase con-

centration (40 Units/ml) and a slightly higher temperature (42°C) in this study was necessitated by the higher resistance of the samples to collagenase attack. However, the studies in Section 3.4 show that changes in these two experimental variables have the effect of changing only the rate of enzymatic degradation without introducing any other additional effects. The rate of the enzymatic attack, as measured by $1/\tau$ is tabulated in Table 4.2 for the uncoated as well as the MPS-coated collagen. It can be seen that the collagen coated with sulfated-MPS are much more resistance to collagenase attack than both the uncoated and HA-coated collagen. This will be discussed and studied further in Section 4.4.

4.2.6. Collagen-Mucopolysaccharide Composite Materials. The preparation of materials in which collagen fibrils are dispersed within a MPS matrix was motivated by the similarity of such materials to the native connective tissue matrix, as pictured in Fig. 2.6. The ease with which these materials can be prepared also make them attractive as probable materials for skin replacement. To date, only collagen-Ch 6-S composite of varying MPS concentration have been prepared and characterized although the techniques for preparation and characterization of these materials, to be described in this Section, appear to be applicable to any collagen-MPS composite material.

The collagen used in the present study was the Ethicon BAT collagen fibrils which had been dispersed in a 0.5M acetic acid

solution (described in Section 4.2.2). The concentration of the collagen dispersion was 1.0%. The Ch 6-S used has also been described and characterized in Section 4.2.2. The buffer solution used was the citric acid-phosphate buffer solution, described in Section 4.2.5 while the crosslinking agent was glutaraldehyde (0.025M), also described in the same Section.

The procedure for preparing the collagen-Ch 6-S composite material is as follows: The Ch 6-S solution was prepared by dissolving various amounts of the MPS (ranging from 5mg to 400 mg) in 20ml of citric acid-phosphate buffer solution, ph 3.2. The collagen dispersion (10 ml) was placed in a Waring Blender and stirred at a moderately fast speed (ca. 10 revs per sec). The Ch 6-S solution was placed in a burette and added dropwise to the well-stirred collagen dispersion. When all the Ch 6-S was added, the mixture of precipitated collagen fibers and Ch 6-S was allowed to stand for ca. 10 min. The mixture was then filtered on a filter papter (No. 576, Schleicher and Schuell, Inc., Keene, New Hampshire) placed on a Buchner funnel by the application of a low negative pressure supplied by a vacuum pump. When all the supernatant had been filtered, the residue was allowed to dry to equilibrium under laboratory conditions (23°C, 50% RH).

Crosslinking of the collagen-Ch 6-S precipitate was done with a two step process similar to that described in Section 4.2.5. The filter paper, with the dried precipitate on top of it was placed in a citric acid-phosphate buffer solution (pH 3.2) con-

taining 0.025M glutaraldehyde at 23°C. After ca. 24 hours immersion, the crosslinked collagen-Ch 6-S precipitate was stripped from the filter paper as a coherent membrane and placed in a citric acid phosphate buffer solution, pH 7.4, containing 0.025M glutaraldehyde at 23°C. After 24h immersion, the collagen-Ch6-S membrane was rinsed three times with distilled water and placed for another 24h in a 0.2 wt-% dimedone solution in order to remove excess, unreacted aldehydes. The membrane was then rinsed five times with distilled water and stored in a citric acid-phosphate buffer solution ph 7.4 at 4°C until required for subsequent characterization.

The reported wt-% of Ch6-S in the collagen-Ch6-S composite materials, prepared by varying the relative concentrations of collagen and Ch6-S in the original solution mixture was the average of the results of the hexosamine and hydroxyproline analyses, obtained as described in Section 4.2.5. The variation of the wt-% of Ch6-S in the composite materials with the relative concentrations of collagen and Ch6-S in the original solution mixture is shown in Fig. 4.6. The Figure shows that for low relative concentrations of Ch6-S in the original solution, most of the Ch6-S interacted with the collagen fibers that were precipitated. However, as the relative concentration of Ch6-S in the original mixture increased, the proportion of interacted Ch6-S decreased. The wt-% of Ch6-S in the composite materials appear to reach an asymptotic value of ca. 16% as large relative concentrations of Ch6-S were used.

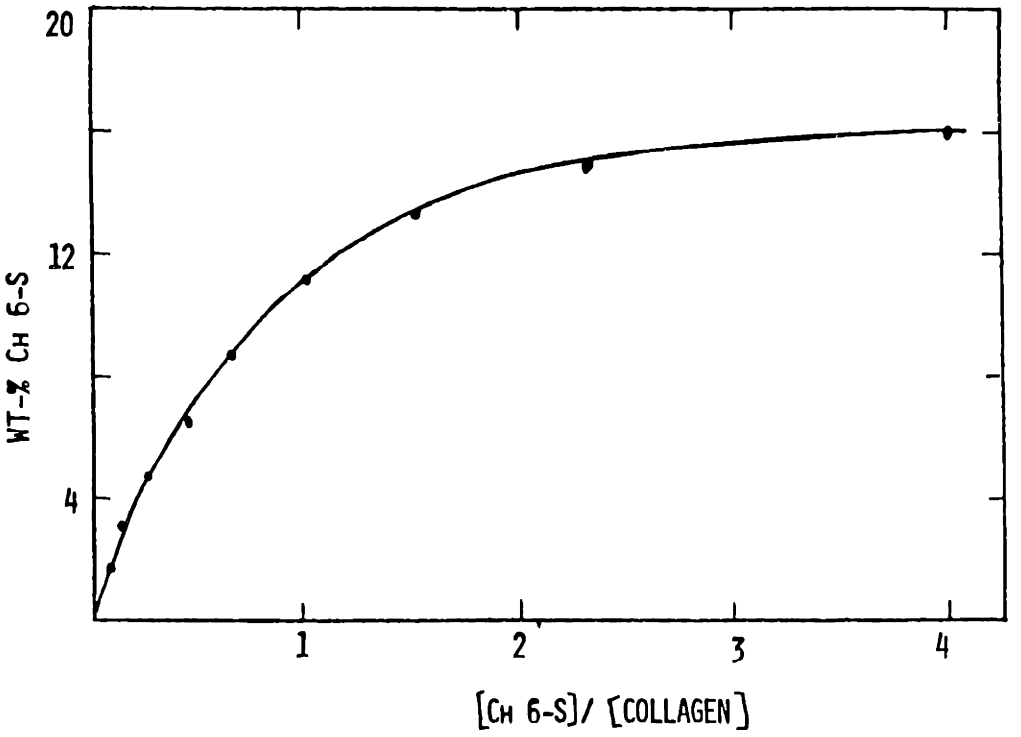


Fig. 4.6. The wt-% of Ch 6-S attached to collagen as a function of the ratio of concentrations of solutions of Ch 6-S and collagen used for the interaction of the two polymers. The medium in which the interaction occurred was citric acid-phosphate buffer, pH 3.2 at 23°C.

TABLE 4.3

PHYSICOCHEMICAL PROPERTIES OF COLLAGEN/CHONDROITIN 6-SULFATE COMPOSITE MATERIALS

wt-%MPS	χ	$M_c \times 10^{-4}$	UTS$\times 10^{-7}$ (dynes/cm^2)	E_B(%)	Swelling Ratio (R)	%-H	$1/\tau^2 \times 10$ (min^{-1})
0	0.53±0.04	1.5±0.1	1.1±0.2	26±1	9.3±0.8	100±7	0.255±0.009
1.8±0.2	0.52±0.04	1.4±0.1	2.1±0.2	30±1	8.9±0.8	98±7	0.149±0.009
3.0±0.2	0.50±0.04	1.2±0.1	2.3±0.2	30±1	7.8±0.8	98±7	0.153±0.009
4.8±0.2	0.55±0.04	1.3±0.1	3.7±0.2	33±1	5.5±0.8	100±7	0.093±0.009
6.5±0.2	0.51±0.04	1.1±0.1	3.6±0.2	32±1	4.6±0.8	99±7	0.084±0.009
8.6±0.2	0.52±0.04	0.9±0.1	3.6±0.2	34±1	4.0±0.8	100±7	0.049±0.009
11.2±0.2	0.50±0.04	1.0±0.1	3.5±0.2	34±1	4.1±0.8	97±7	0.052±0.009
13.3±0.2	0.53±0.04	1.2±0.1	2.3±0.2	32±1	5.1±0.8	100±7	0.047±0.009
14.9±0.2	0.52±0.04	1.1±0.1	1.7±0.2	27±1	6.3±0.8	96±7	0.064±0.009
16.0±0.2	0.54±0.04	1.4±0.1	1.4±0.2	27±1	7.8±0.8	100±7	0.067±0.009

The results of characterization of the collagen-Ch6-S composite materials prepared as described above are tabulated in Table 4.3. The polymer-solvent interaction parameter, χ, determined on the denatured collagen fibers, as described in Section 3.2.3, showed little change over the concentration of Ch6-S studied. However, as the concentration of Ch6-S increases over the range of studied (0 to 16 wt-% MPS), the crosslinking density, the ultimate tensile strength (UTS) and the elongation at break (E_B) appear to go through a maximum at ca. 11 wt-% MPS while the swelling ratio (R) and the rate of enzymatic degradation ($1/\tau$) appear to go through a minimum at this concentration. The mechanical behavior of these composites will be further studied and discussed in Section 4.3 where a probable explanation for the phenomena just described will be made. The enzymatic degradation of the collagen-Ch6-S composite will be further studied and discussed in Section 4.4.

4.3. Mechanical Behavior of Collagen-mucopolysaccharide Composite Materials.

4.3.1. Introduction. Since the skin provides protection for the body against a variety of mechanical injuries (see Section 2.1.5), an assessment of the mechanical behavior of any material chosen for use as skin replacement is important. A study of the mechanical behavior of the materials is also important in assessing their ability to withstand the various surgical operations (such as suturing) which may be necessary to secure the materials to the wound site.

Collagen fibers, free from mucopolysaccharides, are strong enough to be used in a variety of biomedical applications without posing any problems due to their mechanical behavior (Chvapil, 1973). Coating of collagen fibers with MPS apparently do not alter significantly their mechanical behavior (Table 4.2). However, previous studies (Milch, 1966) suggest that the presence of MPS, within a matrix of collagen fibrils may act to soften the matrix while other studies (Jackson, 1953; Kuhnke, 1962; Elden, 1964) suggest an exactly opposite effect (i.e. the MPS can reinforce the collagen fibrils). Bryant and Weeks (1967) have attempted to reconcile these apparent contradictions by suggesting that at low MPS content, the MPS may reinforce the collagen fibrils while at high MPS content, they may soften and weaken the fibrils. Their studies on the tensile strength of secondary wounds of rats and mice (Bryant and Weeks, 1967) appear to support such a hypothesis (see Section 4.1.3).

The availability of collagen-MPS composite materials with varying MPS concentrations (prepared by the method described in Section 4.2.6) coupled with the necessity to assess their mechanical behavior have provided both the means and the motivation for testing the validity of the hypothesis of Bryant and Weeks (1967). We describe here studies on the mechanical behavior of these composite materials under both tensile and compressive loading.

4.3.2. Experimental. The composite material used was the collagen-Ch6-S composite prepared by the method described in Section 4.2.6. Both the tensile and compressive properties were

measured in 0.05M Tris-HCl buffer solutions, pH 7.4 (described Section 3.4.2). The apparatus for determining the tensile stress-strain curve is shown in Fig. 3.10 and the procedure for the determination was described in Section 3.2.3. The ultimate tensile strength (i.e. the stress at which the sample breaks) and the elongation at break were determined and the results are tabulated in Table 4.3.

The apparatus used for studying the compressive properties of the composite materials, shown in Fig. 4.7, has been described fully by Lee (1973) who used it to study the compressive properties of model materials similar in mechanical behavior to cartilage. In brief, the specimen, in the shape of a flat disc is placed on a coarse sintered glass disc through which the appropriate swelling agent permeates freely. The latter is supplied from a reservoir to the underside of the sintered glass disc. A plunger, which rests on the specimen, carries an extension rod which is attached to the transducer core of a linear displacement transducer (7 DCDT-100, Hewlett Packard, Waltham, Mass.). The load is applied on the specimen through the plunger whose displacement is measured by the transducer and recorded on a Hewlett Packard X-Y recorder (Model No. 7001 AM). The time dependence of strain (i.e. creep curve) of a specimen loaded by an initial fixed load can be measured using this apparatus. By using a range of loads, a series of creep curves corresponding to each of the applied loads can be obtained. Isochronous (i.e. fixed time) stress-strain curves can then be determined from the series of creep curves.

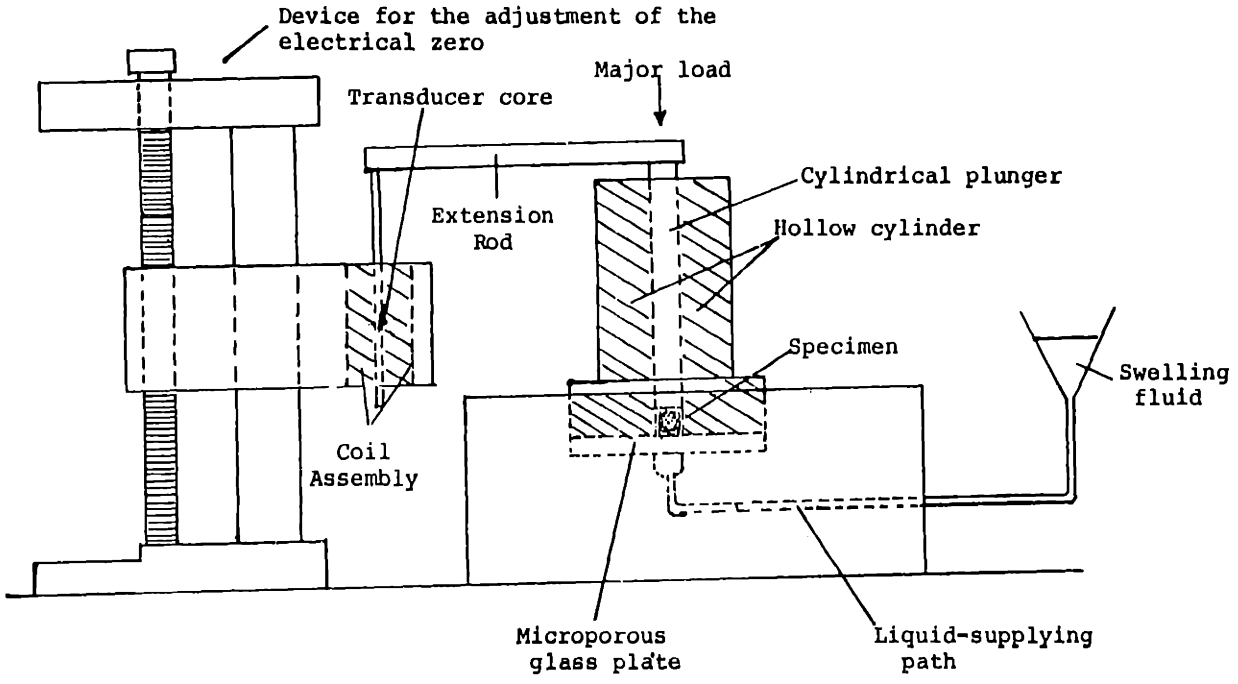


Fig. 4.7. Apparatus for studying the compression property of a material.

The procedure for obtaining the compressive creep curves of the collagen-Ch6-S composite materials is as follows: Circular discs of the samples (diameter, 2.2 mm, thickness ca. 0.1 mm) were cut from a membrane of collagen-Ch6-S composite material using a die. Samples were placed in the center of the testing chamber by means of a centering device which consisted of a hollow tube with an inner diameter equal to that of the specimen and an outer diameter equal to the inside diameter of the hollow cylinder which forms the wall of the testing chamber. By placing the sample first in one end of the hollow tube and inserting that end into the testing chamber, the sample can be placed centrally in the chamber by means of a piston which is inserted into the hollow tube. The plunger was then placed gently on the sample and the displacement of the plunger from its initial position on the sintered glass disc, as measured by the transducer, was taken as the thickness of the sample.

In order to obtain reproducible results, it was found that preconditioning of the sample before testing was necessary. The preconditioning consists of applying a load higher than the maximum load used in the test (2360 gm) for a fixed period of time and then allowing the specimen to recover over another fixed period of time. The load used for preconditioning in the present study was 3360 gm and the loading and recovery times were 1/2 h and 1 h respectively. After such a preconditioning cycle, the samples were found to be capable of recovering fully from the deformations imposed on them by subsequent loadings using loads less then the preconditioning load.

For each sample of collagen-Ch6-S composite material, a series of creep curves with loads of 200, 360, 860, 1360 and 2360 g were obtained. Each creep curve was obtained by the application of the load for 500 secs followed by a recovery period, under the weight of the plunger (ca. 20 g) for 1000 secs before the application of the next load. From these curves, the isochronous (i.e. fixed time) stress-strain curves for each composite material were determined. Finally, the variation of the isochronous-isotonic (i.e. fixed time and stress) strain with the wt-% of Ch6-S in the composite material was determined.

4.3.3. Results and Discussion. The tensile stress-strain curves of a few of the collagen-Ch6-S composite materials, containing varying amount of Ch6-S, are shown in Fig. 4.8. It can be seen from this Figure that over the concentration range of 0 to ca. 11 wt-% MPS, the effect of increasing MPS content is to increase the initial slopes of the stress-strain curves without altering the slopes of the final linear portion of the curves. For concentrations higher than ca. 11 wt-% MPS, the effect of increasing MPS content appears to be to decrease the initial slopes of the stress-strain curves, again not affecting the final slope of the curves. Since the initial slopes cannot be determined accurately, the ultimate tensile strength (UTS) and the elongation at break (E_B) were taken as measures of the mechanical behavior of the collagen-Ch6-S composite materials. The value of ca. 3.5×10^7 dynes cm^{-2} for the ultimate tensile strength of the collagen-Ch6-S composite material (containing ca. 11 wt-% Ch6-S)

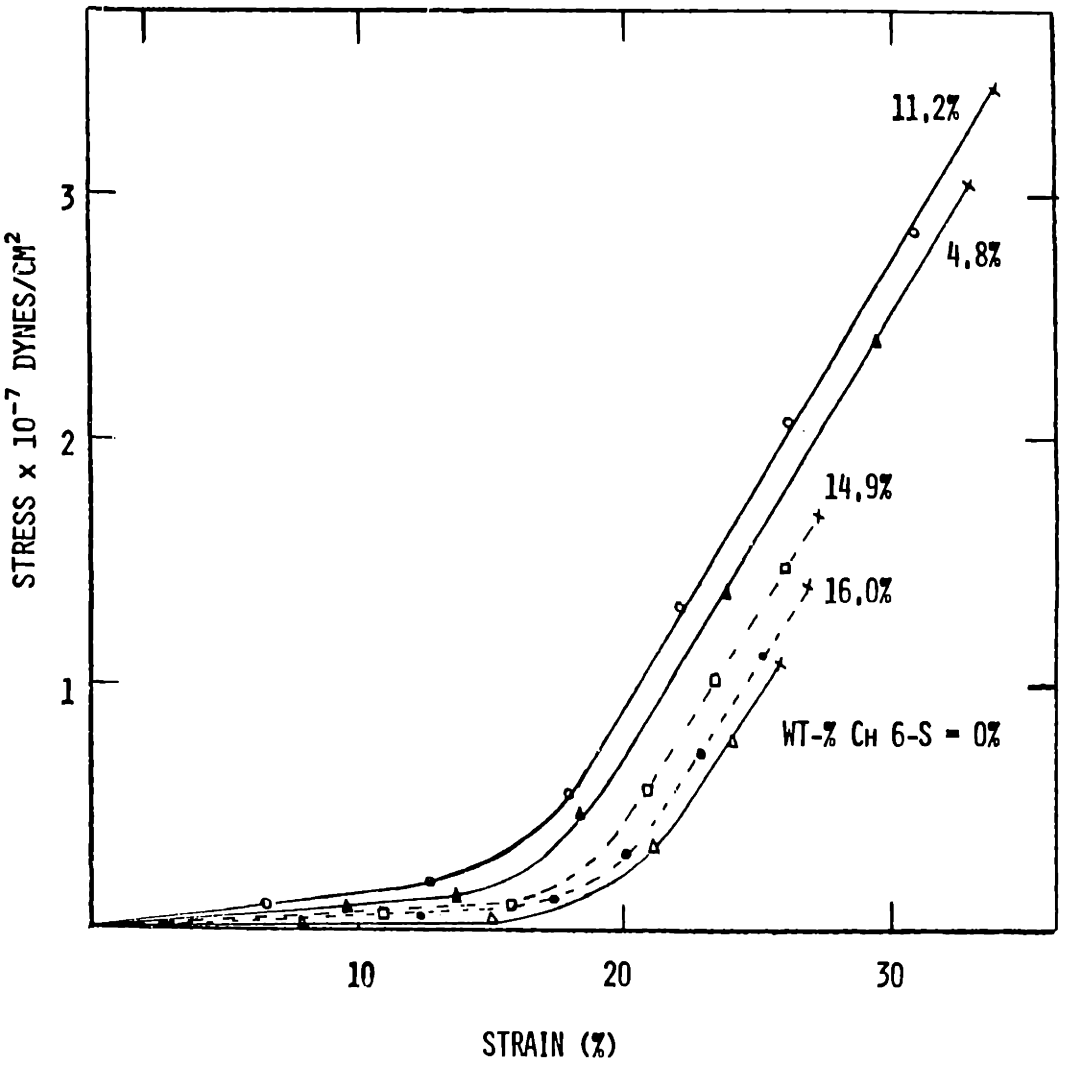


Fig. 4.8. Tensile stress-strain curves of collagen-Ch 6-S composite materials, containing various amount of Ch 6-S, as indicated. The crosses (x) indicate the point at which fracture of the material occur.

is comparable with values of UTS of skin (3×10^7 - 12×10^7 dynes cm^{-2}) from various regions of the human body obtained by Yamada (1970). The markedly lower elongation at break of the composite material (34%) compared to that of normal skin (43%-123%) obtained by Yamada (1970) can probably be explained by the absence of elastin and a higher extent of crosslinking in the composite material.

The variation of the UTS, E_B, swelling ratio (R) and molecular weight between crosslinks (M_c) as a function of MPS content in the collagen-Ch6-S composite materials have been tabulated in Table 4.3 and are shown graphically in Fig. 4.9. Over the range of concentration studied (0 to 16 wt-% Ch6-S), it appears that as the concentration of Ch6-S increases, the UTS, E_B and crosslink density (which is inversely proportional to M_c) all go through a maximum at ca. 11 wt-% Ch6-S, while the swelling ratio goes through a minimum at that composition level. In the studies to be reported below, the compressibility of the composite materials also appears to go through a minimum at this concentration of Ch6-S (See Fig. 4.14).

A probable explanation for these observations is shown diagrammatically in Fig. 4.10 where various states of the composite materials (A, B and C) containing different concentration of Ch6-S are shown. The approximate concentrations of Ch6-S corresponding to the states of the materials depicted in Fig. 4.10 are shown in Fig. 4.9. Collagen contains a greater amount of basic groups than acid groups (Eastoe, 1967) and is generally considered as a cationic polymer, i.e. it possesses a net positive charge. Although the

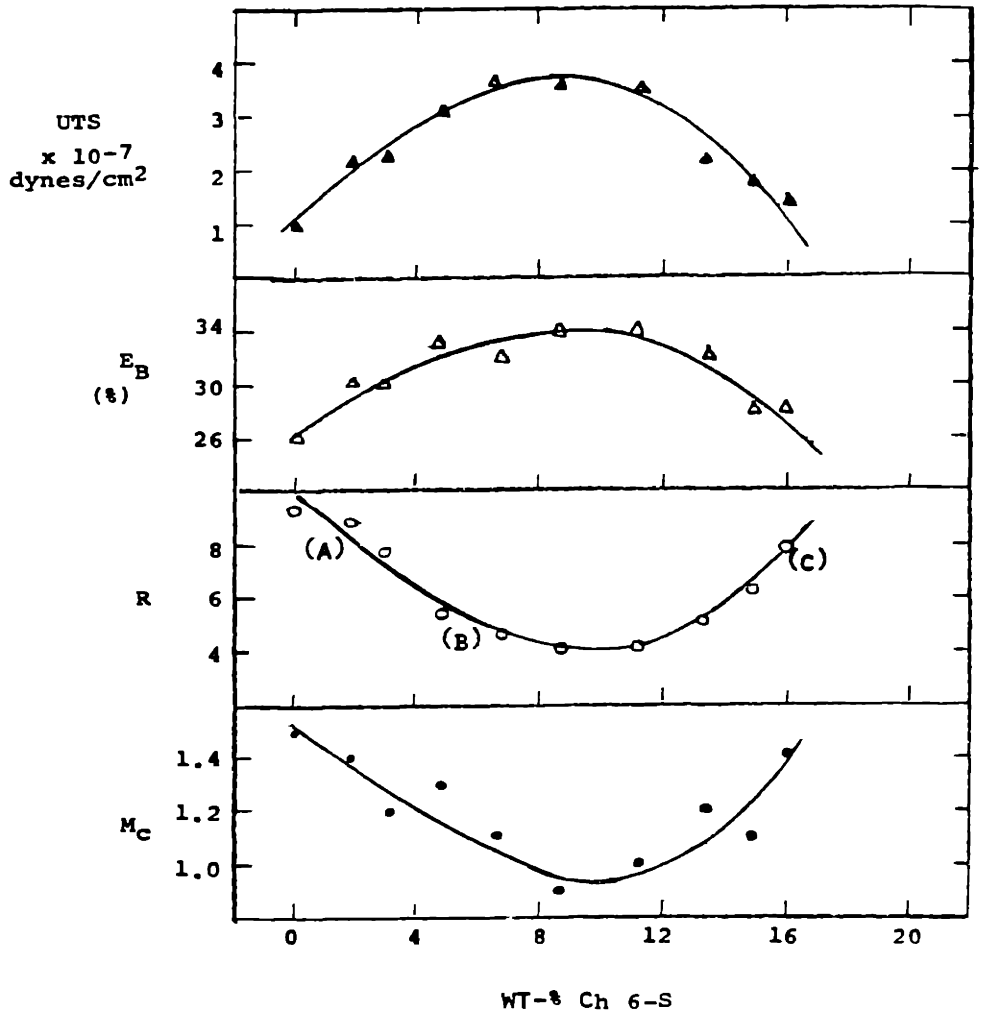


Fig. 4.9. Comparison of a number of physicochemical properties of collagen-Ch 6-S composite materials containing varying amount of Ch 6-S. The properties compared are the ultimate tensile strength (UTS), elongation at break (E_B), swelling ratio (R) and number average chain molecular weight (M_c).

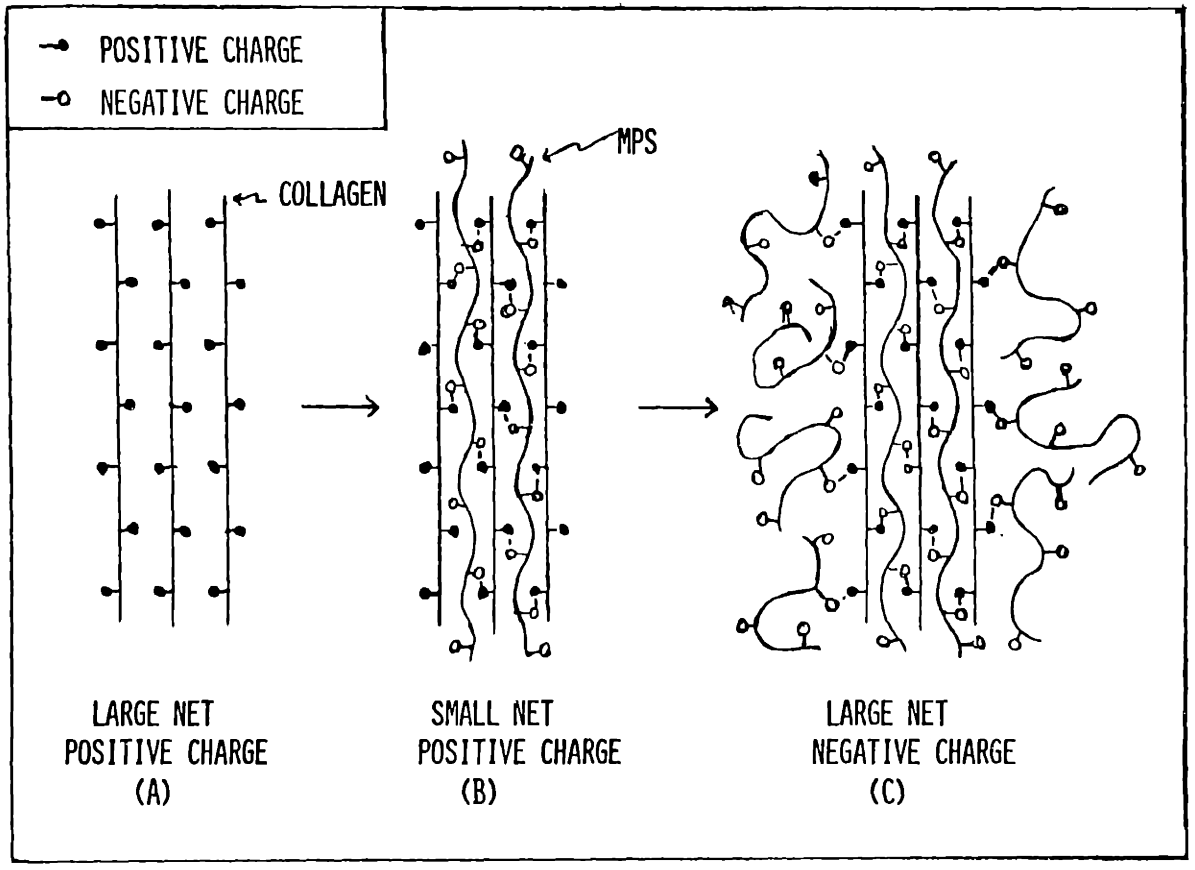


Fig. 4.10. A model for explaining the variation in physicochemical properties of the collagen-Ch 6-S composite materials with the Ch 6-S content as shown in Fig. 4.9. See text for detailed description.

isoionic point of collagen has often been quoted to be ca. pH 4-5 (Anderson and Eriksson, 1968) which suggests that the collagen is an anionic polymer, the true isoionic point of collagen, according to Eastoe (1967), is ca. pH 9.4 - a value which is consistent with its cationic nature. The substantially lower reported value for the isoionic point of collagen (Anderson and Eriksson, 1968) is probably due to ion-binding of the collagen to salts present in the solution in which it is immersed (Eastoe, 1967). Mucopolysaccharides, on the other hand, are anionic polymers (i.e. they possess a net negative charge) by virtue of the carboxylic and sulfate groups present in the polymers (see Section 2.2.4).

The interaction of collagen and mucopolysaccharides is caused by the interaction of the cationic and anionic groups present respectively in these polymers (Podrazky et al., 1971). Fig. 4.10 shows that in the absence of any MPS, the collagen (A) has a net positive charge and hence tends to be swollen because of the repulsion of like charges on the polymer chains. As the concentration of MPS increases, interaction of the charges of the collagen and MPS occur, resulting in a reduced net positive charge on the collagen (B). This results in a decreased swelling of the collagen because of a reduction in the repulsion of like charges. However, as the concentration of MPS is further increased, a large net negative charge is developed and hence the composite material will progressively increase in swelling (again due to the repulsion of like charges). The point where minimum swelling is observed (Fig. 4.9) therefore

probably corresponds to the situation where the no net charges occur on the collagen-Ch6-S composite.

The effect of increasing MPS concentration on the other properties shown in Figure 4.9 can probably be explained by the effect on swelling of the composite materials described above. A decrease in swelling of the material would result in a closer interaction between collagen molecules and hence a greater probability of crosslinking by a chemical agent. A minimum in the swelling of the material thus result in a maximum crosslink density, as shown in Fig. 4.9. A decrease in swelling also tend to strengthen the composite material because of the closer interaction of the macromolecules present in the material. A maximum in the UTS and E_B of the composite materials occurs therefore at the point where a minimum in swelling of the material is observed. The increased crosslink density of the materials at this point may also contribute to the strengthening of the materials.

The results obtained in the present study are in general agreement with the hypothesis of Bryant and Weeks (1967) which postulate that there is a critical MPS concentration at which the strength of the collagen-MPS composite material is maximum. Below this critical concentration the strengthening effect of the MPS (Jackson, 1953) is reduced and the strength of the composite material decreases. Above this critical concentration of MPS, the softening effect of the MPS (Milch, 1966) predominates and the strength of the composite material is again reduced. The critical concentration for the composite

materials studied here is ca. 11 wt-%. This correlates very well with the results of Bryant and Weeks (1967) who showed that the strength of secondary wounds in rats (which contain ca. 11 wt-% MPS) is about four times higher than the strength of wounds in mice (which contain ca. 22 wt-% MPS).

The results of the present study also agree with those obtained by Kempson et al. (1973) who studied the tensile properties of the cartilage of human femoral condyles and related them to the MPS content of the cartilage. They showed that the effect of increasing the MPS content of the cartilage, starting from ca. 11 wt-% MPS, was to decrease the initial slope of the stress-strain curve without affecting the final slope of the curve. They further showed that the effect of increasing collagen content over the range of concentration studied (40 to 90 wt-% collagen) was to increase the ultimate tensile strength of the cartilage. These results agree substantially with the results obtained in the present study (see Fig. 4.8). Kempson et al. (1973) further showed that the removal of MPS (by enzymatic treatment) from cartilage containing ca. 10 wt-% MPS resulted in a decrease in the initial slope of the stress-strain curve without affecting the final slope of the curve. This is again in agreement with the results shown in Fig. 4.8.

The observations made in the present study may be used to explain a number of pathological conditions which result in loss in strength of connective tissues. The decrease in MPS content of cartilage inflicted with osteoarthritis (Hjertquist and Lemperg, 1972)

and rheumatoid arthritis (Caygill, 1969) may explain the loss in strength of the cartilage under these pathological conditions. The removal of MPS and PPS from cartilage is probably caused by the release of lysosomal enzymes (see Section 2.4.4). A loss in strength of a collagen-MPS composite material due to the loss of MPS has been demonstrated in the present studies as well as in the studies of Jackson (1953), Elden (1964), and Kempson et al.(1973). On the other hand, the loss in strength of connective tissues inflicted with scurvy has been explained (Robertson, 1961) by the excessive amount of MPS present in these tissues (Robertson and Hinds, 1956). This accumulation of MPS is thought (Robertson, 1961) to be due to the retardation of collagen biosynthesis caused by a deficiency of ascorbic acid (Gould and Woessner, 1957). The inability of the collagen in the scorbutic connective tissue to form fibrils have also been used to explain the loss in strength of these tissue (Cmuchalova and Chvapil, 1963; McCandlish and Tristram, 1963; Caygill, 1969). The results obtained in the present study (Figs. 4.8 and 4.9) support the notion that the presence of an excessive amount of MPS can decrease the strength of a collagen-MPS composite material. In all of the pathological conditions just discussed, a loss in strength of the materials may also be due to an accelerated enzymatic degration of the collagen fibers in the materials. This will be discussed in Section 4.4.3.

The results of compression studies on the collagen-MPS composite materials demonstrate other interesting aspects of the effect

of MPS on the mechanical properties of collagen fibers. Figure 4.11 compares the compression creep curves of pure collagen with that of a collagen-Ch6-S composite material containing ca. 11.2% Ch6-S. Two major differences can be distinguished between these two sets of creep curves. Firstly, for equivalent stresses on the materials, the strain level of the pure collagen at any fixed time is considerably higher than that of the collagen-Ch6-S composite material (i.e. the pure collagen is more compressible than the composite material). Secondly, the time required for the pure collagen to attain equilibrium strain level (ca. 500 secs) is considerably longer than that required by the composite material to attain equilibrium strain level (ca. 75 secs). To further illustrate this point, the initial portion of the creep curves of a number of collagen-Ch6-S composite materials are presented in Fig. 4.12, where the strain levels $\varepsilon(t)$ have been normalized by dividing with the equilibrium strain level at 500 secs. It can be seen from this Figure that as the concentration of Ch6-S increases (up to ca. 11 wt-% Ch6-S), the time required to reach equilibrium strain level decreases. Above the concentration of ca. 11 wt-% Ch6-S, the time constant for the attainment of equilibrium strain is slightly increased by increasing concentration of Ch6-S. An identical pattern of behavior occurs for the recovery portion of the compression test (see Fig. 4.11).

The results obtained above illustrate that a probable function of the MPS present in connective tissues is to modify the dynamic mechanical properties of the tissues. Since a major part of

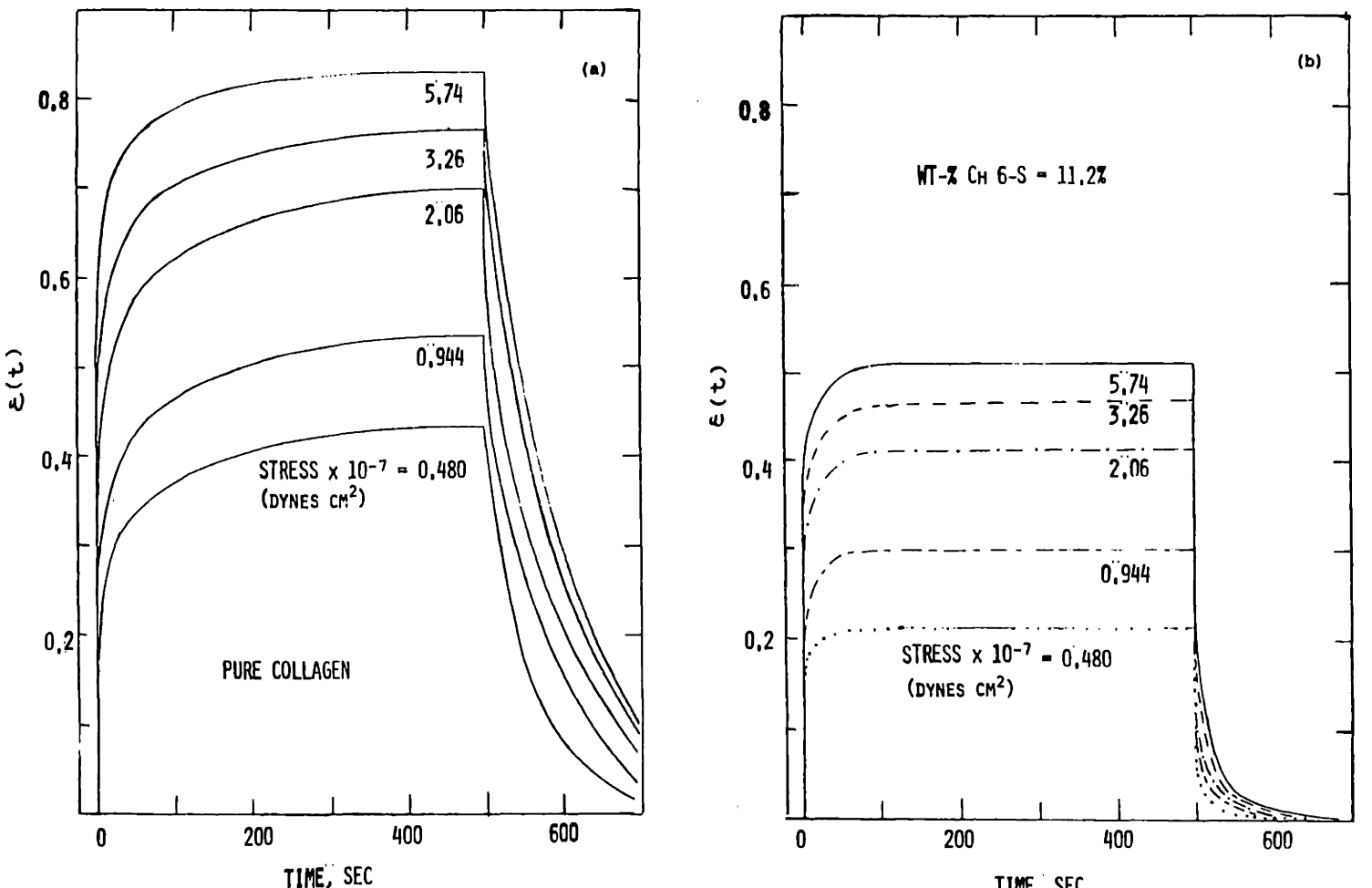


Fig. 4.11. A comparison of the compression creep curves of pure collagen and a collagen-Ch 6-S composite material containing 11.2 wt-% Ch 6-S. Stresses used in each creep determination are as indicated.

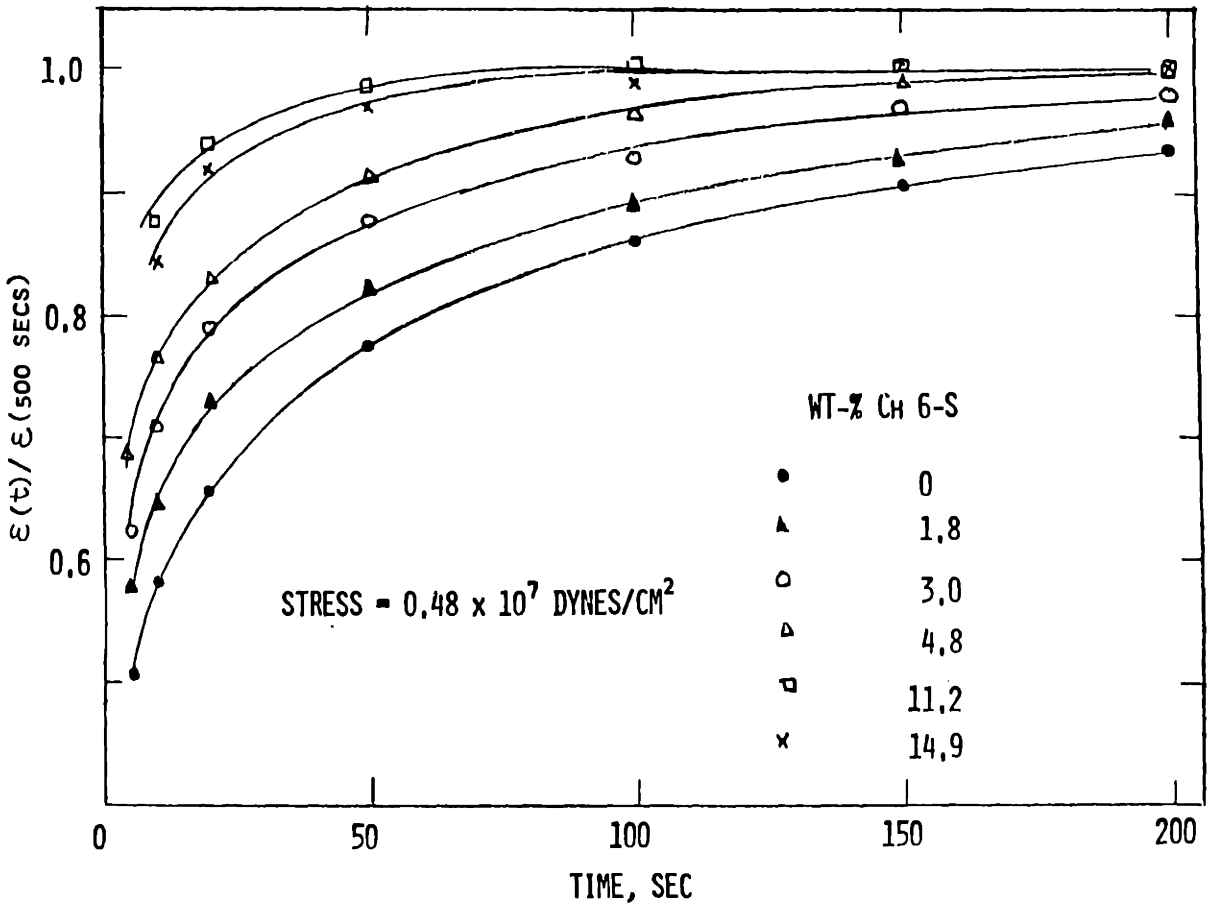


Fig. 4.12. Comparison of the initial portion of the compression creep curves of a series of collagen-Ch 6-S composite materials, containing varying amount of Ch 6-S, as indicated. The strain at any time, $\varepsilon(t)$ have been normalized by division with the equilibrium strain level at 500 secs, $\varepsilon(500 \text{ secs})$.

the mechanical forces experienced by the tissues are dynamic, this modification may be of some physiological significance. The aging of skin results in a loss of its ability to recover quickly its original form when it is subjected to an intermittent deformation (Kirk and Kvorning, 1949). The observation that the total MPS content in skin decreases with age (Pearce, 1965) coupled with the observation, made in this study (that a decrease in MPS content can result in a decrease in the ability of the material to recover quickly from a deformation) may be used to explain the effect of aging on the dynamic mechanical behavior of skin.

From the creep curves shown in Fig. 4.11, the isochronous stress-strain curves in compression of the collagen-Ch6-S composite materials can be determined. The equilibrium stress-strain curves (at 500 secs) of these materials are shown in Fig. 4.13. It can be observed that the effect of increasing MPS concentration (up to a concentration of ca. 11 wt-% CH6-S) is to increase the initial slope of the stress-strain curve without affecting the final slope. Above the concentration of ca. 11 wt-% CH6-S, the initial slope of the stress-strain curve is reduced by increasing concentration of Ch6-S. This behavior can be seen more clearly in Fig. 4.14 where the isochronous-isotonic strain is plotted against the wt-% of Ch6-S. Comparison of Fig. 4.14 with Fig. 4.9 shows that the minimum compressibility of the composite material occurs at the same concentration (ca. 11 wt-% CH6-S) where minimum swelling occurs. The explanation given earlier for the minimum swelling of the composite material can, therefore, be used to explain the minimum compressibility observed

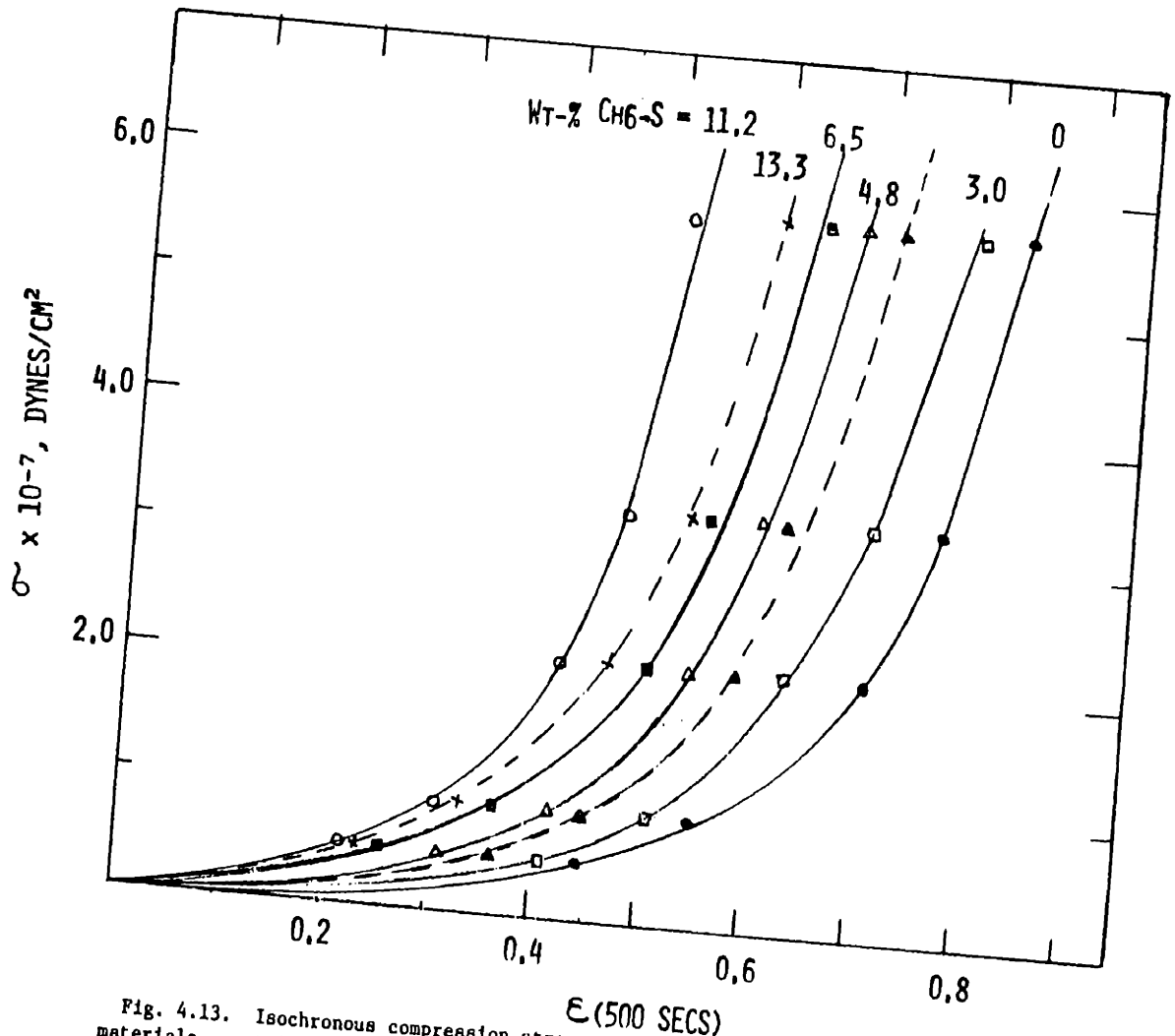


Fig. 4.13. Isochronous compression stress-strain curves of collagen-Ch 6-S composite materials, containing varying amount of Ch 6-S, as indicated.

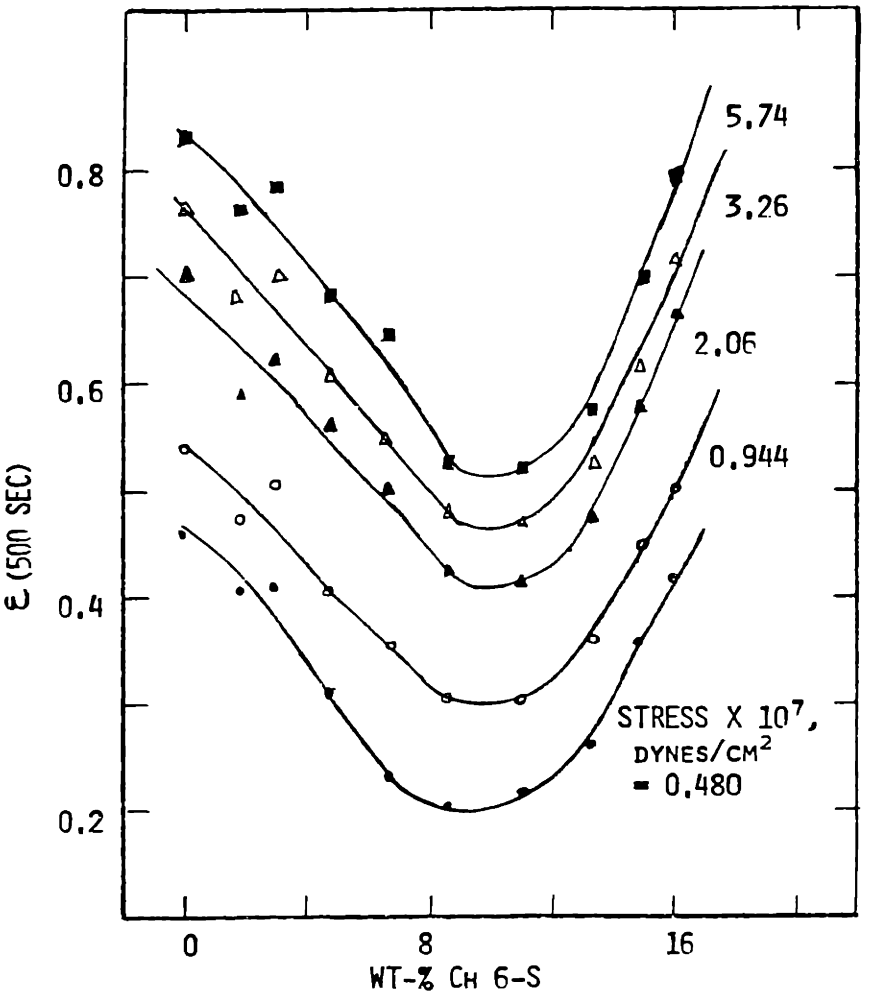


Fig. 4.14. Variation of the isochronous-isotonic strain in compression with the Ch 6-S content in collagen-Ch 6-S composite materials. Calculated from Fig. 4.13.

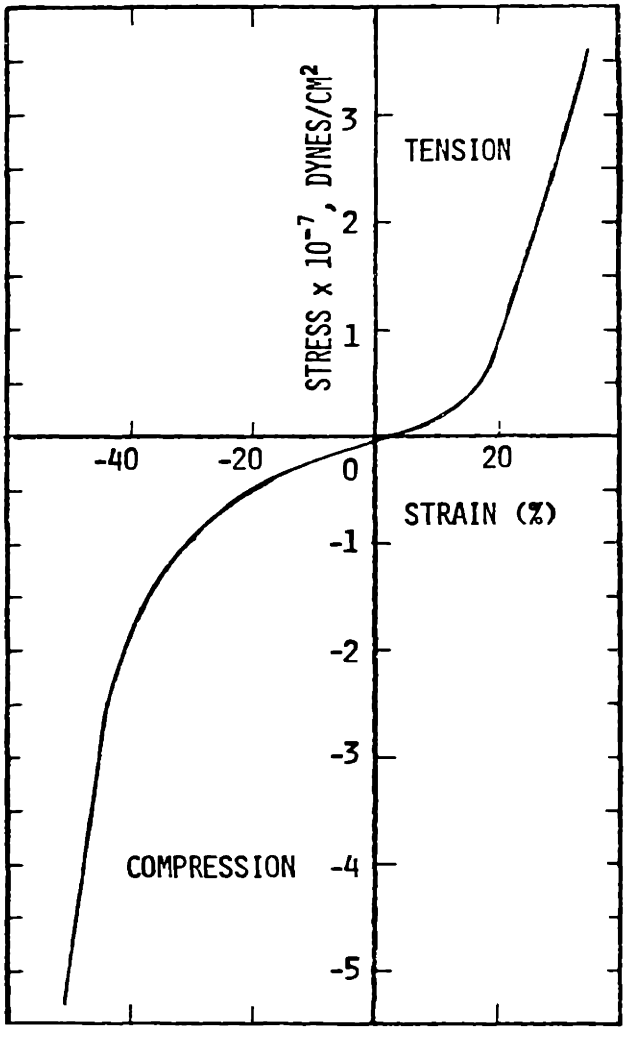


Fig. 4.15. A comparison of the equilibrium stress-strain curves of a collagen-Ch 6-S composite material (ca. 11.2 wt-% Ch 6-S) under tensile and compressive loading.

in the present study (Fig. 4.14).

A comparison of the stress-strain curves of the composite material (11.2 wt-% CH6-S) under tensile (Fig. 4.8) and compressive (Fig. 4.13) loadings is shown in Fig. 4.15. It can be seen from the latter Figure that the strain at which the high modulus portion (i.e. the straight portion) of the stress-strain curve begins is higher in the case of compressive loading (ca. 40% strain) than in the tensile loading (ca. 20% strain). This probably indicates that the collagen fibers in the composite material are in a state of tension, due, perhaps to the manner it was prepared. The modulus of the composite material (as deduced from the slope of the straight portion of the stress-strain curve) is higher in compression (ca. 3.7×10^8 dynes/cm^2) than in tension (ca. 1.9×10^8 dynes/cm^2).

4.4. Enzymatic Degradation of Collagen-Mucopolysaccharide Composite Materials.

4.4.1. Introduction. A study on the enzymatic degradation of the MPS-coated collagen (described in Section 4.2.5) and of the collagen-MPS composite materials (described in Section 4.2.6) was made in order to assess the potential use of these materials for skin replacement as well as to answer the more fundamental question of whether the MPS has any effect on the degradation of collagen by collagenase. The results of such a study will be reported in this Section.

Previous studies by Quintarelli and Dellovo (1970) showed that a coat of glycoproteins surround the collagen fibrils in

connective tissues and that such a coat inhibits the attack of the collagen by collagenase. The removal of this glycoprotein by treatment with a crude bacterial α-amylase (CBA) resulted in the connective tissue being markedly more susceptible to attack by collagenase. In order to test this idea, as well as to examine the possibility of the MPS having a similar effect, a study of the enzymatic degradation of rat tail tendon, treated with CBA and with hyaluronidase was made. The results of these studies, to be reported in this Section, appear to answer some of the questions raised in Section 2.4 regarding the in vivo degradation of the connective tissue and also to contribute to a better understanding of the ultrastructure of RTT which was studied by the scanning electron microscope in Section 3.1.

4.4.2. Experimental. The MPS-coated collagen tapes, prepared and characterized as described in Section 4.2.5 were tested for their susceptibility to collagenase degradation by the method described in Section 3.4.3. Briefly, the tapes were extended to a strain of 4.0 ± 0.5% in the presence of a solution of collagenase (40 units/ml) and the force induced on the tape was recorded as a function of time. As with the case of uncoated collagen tape, the force was found to be respresentable by a single negative exponential of the time (Equation 3.14 in Section 3.4.3) and hence a plot of the logarithmic force versus time yields a straight line. The slope of the straight line yields $1/\tau$ - a value which is taken as a measure of the rate of enzymatic degradation of the collagen by the collagenase.

Similarly, the collagen-mucopolysaccharide composite materials, prepared and characterized as described in Section 3.2.6 were tested for their susceptibility to collagenase degradation. However, the strain imposed on the composite materials was 20 ± 2% since this strain level probably corresponds to the point where uncrimping of the collagen fibers in the composite material ceases (see Fig. 4.8). The substantially higher strain level (20 ± 2%) required to uncrimp the fibers in the composite material, compared to that in the collagen tape (4.0 ± 0.5%), is probably due to the random distribution of the fibers in the composite materials compared to the oriented fibers present in the tape. As was discussed in Section 3.4.3, at this strain level, the error incurred by small variations in strain levels that invariably occurs from one experiment to another is minimal.

In the studies involving rat tail tendon (RTT) collagen, the following samples were prepared for the collagenase test:

(1) Untreated RTT. The tendons were teased out of tails of 3-month-old Sprague-Dawley rats by the method of Dumitru and Garrett (1947) and stored in a 0.05M Tris-HCl buffer solution, pH 7.4 at 4°C.

(2) RTT treated with 0.5M NaH_2PO_4. The tendons were teased out of rat tails as in (1) and placed in a 0.5M NaH_2PO_4 solution at 23°C for 3 hour. Such a treatment has been shown (see Section 3.1.3) to remove the reticular membrane which surrounds the tendon (Fig. 3.1).

The tendons were stored in a 0.05M Tris-HCl buffer solution, pH 7.4 at 4°C.

(3) RTT treated with hyaluronidase. The RTT was teased out of rat tails as in (1) and treated with 0.5M NaH_2PO_4 as in (2). The tendons were then placed at room temperature in a 0.2M phosphate buffer solution, pH 4.6, containing 1 mg/10 ml of bovine testicular hyaluronidase (Worthington Biochemical Corp., Freehold, N. J.) which had an activity of 14,000 units/mg. After treatment with the hyaluronidase for 24 hours, the tendons were removed, rinsed three times with distilled water and stored in a 0.5M Tris-HCl buffer solution, pH 7.4 at 4°C.

(4) RTT treated with hyaluronidase and α-amylase. The RTT was treated as in (3) and then placed in a 0.2M phosphate buffer solution pH 5.5 containing 1 mg/ml of a crude preparation of Bacillus subtilis α-amylase (Nutritional Biochemicals Corp., Cleveland, Ohio) for 24 hours at room temperature. The tendon was the rinsed three times with distilled water and stored in a 0.05M Tris-HCl buffer solution, pH 7.4 at 4°C.

All the RTT were subsequently crosslinked with a 0.025M solution of formaldehyde (citric acid-phosphate buffer pH 7.4) for 24 hours at room temperature. This was necessary because the uncrosslinked tendons were found to be too weak to be suitable for the collagenase test. A determination of the crosslink densities of the tendons using the method described in Section 3.2.3 showed that in all case, the tendons were crosslinked to about the same extent

(M_c = 14,500 ± 500). The tendons were then treated with a 0.2 wt-% dimedone solution to remove excess aldehydes (MacFayden, 1945), rinsed five times with distilled water and stored in a 0.05M Tris-HCl buffer solution, ph 7.4 at 4°C until required for the collagenase test. The latter was conducted on all of the above tendons using the method described in Section 3.4.3.

4.4.3. Results and Discussion. The rate of enzymatic degration of the MPS-coated collagen, compared to that of the uncoated collagen are tabulated in Table 4.2 and illustrated graphically in Fig. 4.16. It can be seen that the sulfated MPS (Ch4-S, Ch6-S, DS, H, HS) reduce the rate of degradation of the collagen to a much greater extent (ca. 85-90% reduction) than the unsulfated HA (ca. 35% reduction). Since the interaction of the MPS with collagen is known to involve the sulfate groups of the MPS (Podrazky et al., 1971), it appears that this interaction is the major source of inhibition of the degradation of collagen by collagenase. The volume exclusion effect which occurs in both the sulfated MPS and unsulfated HA (see Section 4.1) appears to contribute to a lesser extent to this inhibition.

The rate of enzymatic degradation of collagen-Ch6-S composite materials (containing varying amount of Ch6-S), compared to that of the pure collagen are tabulated in Table 4.3 and shown graphically in Fig. 4.17. The rate of degradation of the composite materials, plotted as a function of wt-% of Ch6-S in the materials is shown graphically in Fig. 4.18. For the concentration range of 0 to

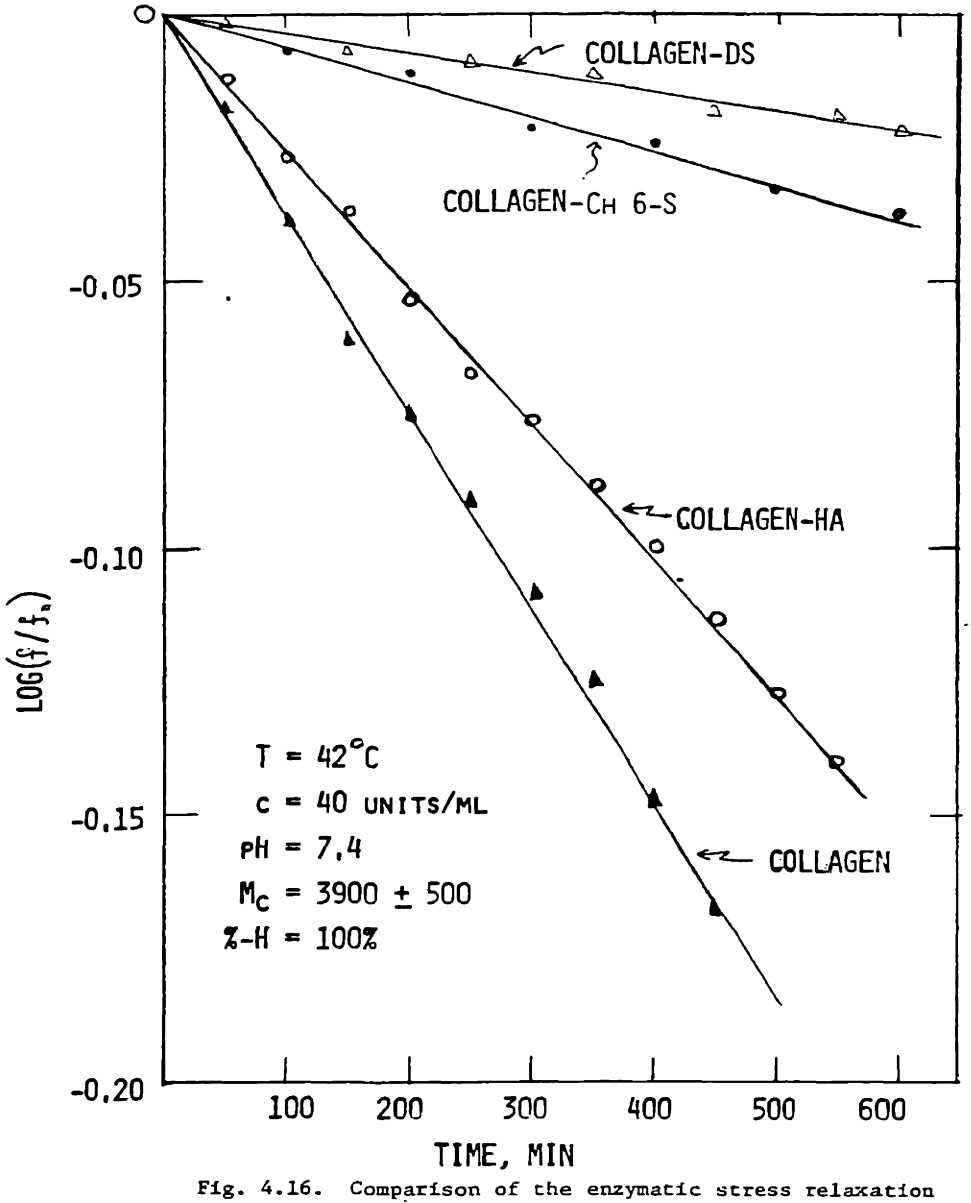


Fig. 4.16. Comparison of the enzymatic stress relaxation of collagen fibers coated with the indicated mucopolysaccharides and an uncoated collagen fiber. See legends to Fig. 3.30 for explanation of symbols.

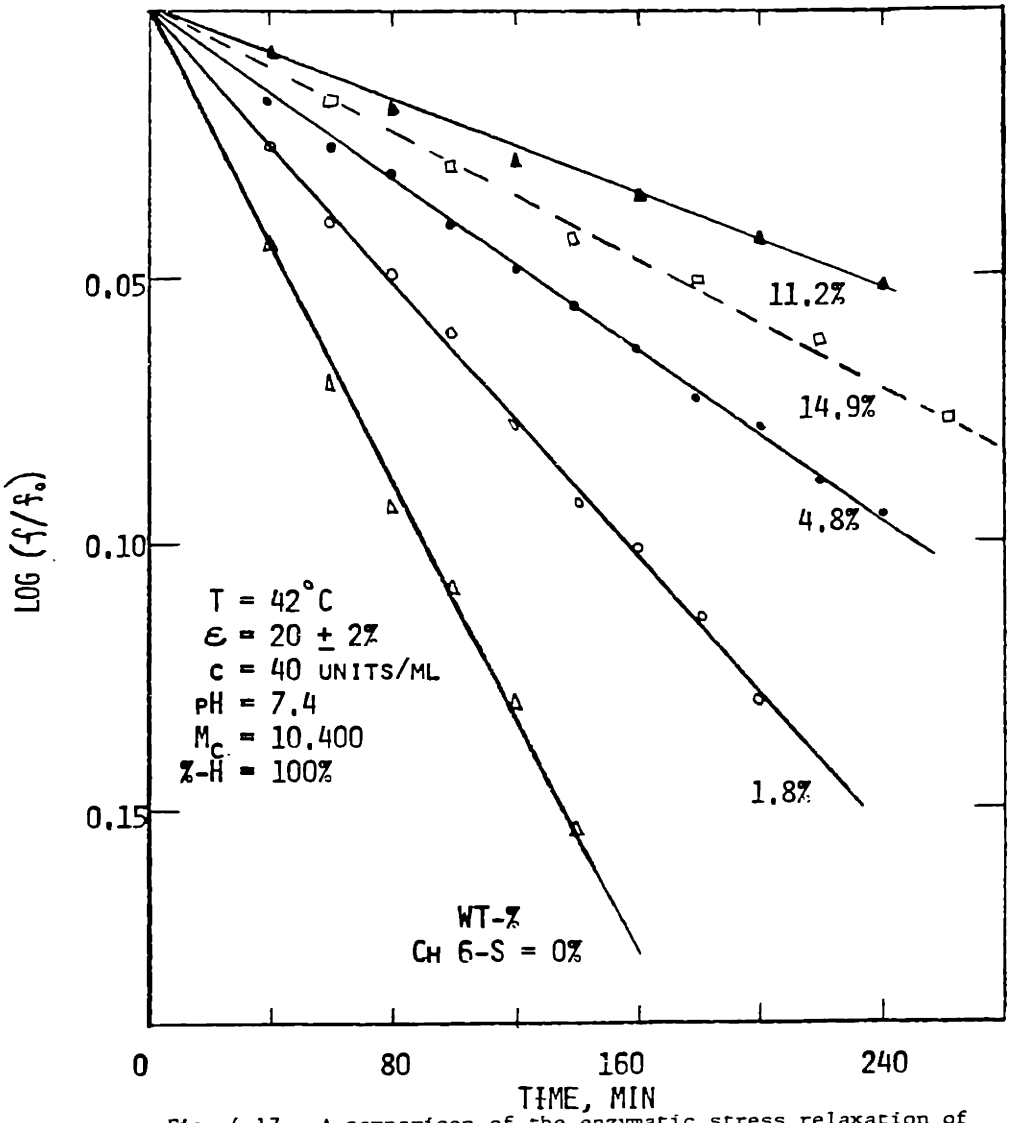


Fig. 4.17. A comparison of the enzymatic stress relaxation of collagen-Ch 6-S composite materials containing varying amount of Ch 6-S, as indicated. See legends to Fig. 3.30 for explanation of symbols.

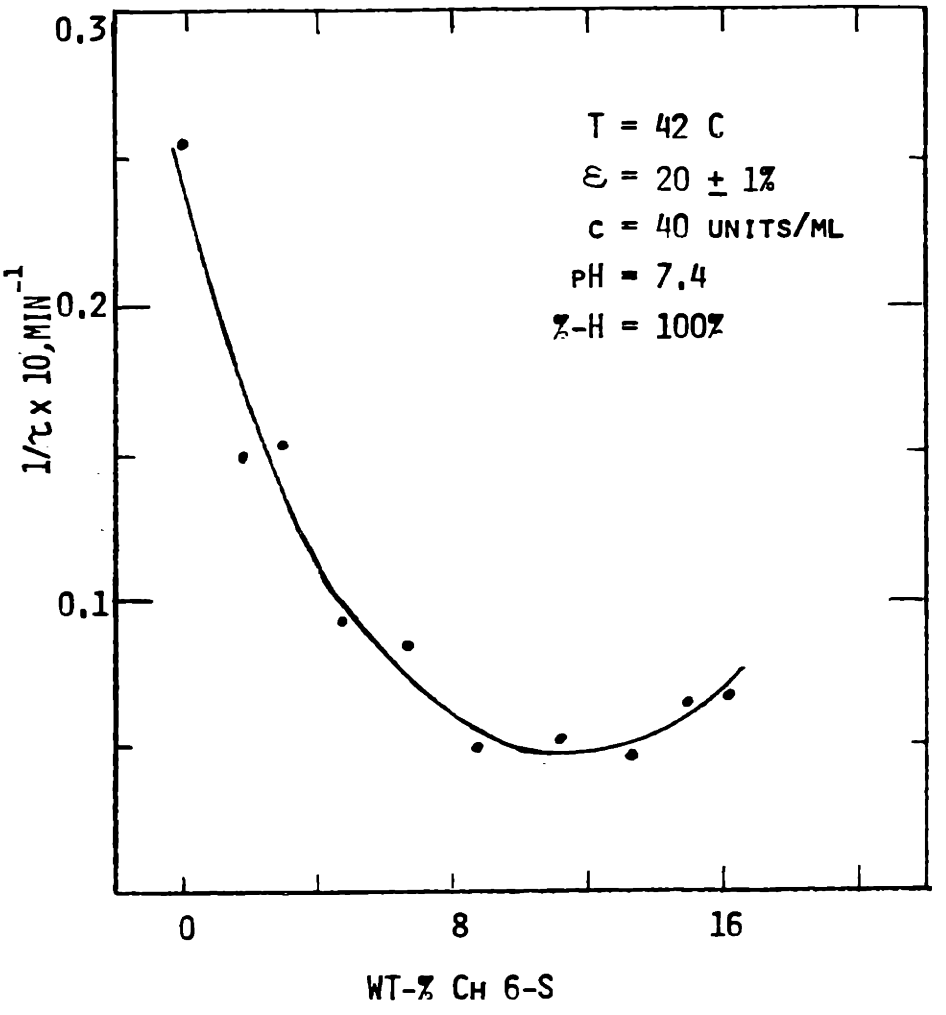


Fig. 4.18. The effect of varying Ch 6-S content on the rate of enzymatic degradation of collagen-Ch 6-S composite materials. See legends to Fig. 3.30 for explanation of symbols.

ca. 11 wt-% CH6-S, the effect of increasing CH6-S content in the composite material is to reduce the rate of enzymatic degradation of the collagen in the material. This is in agreement with the results obtained above for the MPS-coated collagen. However, above the concentration of 11 wt-% CH6-S, the effect of increasing the CH6-S content in the composite material appears to be a slight increase in the rate of degradation.

The concentration of Ch6-S at which the minimum enzymatic degradation occurs (ca. 11 wt-%) is close to that at which a minimum in the swelling of the composite materials occurs (see Fig. 4.9). The explanation given in Section 4.3.3 for the minimum in swelling of the composite materials can probably be used to explain the minimum in enzymatic degradation of the materials in the present study. For the lower concentration range (0 to 11 wt-% CH6-S), the effect of increasing CH6-S is to bind the collagen fibers closer together and thus reduce the rate of penetration of the collagenase molecule into the matrix. However, at the high concentration range (above 11 wt-% CH6-S), the effect of an excessive amount of Ch6-S is to cause the material to swell and hence facilitate the penetration of the collagenase molecule.

The results obtained in the present study may be used to explain a number of pathological conditions of connective tissues in which degradation of the collagen fibers followed by a loss in strength of the tissue occur. In osteoarthritis, the loss in strength of the cartilage has been associated with an attack of the collagen

fibers by collagenase present in the synovial fluid (Kempson et al., 1970). Since the total MPS content in the osteoarthritic cartilage has been found (Hjertquist and Lemperg, 1972) to be lower than that in normal cartilage, it can be speculated, in view of the present study, that the decrease in MPS content in the osteoarthritic cartilage render the collagen fibers in it more susceptible to attack by the collagenase. Similarly, in rheumatoid arthritis, a decrease in the MPS content of the cartilage (Schubert and Hamerman, 1965) may explain the observed (Harris et al., 1970) increase in enzymatic degradation of the cartilage. In the case of herniated intervertebral disks, which involves rupture of the annulus fibrosus, a dramatic loss in the MPS content has also been observed (Davidson and Woodhall, 1959). This loss in strength of the intervertebral disks may again be explained by the accelerated enzymatic degradation caused by the loss of MPS.

In arteriosclerosis, which results in deformation of the lumen and brittleness in the walls of arteries, a loss in MPS has also been observed (Balo, 1963). As the MPS has been demonstrated to inhibit both the degradation of elastin by elastase (Yu and Bluementhal, 1958) and the degradation of collagen by collagenase (in the present study), a loss of MPS would result in an accelerated enzymatic attack on both elastin (leading to a loss in elasticity of the artery) and collagen (leading to a loss in the strength of the artery).

In a study on immune injury of the cornea, Mohos and Wagner (1969) provided evidence to support the postulation that enzymatic degradation of the collagen fibers occur during the injury.

Their studies further suggested that the removal of MPS from the cornea was necessary for the degradation of collagen. In a later study, Wagner, (1972) provided additional evidence to support this hypothesis. The results of our present study is in agreement with this hypothesis.

Although the examples presented so far suggest that a loss of MPS can result in an increased enzymatic degradation, other studies suggest that an excessive amount of MPS can produce the same effect. Disaggregation of collagen fibers in the repair of split skin donor sites (Flint, 1971), in precancerous changes in dermis (Prodi and Maltoni, 1957; Dobson and Griffin, 1962) and in many hormone-dependent situations (Storey, 1957; Bryant et al., 1968) have been attributed to an increased enzymatic degradation of the collagen caused by the presence of an excessive amount of MPS. Robertson (1961) found an excessive accumulation of MPS in connective tissues inflicted with scurvy and suggested that the loss in strength of these tissues may be due to an accelerated enzymatic degradation of the collagen fibers. All these observations are in agreement with the results of the present study which indicate that an excessive amount of MPS (> 11 wt-%) can cause an increase in enzymatic degradation of the collagen (Fig. 4.18) and thus presumably result in disaggregation of the collagen fibers.

The arguments presented above are, by necessity, highly speculative since quantitative data on the amount of MPS present in each of the cases cited are not available. The wt-% of MPS at which a minimum enzymatic degradation can occur in a connective tissue must depend on a number of factors such as the nature of the MPS, the

presence of glycoproteins, the extent of crosslinking of the collagen, the size of collagen fibrils and the state of aggregation and orientation of these fibrils.

The rate of enzymatic degradation of RTT treated in various ways are compared with that of the untreated RTT in Fig. 4.19. The untreated RTT is very resistant to collagenase degradation ($1/\tau=0.25 \times 10^{-3} min^{-1}$). However, the removal of the reticular membrane which surrounds the RTT (Fig. 3.1) by immersion in 0.5M NaH_2PO_4 resulted in a substantial increase in the degradation ($1/\tau = 0.85 \times 10^{-3} min^{-1}$). Treatment of the denuded tendon with hyaluronidase further increased the enzymatic attack of the collagen by collagenase ($1/\tau=1.61 \times 10^{-3} min^{-1}$). Finally, treatment of the tendon with both hyaluronidase and α-amylase produced a material which was easily degraded by the collagenase ($1/\tau = 4.74 \times 10^{-3} min^{-1}$).

These studies, together with the ultrastructural studies made in Section 3.1 suggest the following model for the RTT: The tendon (ca. 200 in diameter) is covered by a non-collagenous reticular membrane which is resistant to collagenase degradation. Within the membrane, the tendon is made up of fibers (5-10μ in diameter) which are probably held together by protein-polysaccharides in a way similar that is shown in Fig. 2.6. The MPS of the PPS form a coating over the fibers, making them more resistant to collagenase attack. The fibers are in turn made up of fibrils (ca. 0.1μ in diameter) which are held together by glycoproteins. Removal of the glycoprotein (with CBA) render the fibrils easily susceptible to

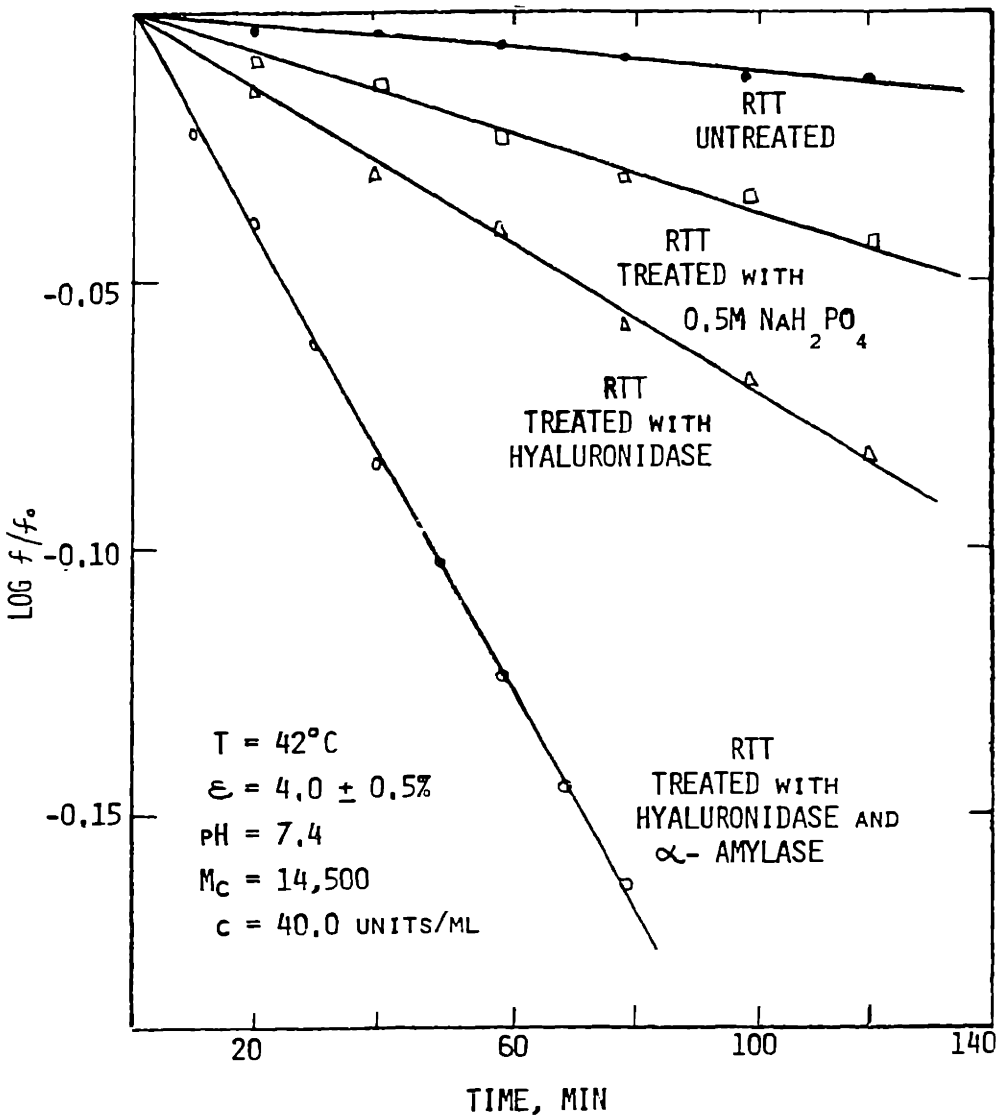


Fig. 4.19. The effect of various treatment of rat tail tendon collagen on its rate of enzymatic degradation by collagenase. See legends to Fig. 3.30 for explanation of symbols.

collagenase degradation. Finlay et al. (1971) have studied the treatment of human skin with CBA by means of a high resolution scanning electron microscope and their micrographs revealed distinctly the presence of collagen fibrils which were ca. 0.1μ in diameter - similar to those observed (Fig. 3.6) in RTT.

Chapter 5

IMPLANTATION STUDIES

In this Chapter, the results of a number of implantation studies, involving some of the materials that were prepared and characterized in Chapters 3 and 4 will be reported. Special emphasis will be given to the use of various characterization methods described in these Chapters to understand (and hopefully predict) the fate of materials that are implanted in the experimental animals. Specifically, a correlation of *in vitro* enzymatic degradation (determined by the method described in Section 3.4.3) with *in vivo* enzymatic degradation (determined by the fractional weight loss of implanted specimens) was sought for in order that a prediction of the *in vivo* degradation can be made based on the relatively simpler *in vitro* degradation studies. The results of such a correlation study will be reported in Section 5.2.

The studies on *in vitro* degradation of mucopolysaccharide-coated collagen (Section 4.3) and collagen-mucopolysaccharide composite materials (Section 4.4) showed that the presence of MPS can inhibit the degradation of the collagen by bacterial collagenase. In this Chapter, the *in vivo* degradation of these materials was studied by characterizing them prior to and after implantation in the experimental animals (Sections 5.3 and 5.4). The properties studied were the fractional weight change, swelling characteristics, modulus, extent of crosslinking, helical content and composition of the materials. From the change in

these properties of the explanted materials by the 4th, 10th and 20th post-implantation day, the extent of degradation of the materials was deduced and compared with the extent of *in vitro* degradation obtained with the same materials prior to their implantation. Attempts were also made to correlate the events that occurred in the tissue surrounding the implanted samples (deduced from histological studies) with the change in properties of the samples.

5.1. Experimental Methods

5.1.1. *Sterilization of samples.* A variety of agents have been previously used for sterilizing collagenous products made for biomedical purposes (see Chvapil, 1973 for a comprehensive review). The commercial processes currently used to sterilize collagen include: (1) application of moist heat; (2) sterilization with gaseous ethylene oxide; and (3) irradiation of the collagen product. Sterilization by heat or irradiation invariably results in partial denaturation of the collagen and a consequent accelerated resorption when the collagen is implanted in tissue (Chvapil, 1973). The use of ethylene oxide usually requires elevated temperatures and pressures to enhance the efficiency of the sterilizing process (George and Eberl, 1957); slight denaturation of the collagen is, therefore, possible in this process as well. Furthermore, there is evidence (Chvapil, 1973) that the ethylene oxide reacts chemically with the collagen and alters both its physical and biological properties.

In the present investigation, sterilization was achieved by immersion of the sample (1cm x 1cm) in 70/30 isopropanol/water for 24h at room temperature. This step was followed by rinsing the sample five times with sterile distilled water and immersion of the sample for 48 h in the sterile distilled water, with three changes of the water. Finally the sample was transferred to sterile Dulbecco solution (Grand Island Biological Co., Grand Island, N.Y.) and stored under refrigeration in a sterile cup with a tightly fitted lid (Lab-Tek Specimen containers; Miles Lab. Inc., Westmont, Illinois). Throughout the sterilization process, transferring of samples from one solution to another was done in a sterile atmosphere, supplied by a laminar flow bench (Relialab; Tenney Engineering Inc., Union, N.J.). All instruments used were sterilized in an autoclave (Steroclave No. 25-X; Wisconsin Aluminium Foundry Co., Inc., Manitowoc, Wisconsin) for 1h at a temperature of 259°F and a pressure of 20 psi.

At the end of such a sterilization treatment, the extent of crosslinking (determined by the method described in Section 3.2.3) and the helical content of the collagen (determined by the IR absorbance at $340cm^{-1}$; see Section 3.3.4) remained unchanged. Cultures of the sample for bacterial and fungal growth (performed at Shriners Burns Institute, Boston, Mass.) showed that the materials were aseptic. For collagen-MPS composite materials, the composition of the materials, as measured by hydroxyproline analysis (Section 4.3.3) and hexosamine analysis (Section 4.3.4) remained unaltered after the sterilization treatment.

5.1.2. Implantation procedure. The implantation of samples into experimental animals was performed in the laboratory of Dr. J. F. Burke at the Shriners Burns Institute, Boston, Mass. Although the long-term objectives of our study is to design a suitable skin replacement, initial stages of our experiments were performed with the materials implanted subcutaneously (i.e., between the dermis and panniculus carnosus). When finally developed, the skin replacement is envisaged as a membrane which (1) bonds intimately with tissues of the subcutaneous area on its one side, while (2) maintaining its physicochemical and biochemical properties during exposure of its opposite side to the atmosphere. The results obtained from the subcutaneous implantation would obviously assist in the design of a membrane which would meet the first specification. The results to be reported in this Chapter involve only the subcutaneous implantation.

The experimental animals used in our study were Hartley albino female guinea pigs, weighing ca. 500g (purchased from Camm Research Institute, Wayne, N.J.). The procedure for implantation is illustrated in Figs. 5.1 and 5.2. Prior to implantation, the back of each animal was shaved with electric clippers over an area of approximately 6" x 5" (Fig. 5.1A). Precautions were taken to remove and discard loose hair clippings (with vacuum suction) in order to eliminate the possibility of hair falling into the implant cavity.

The animal was then anesthezied (Fig. 5.1B) by exposure to a mixture of oxygen and Halothane (Ayerst Lab Inc., New York, New York). The experimental set-up for supplying the mixture is shown

in Fig. 5.2. The animal was initially anesthesized in an enclosed chamber filled with the mixture (Fig. 5.2). When the animal became apparently immobile, it was placed on a warm pad (kept at 37°C) and kept anesthesized through-out the implantation by means of a hood which was filled with a gentle flow of the mixture of oxygen and Halothane and which was fitted over the animal's head (Fig. 5.2).

The back of the guinea pig was then sterilized by wiping it with 70/30 propanol/water and a one-inch incision was made on one side of the back. The incision was made such that a pocket between the dermis and the paniculus carnosus was created (Fig. 5.1C). The sample was inserted into this pocket (Fig. 5.1D) such that the whole sample lay flat within the pocket. The incision was then sutured (Fig. 5.1E) with nylon sutures. A total of ca. 5 to 6 stitches were made to close the incision (Fig. 5.1F). The procedure was repeated with the other side of the guinea pig back, using an identical sample. The right side was subsequently used for histological studies (Section 5.1.5) while samples from the left side, after their explantation (see Section 5.1.3), were used for physicochemical characterization (Section 5.1.4). Three animals were used for each type of material in order to obtain samples implanted for 4, 10 and 20 days. Each animal was covered with a bandage around its body in such a way that slippage of the bandage was prevented. This was achieved by securing one side of the bandage around the neck of the guinea pig. The animals were kept in separate cages in order to prevent them from biting the bandage and the wounded sites of each other.

Fig. 5.1. Procedures involved in subcutaneous implantation of materials in the guinea pig:

(A) The animal was shaved with an electric clipper. Loose hair clippings were removed by vacuum suction.

(B) The animal was anesthesized by placing it in a enclosed chamber filled with a mixture of oxygen and Halothane. See Fig. 5.2 for the detailed set-up used in the application of the anesthesia.

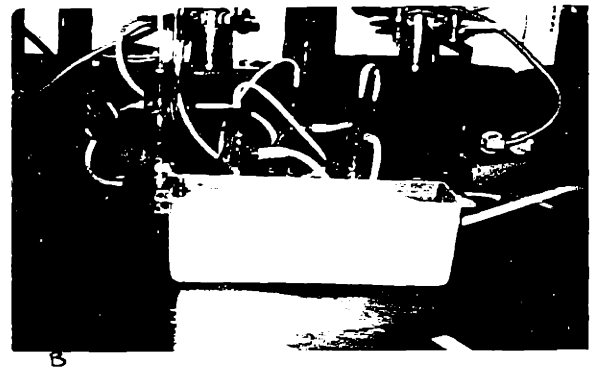
(C) An incision was made on the back of the animal such that a pocket was created between the dermis and paniculus carnosus.

(D) The sample was inserted into the pocket created in (C). Note that the animal was kept anesthesized throughout the implantation by a hood, filled with a gentle flow of a mixture of oxygen and Halothane, fitted over its head.

(E) The incision was then closed by suturing it with nylon sutures.

(F) A view of the closed incision is shown here. Note that a new incision had been made on the other side of the guinea pig back (covered by a swab). Samples from the left side were subsequently explanted and used for physicochemical characterization. Tissues on the right side were examined histologically.

A



B

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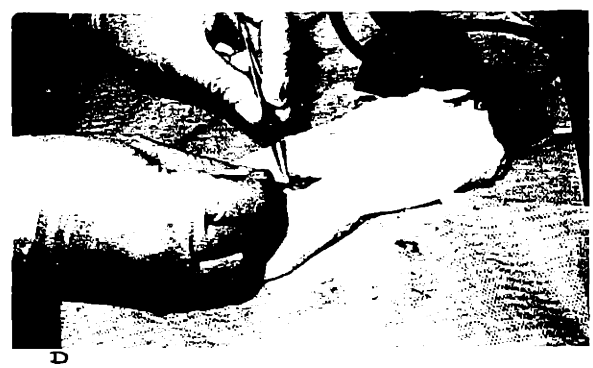

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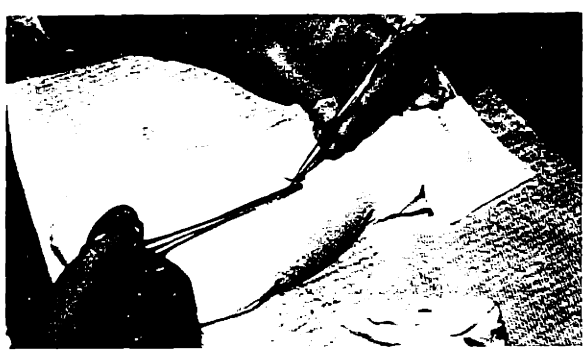


E

F

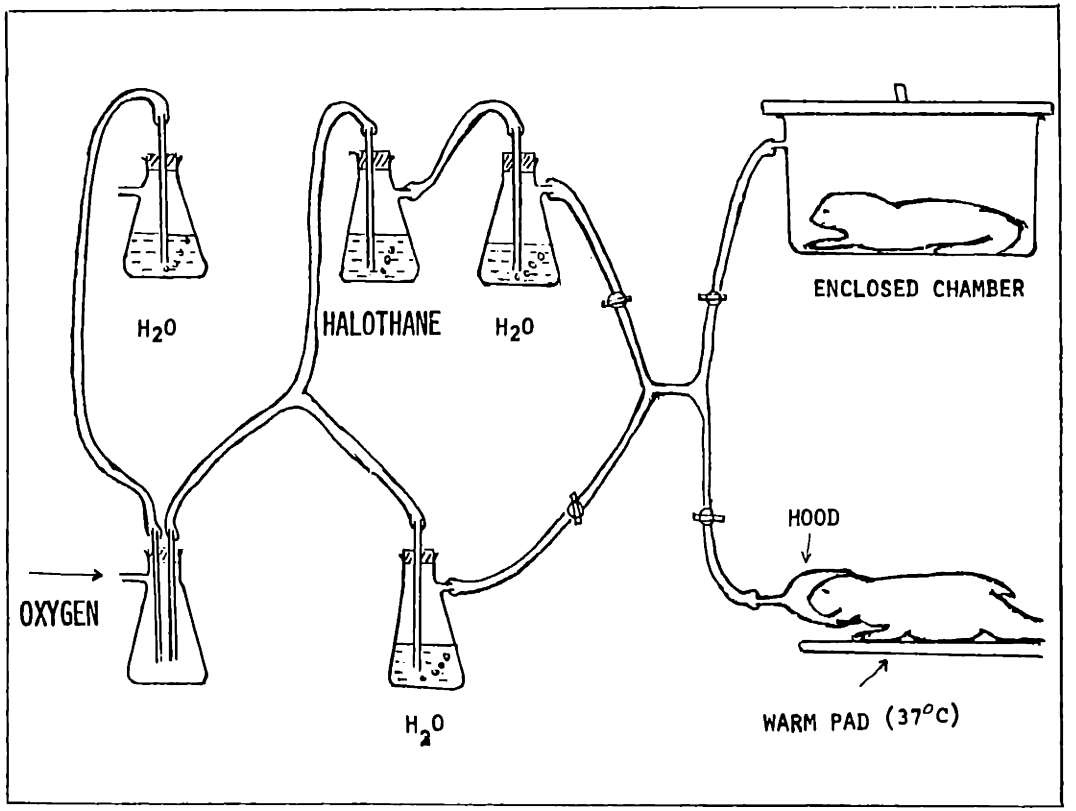


Fig. 5.2. Experimental set-up for the application of anethesia to the guinea pig during implantation.

5.1.3. Explantation Procedure. On the 4th, 10th, and 20th post-implantation day, the animals were sacrificed by placing them in a desiccator containing ether. From both the left and right implantation sites, 1 1/2" x 1 1/2" squares of the tissues were cut below the subcutaneous layer such that the implanted specimens remained in the tissue. The tissue from the right side was placed in a 10% formalin solution and was subsequently used for histological studies as described in Section 5.1.5. The tissue from the left side was immersed in a sterile Dulbecco solution (50 ml) containing a few drops of chloroform (which acts as a bactericide) and stored under refrigeration for not more than 24 hours before the sample within it was removed.

Removal of the sample within the tissue was done by placing the tissue on the stage of a low powered microscope (equipped with a camera) and stripping the subcutaneous tissue from the dermis in such a way that the state of the sample within the tissue could be examined clearly with the microscope. This could be achieved by first cutting between the dermis and the subcutaneous tissue and gently separating the two parts by means of forceps. When viewed on the microscope, the state of the tissue and the sample embedded within it can be examined to reveal such features as the presence of acute inflammation (Fig. 5.6C) and the attachment of tissues to the implanted material (Fig. 5.6E). Removal of the sample from the tissue was done by means of forceps and examined at the same time with the microscope. When attachment of the tissues to the implanted materials were apparent during the removal, photographs were taken to reveal such features (see

Fig. 5.6F). After the materials have been removed from the tissue they were stored in Dulbecco solution at 4°C until required for the various physicochemical characterizations to be described in the next Section.

5.1.4. Characterization of Samples. The samples characterized included the preimplantation specimens and the specimens explanted on the 4th, 10th and 20th post-implantation day. The properties characterized are as follows:

(1) The wt-% of collagen W_c in the vacuum dried sample. This was determined by the hydroxyproline analysis described in Section 4.2.3.

(2) The wt-% of mucopolysaccharide in the sample, W_m. This was determined by the hexosamine analysis described in Section 4.2.4.

(3) The fractional weight change of the sample, ΔW. This was obtained by determining the dry weight of the samples (after dehydration at 105°C at a pressure of 10^{-3}mm Hg for 48 hours). The fractional weight change is then given by:

$$\Delta W = \frac{w_e - w_i}{w_i} \qquad (5.1)$$

Where w_e = dry weight of the explanted sample,

and w_i = dry weight of the preimplantation sample.

(4) Swelling ratio in Dulbecco solution, R. This was found by determining the wet weight of the specimen (equilibrated in

Dulbecco solution at 23°C) and the dry weight. The wet weight was obtained by removing the specimen and blotting it lightly with a filter paper (ca. 2 secs) to remove excess solution before placing it immediately on the weighting balance. The dry weight of the specimen was obtained as in (3). The swelling ratio is then given by:

$$R = \frac{w_s}{w_d}$$

where w_s = weight of the swollen sample,

and w_d = dry weight of the sample.

(5) Tensile modulus, E, (in dynes/cm^2). This was obtained by the method described in Section 3.2.3 using the apparatus shown in Fig. 3.10. The modulus is determined by the slope of the straight portion of the stress-strain curve.

(6) Molecular weight between crosslinks, M_c (in daltons). This was determined by measuring the swelling ratio of the denatured collagen sample and using Equation 3.4 as described in Section 3.2.2. The χ values required for determining M_c through the swelling ratio measurements are tabulated in Tables 4.2 and 4.3.

(7) Helical content of collagen, %-H. This was determined by the IR absorbance at 340cm^{-1}, normalized for thickness and concentration of collagen, as described in Section 3.3.4.

5.1.5. Histological studies. The procedures for preparing and examining histological slides of the explanted sample and surrounding tissue are as follows:

(1) The tissue was fixed in 10% formalin (Fischer Scientific Co., N.J.) for at least 24h at room temperature.

(2) It was then dehydrated by sequential immersion in water-ethyl alcohol mixtures containing 50%, 70%, 85%, 95% and 100% alcohol, the time of immersion being 1h per mixture.

(3) The tissue was then immersed in dioxane for 2 hours before it was embedded in a tissue-embedding medium (Paraplast, Mpt. 56-57°C; Curtin Scientific Co., Houston, Texas). Embedding was achieved by first placing the tissue in the molten paraffin kept at 58°C for 4 hours, with hourly exchanges of the paraffin. Finally the tissue was placed in a mould and embedded with a fresh supply of paraffin.

(4) The paraffin block containing the tissue was then cooled to 0°C in a bath containing chipped ice for 20 min and was then mounted on a microtome (Minot Custom Microtome; International Equipment Co., Needham Heights, Mass.). Slices of the paraffin containing the tissue were microtomed to thicknesses of ca. 6 μ.

(5) The microtomed specimen was then mounted on a clear microscope slide and deparaffinization was achieved by immersing the mounted specimen in two exchanges of xylene for 3 min each.

(6) The specimen was then rehydrated by sequential immersion in water-ethyl alcohol mixtures containing 100%, 95%, 85%, 70%, 50% and 0% alcohol, the time of immersion being 1h per mixture. The specimen was finally rinsed thoroughly with distilled water.

(7) The specimen was then stained with hematoxylin for 5 min and rinsed briefly with distilled water. Excess stain was removed by rinsing the specimen with 0.5% acid alcohol

(70% ethyl alcohol in concentrated HCl). The acid alcohol was finally removed by rinsing the specimen and immersing it in water for 1/2h.

(8) The specimen was then stained with 0.5% aqueous eosin for 3 min and then rinsed with 5 exchanges of water.

(9) The specimen was dehydrated as in (2) above and then rinsed a few times with xylene.

(10) It was then mounted on a clean cover slip with a permanent mounting medium (Harleco Synthetic Resin; Hartman-Leddon Co., Philadelphia, Pa.).

(11) The cover slip containing the stained specimen was examined with a microscope by Dr. J. B. Caufield, Shriners Burns Hospital, Boston, Mass. and the results of such examinations were then reported to us.

5.2. Correlation of in vivo and in vitro Enzymatic Degradation.

5.2.1. Introduction. In Section 3.4, the *in vitro* degradation of collagen which had been crosslinked to varying extent was studied and it was shown (Fig. 3.38) that the degradation decreased considerably as the crosslinking density of the collagen was increased. In order to find a correlation between the *in vitro* degradation as studied in Section 3.4, and the *in vivo* degradation of collagen, a number of collagen samples of varying crosslink density was implanted in the experimental animals for a fixed period of time (10 days). The

extent of *in vivo* degradation was determined by the fractional weight changes of the explanted samples. Histological study of the implant sites was also done in order to compare the nature of tissue response induced by collagen samples which have been crosslinked to different extent.

5.2.2. *Experimental*. Collagen samples of varying crosslinking density were prepared and characterized as described in Section 3.2. The rate of *in vitro* degradation of the collagen by bacterial collagenase was determined as described in Section 3.4.3. The extent of crosslinking (as measured by M_c) and the rate of *in vitro* degradation of the collagen used in the present study are tabulated in Table 5.1. The collagen samples were then sterilized (as in Section 5.1.1) and implanted for 10 days in the guinea pigs, using the method described in Section 5.1.2. The samples were explanted as in Section 5.1.3 and the fractional weight changes were determined as in Section 5.1.4. Histological study of the implant sites was done by the pathology group at the Shriners Burns Institute and reported to us in the form shown in Table 5.1.

5.2.3. *Results and discussion*. A correlation of the *in vitro* degradation (as determined by $1/\tau$) and *in vivo* degradation (as determined by Δw) of the collagen samples are tabulated in Table 5.1 and shown graphically in Fig. 5.3. There is clearly a correlation between these two forms of degradation: the extent of degradation increases in both cases as the extent of crosslinking of the collagen is decreased.

TABLE 5-1

FRACTIONAL WEIGHT CHANGE (ΔW) AND REPORT FROM HISTOLOGICAL STUDIES ON THE IMPLANTATION OF COLLAGEN FIBERS CROSSLINKED TO VARYING EXTENT

M_c	$1/\tau$	ΔW on 10th post-implant day	Histological Studies
29,000	0.740	0.950	Severe acute inflammation; extensive fragmentation; marked dense granulation tissue response.
24,600	0.305	0.605	(Sample lost)
16,200	0.125	0.180	Moderate fragmentation of tape; slight acute inflammation; moderate chronic inflammation; moderate dense granulation tissue response.
4,500	0.020	0.155	Moderate fragmented tape; tape surrounded by thin layer of granulation tissue.

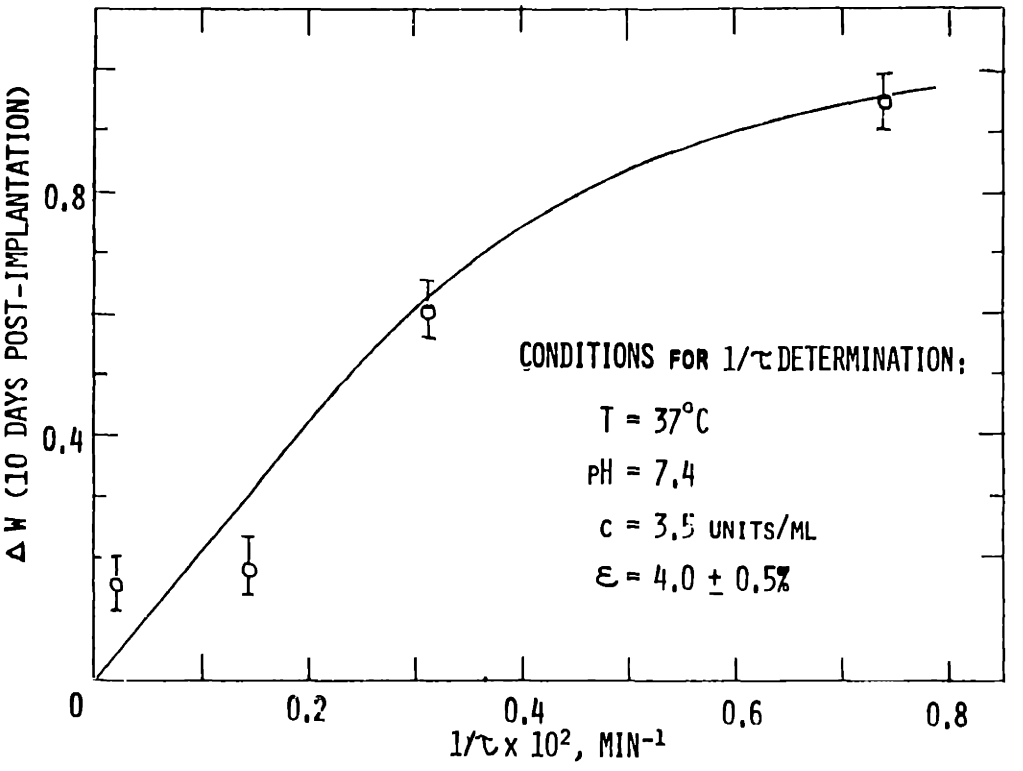


Fig.5.3. Comparison of the extent of in vivo enzymatic degradation (as deduced by the fractional weight loses, ΔW, of materials implanted in the guinea pig for 10 days) and in vitro enzymatic degradation (as deduced by $1/\tau$, measured as described in Section 3.4.3). See legends to Fig. 3.30 for explanation of symbols.

Further support for the increase in extent of degradation *in vivo* of the collagen with decreasing crosslink density is shown in report from the histological studies tabulated in Table 5.1. While the collagen with high crosslink density (M_c = 4,500) was moderately fragmented, the collagen with low crosslink density (M_c = 29,000) was extensively fragmented.

There is also a clear difference in the tissue response induced by samples which are crosslinked to different extent (see Table 5.1). The lightly crosslinked sample (M_c = 29,000) caused a severe acute inflammation with a dense granulation tissue response; the moderately crosslinked sample (M_c = 16,200) caused a slight acute inflammation with a moderately dense granulation tissue response; and the highly crosslinked sample (M_c = 4,500) apparently did not cause any acute inflammation and had only a thin layer of granulation tissue. It appears, therefore, that the severity of inflammation and the extent of granulation tissue response decreases with increasing crosslinking of the collagen.

The results obtained in the present study are in agreement with the conclusions reached by Chvapil (1973) and Woessner (1968) that the extent of resorption of the collagen is reduced by crosslinking. The mechanism for resorption of collagen has been studied previously by a number of researchers. From these studies, it became clear that the resorption occurs in two stages (Jenkins *et al.*, 1942, Lawrie *et al.*, 1959). First, polymorphonuclear (PMN) leucocytes (see Section 2.3.3)

invade the collagen fiber and break it into smaller bundles and fragments. The cytoplasm of the PMN leucocytes is studded with granules which contain a large variety of hydrolytic enzymes -- the so-called lysosomal enzymes (Hirsch, 1965; Woessner, 1965). The ability of these granules to fragment and hence secrete the enzymes extracellularly has been observed by Dingle (1969) and Ross (1970). A "collagenolytic cathepsin", capable of attacking collagen has been isolated by Bazin and Delaunay (1970) from the granular tissues produced in acute inflammation. The first stage of collagen resorption appears, therefore, to occur extracellularly (see Section 2.4.1 for further discussions on this aspect of degradation of collagen).

The second stage of the degradation involves the macrophages (see Section 2.3.4). These cells cause further fragmentation of the collagen fibers and finally digest them intracellularly by a mechanism known as the "lysosomal concept" (described in Section 2.3.3). Giant cells may also participate.

Additional features of the tissue response to the collagen implant was provided by Ungar and Feldman (1953) who showed that an early fibroblast response occurred which resulted in the collagen fiber being walled off by a dense granulation tissue. While this process was going on, capillaries, PMN neutrophils and macrophages (histiocytes) invade the collagen fiber. Not only was the collagen fiber resorbed, but so also was the new granulation tissue (Ungar and Neuman, 1953).

Based on these previous studies, the results of the present study can be interpreted as follows: The extent of fibroblast response

to the implanted collagen appears to be dependent on the extent of crosslinking of the collagen. The higher the crosslinking, the lower is the fibroblast response and, hence, the thinner is the granulation tissue formed around the sample. Furthermore, the extent of histiocyte invasion appears to be dependent on the extent of crosslinking of the collagen and/or the extent of fibroblast response. The higher the crosslinking, the more difficult it is for the histiocytes to invade and fragment the collagen fibers. Since the invasion of histiocytes is believed to be caused by the possible secretion of collagenolytic enzymes by the cells (Salthouse et al., 1969), the lower extent of degradation by these enzymes arising from an increased crosslinking of the collagen correlates very well with similar results obtained in the in vitro degradation (Fig. 3.38).

The correlation curve obtained in the present study relating the in vitro degradation to the in vivo degradation has significant practical uses. Since the in vivo studies are generally more time consuming and involve a much more complicated procedure, the correlation curve enables one to predict the extent of in vivo degradation by the relatively simpler determination of in vitro degradation. Materials which are likely candidates for skin replacements can, therefore, be screened by the in vitro studies before they are implanted into experimental animals.

5.3. Implantation of Mucopolysaccharide-coated Collagen.

5.3.1. Introduction. In Section 4.4, it was demonstrated that a coat

of sulfated-MPS on collagen fibers can retard substantially the *in vitro* enzymatic degradation of the collagen (see Fig. 4.15). In view of the correlation between *in vivo* and *in vitro* enzymatic degradation, obtained in the previous Section, it can be postulated that MPS-coated collagen must also be resistant to *in vivo* degradation. In this Section, the validity of such a hypothesis was tested. At the same time, the usefulness of the MPS-coated collagen as materials for skin replacement was assessed.

5.3.2 Experimental. MPS-coated collagen were prepared as described in Section 4.2.5. Characterization of these materials prior to implantation are tabulated in Table 4.2. The samples were implanted in guinea pigs for 4, 10 and 20 days as described in Section 5.1. Characterization of the explanted samples for collagen and MPS content, fractional weight change, swelling ratio, tensile modulus, crosslinking density and helical content were made as described in 5.1.4. Histological studies of the implant sites were made by the pathology group at Shriners Burns Institute, Boston, as described in Section 5.1.5.

5.3.3 Results and discussion. A tabulation of the results on characterization of the explanted samples, compared to that of the pre-implantation samples is shown in Table 5.2. A comparison of the fate, following implantation, of a collagen control with that of collagen coated with a sulfated-MPS is shown in Fig. 5.4. A similar comparison between collagen coated with a non-sulfated MPS (HA) and collagen coated with a sulfated MPS is shown in Fig. 5.5.

TABLE 5.2

CHARACTERIZATION OF MUCOPOLYSACCHARIDE-COATED COLLAGEN BEFORE AND AFTER IMPLANTATION

Properties	Preimplantation	In vivo residence time, days: 4 days	10 days	20 days
(1) Collagen Control ($1/\tau = 8.48 \times 10^{-4}$ min^{-1})				
ΔW	0.00±0.04	-0.16±0.04	-0.15±0.04	-0.31±0.04
R	3.5±0.5	4.7±0.5	6.2±0.5	6.2±0.5
Ex 10^{-9} (dynes cm^{-2})	3.3±0.3	2.5±0.3	1.4±0.3	1.6±0.3
$M_c \times 10^{-3}$	3.8±0.5	6.6±0.5	6.1±0.5	8.6±0.5
%-H	100 ± 7	93 ± 7	103 ± 7	87 ± 7
wt-% Collagen	100 ± 0.5	98.4±0.5	97.3±0.5	99.5±0.5
(2) Collagen-Hyaluronic Acid ($1/\tau = 5.38 \times 10^{-4}$ min^{-1})				
ΔW	0.00±0.04	-0.12±0.04	-0.30±0.04	-0.28±0.04
R	3.6±0.5	5.3±0.5	4.6±0.5	6.7±0.5
Ex 10^{-9} (dynes cm^{-2})	3.5±0.3	2.3±0.3	1.8±0.3	-.9±0.3
$M_c \times 10^{-3}$	4.2±0.5	7.2±0.5	6.7±0.5	8.5±0.5
%-H	100 ± 7	88 ± 7	95 ± 7	83 ± 7
wt-% Collagen	91.8±0.5	91.5±0.5	91.7±0.5	93.1±0.5

TABLE 5.2 (CONTINUED)

Properties	Preimplantation	In vivo residence time, days 4 days	10 days	20 days
(3) Collagen - Heparan Sulfate ($1/\tau = 1.10 \times 10^{-4}$ min^{-1})				
ΔW	0.00±0.04	-0.06±0.04	+0.04±0.04	+0.38±0.04
R	3.2±0.5	4.9±0.5	3.8±0.5	3.1±0.5
E x 10^{-9} (dynes cm^{-2})	4.0±0.3	3.5±0.3	3.8±0.5	3.6±0.5
M_c x 10^{-3}	3.8±0.5	4.6±0.5	4.7±0.5	4.5±0.5
%-H	100 ± 7	93 ± 7	103 ± 7	98 ± 7
wt-% Collagen	89.5±0.5	90.5±0.5	90.7±0.5	96.2±0.5
(4) Collagen - Heparin ($1/\tau = 0.98 \times 10^{-4}$ min^{-1})				
ΔW	0.00±0.04	-0.02±0.04	+0.08±0.04	+0.32±0.04
R	2.5±0.5	3.6±0.5	3.4±0.5	2.2±0.5
E x 10^{-9} (dynes cm^{-2})	4.2±0.3	3.9±0.3	4.2±0.3	4.3±0.3
M_c x 10^{-3}	3.8±0.5	4.3±0.5	4.3±0.5	3.6±0.5
%-H	100 ± 7	95 ± 7	99 ± 7	105 ± 7
wt-% Collagen	91.3±0.5	90.8±0.5	90.7±0.5	97.1±0.5

TABLE 5.2 (CONTINUED)

Properites	Preimplantation	In vivo residence time, days 4 days	10 days	20 days
(5) Collagen - Dermatan Sulfate ($1/\tau = 0.90 \times 10^{-4}$ min^{-1})				
ΔW	0.00±0.04	-0.09±0.04	-0.05±0.04	+0.31±0.04
R	2.7±0.5	3.6±0.5	4.8±0.5	4.1±0.5
$E \times 10^{-9}$ (dynes cm^{-2})	3.9±0.3	4.0±0.3	3.0±0.3	3.1±0.3
$M_c \times 10^{-3}$	4.0±0.5	4.9±0.5	5.3±0.5	5.2±0.5
%-H	100 ± 7	93 ±7	100 ± 7	102 ± 7
wt-% Collagen	91.8±0.5	92.5±0.5	92.7±0.5	96.8±0.5
(6) Collagen-Chondroitin 6-sulfate ($1/\tau = 1.46 \times 10^{-4}$ min^{-1})				
ΔW	0.00±0.04	-0.02±0.04	-0.07±0.04	+0.40±0.04
R	2.7±0.5	3.4±0.5	4.4±0.5	3.2±0.5
$E \times 10^{-9}$ (dynes cm^{-2})	4.0±0.3	3.6±0.3	3.2±0.3	3.3±0.3
$M_c \times 10^{-3}$	4.1±0.5	4.3±0.5	5.7±0.5	5.4±0.5
%-H	100 ± 7	97 ± 7	102 ± 7	95 ± 7
wt-% Collagen	88.1±0.5	89.0±0.5	92.1±0.5	97.2±0.5

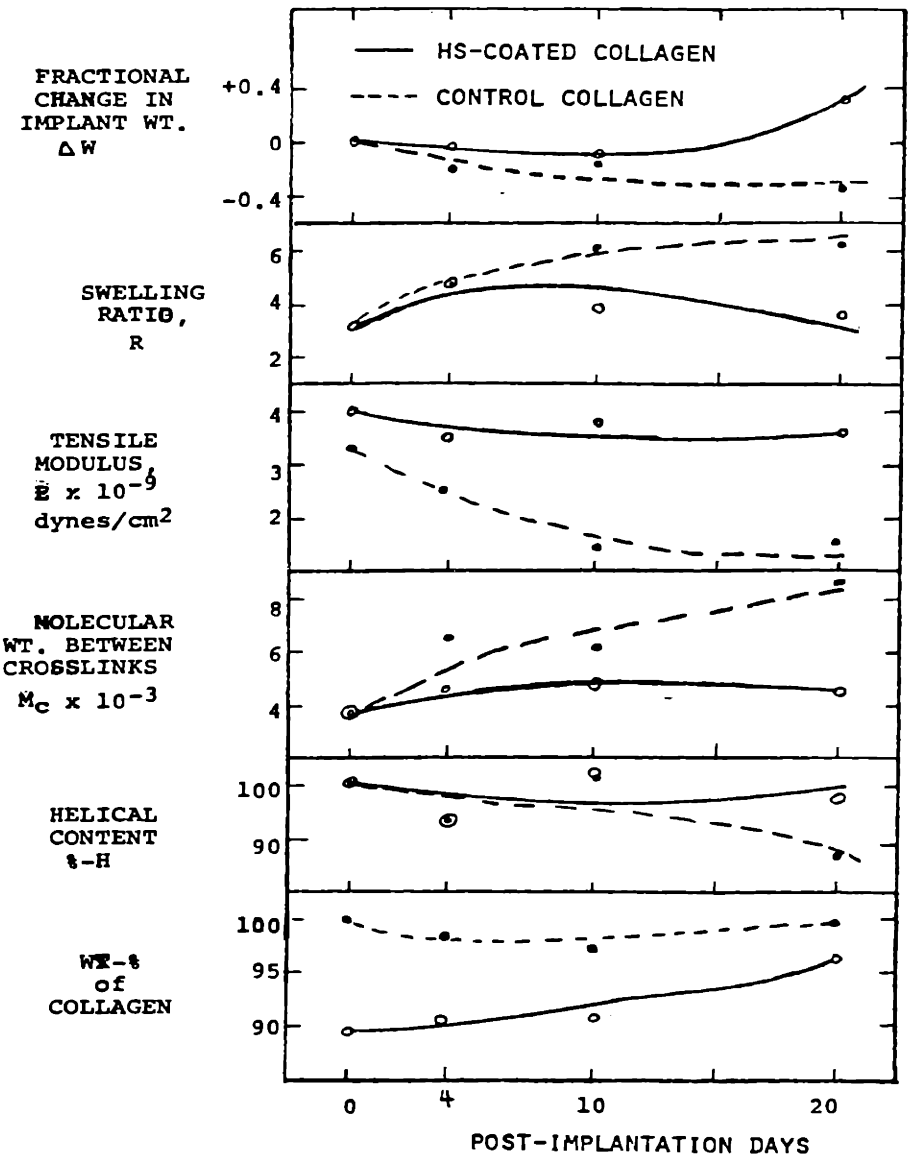


Fig. 5.4. The variation in physicochemical properties with post-implantation time of a collagen coated with heparan sulfate (HS) compared to that of an uncoated collagen fiber.

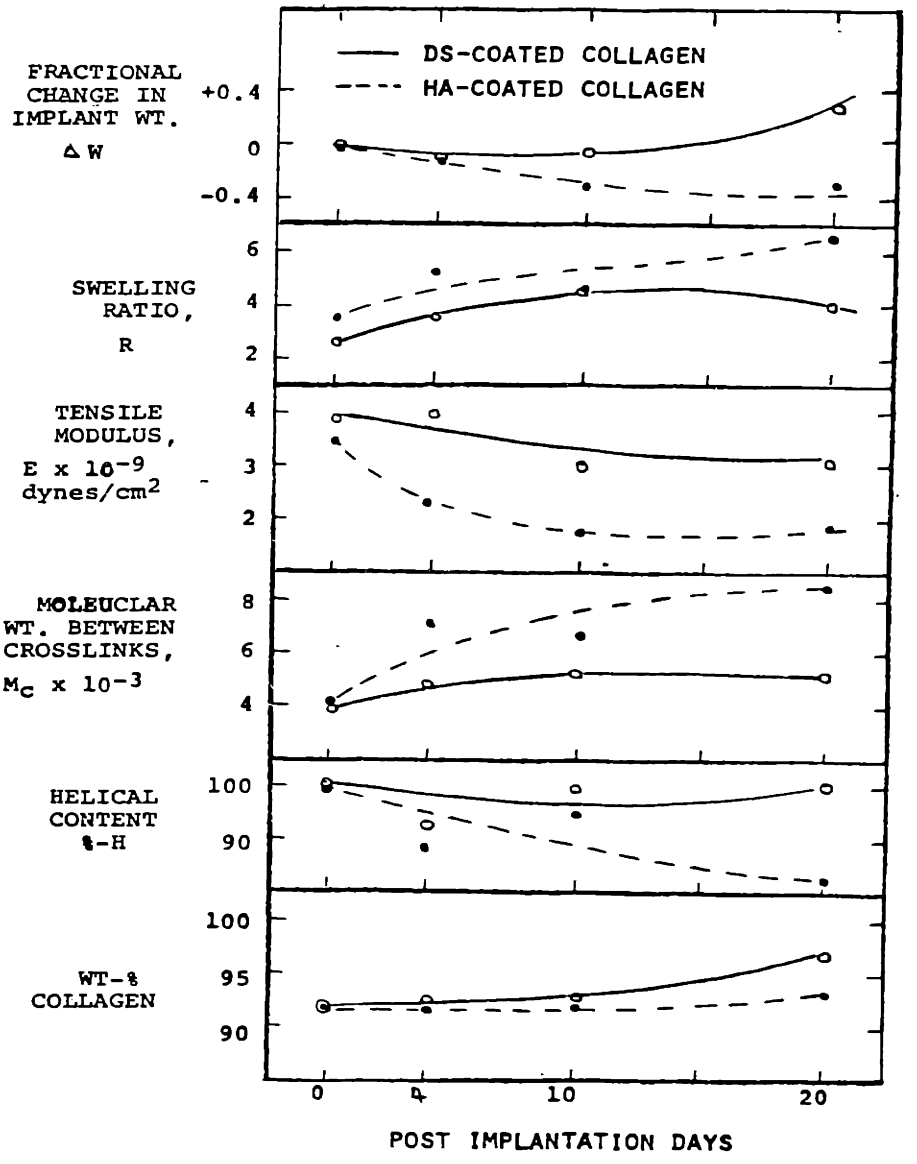


Fig. 5.5. The variation in physicochemical properties with post-implantation time of a collagen fiber coated with a sulfated mucopolysaccharide (DS) compared to that of a fiber coated with a non-sulfated mucopolysaccharide (HA).

The most characteristic comparative results which are illustrated in Fig. 5.4 are (a) the negative fractional change in implant weight, Δw, with uncoated collagen contrasting with the long-term positive change with MPS-coated specimens; (b) the monotonous increase in the swelling ratio, R, with uncoated collagen and the eventual reversal of such increase with coated specimens; (c) the somewhat greater drop in tensile modulus, E, of uncoated specimens; (d) the sharper increase in molecular weight between crosslinks, M_c, observed with uncoated specimens; (e) the slightly greater loss in helical content, %-H, observed with the uncoated specimens; and (f) the monotonous increase in the weight percentage of collagen, W_c, with the coated specimen contrasting with the relative invariance in collagen content of the uncoated specimen.

These results strongly suggest that the uncoated collagen is degraded <u>in vivo</u> to a greater extent than the coated specimens. This is further confirmed by direct observation of the two specimens with a low-powered microscope as shown in Fig. 5.6A, where the left specimen is the uncoated collagen and the right specimen is collagen coated with HS, both specimens having been implanted in the animal for 10 days. As can be observed in Fig. 5.6A, the uncoated collagen is more fragmented and highly swollen than the coated specimen. The report from histological studies of the implanted site (Table 5.3) also support this conclusion. The uncoated collagen became progressively fragmented. By contrast, the HS-coated collagen was only slightly fragmented on the 20th post-implantation day (Table 5.3). These results are con-

sistent with our previous studies which showed that MPS-coated collagen is more resistant to *in vitro* enzymatic degradation than the uncoated collagen (Section 4.4) and that there is a direct correlation between the *in vitro* and *in vivo* enzymatic degradation (Section 5.2).

The increase in weight of the HS-coated collagen at the 20th post-implantation day, shown in Fig. 5.4, is probably due to the adherence of some of the surrounding tissue on to the implant as it was removed from the tissue. A magnified view of the implant, shown in Fig. 5.6B, reveals the presence of some extraneous materials attached to the collagen fibers. These materials appear to be mainly collagen since the wt-% of collagen in the implant increased significantly on 20th post-implantation day (Fig. 5.4). The absence of a similar increase in weight of uncoated collagen (Fig. 5.4) suggests that the coat of HS on the collagen fibers probably served as a bond between the implanted collagen and the collagen synthesized by the cells of the granulation tissue. Such a bonding can be seen clearly with implanted collagen-MPS composite materials (Fig. 5.6F) which will be described in the next Section.

The reversal in the increase in swelling ratio of the coated specimen on the 20th post-implantation day (Fig. 5.4) is probably caused by the interaction of the specimen with freshly synthesized collagen. The decrease in swelling ratio caused by such an interaction has been discussed in Section 4.3.3 (see Fig. 4.9).

The results illustrated in Fig. 5.5 suggest that the sulfated MPS can retard the *in vivo* degradation of the collagen to a

Fig. 5.6. Examination of explanted materials and the tissue surrounding them with a low powered microscope.

(A) Comparison of the fate, following implantation for 10 days, of a collagen fiber coated with heparan sulfate (right hand side specimen) and an uncoated collagen fiber (left hand side specimen). Note the substantially larger fragmentation of the uncoated collagen fiber. Magnification, 10X.

(B) A magnified view of the collagen fiber coated with heparan sulfate following implantation for 10 days (from A). Note the presence of extraneous materials attached to the collagen fibers. Magnification, 70X.

(C) A view of the tissue surrounding a pure (control) collagen after implantation for 10 days. Note the presence of pus surrounding the sample and the inflammed nature of the surrounding tissue. Magnification, 15X.

(D) A view of the tissue surrounding a collagen-Ch 6-S composite material (ca. 4.8 wt-% Ch 6-S) after implantation for 10 days. The tissue surrounding the sample appeared to be healthy. Magnification, 15X.

(E) Attempts to remove the sample described in (D) from the surrounding tissue revealed signs of attachment of the tissue to the sample. Magnification 15X.

(F) After partial detachment of the sample in (E) from the surrounding tissue, the underside of the sample revealed strong attachment of fibers from the surrounding tissue to the sample. Chemical analyses (Fig. 5.7) suggests that the fibers are collagenous in nature. Magnification, 20X.

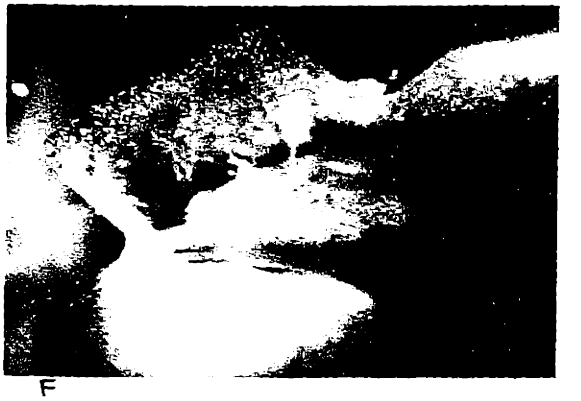
A

B

C

D

E



F

TABLE 5.3

REPORT FROM HISTOLOGICAL STUDIES ON THE IMPLANTATION

OF MUCOPOLYSACCHARIDE-COATED COLLAGEN

Samples	In vivo residence time, days		
	4 days	10 days	20 days
(1) Collagen Control	Slight framentation; chronic and acute inflammation.	Moderate fragmentation; sample surrounded by thin granulation tissue.	Total fragmentation; slight granulation tissue.
(2) HA-coated Collagen	Slight fragmentation; severe acute inflammation.	Slight fragmentation; slight granulation tissue.	Moderate fragmentation; moderate chronic and acute inflammation; moderate granulation tissue.
(3) HS-coated Collagen	Slight fragmentation; slight acute and chronic inflammation.	Marked fragmentation; slight chronic inflammation. Moderate granulation tissue.	Slight fragmentation; slight acute and chronic inflammation. Loose granulation tissue.

TABLE 5.3 (CONTINUED)

REPORT FROM HISTOLOGICAL STUDIES ON THE IMPLANTATION

OF MUCOPOLYSACCHARIDE-COATED COLLAGEN

Samples	*In vivo* residence time, days		
	4 days	10 days	20 days
(4) H-coated Collagen	Moderate fragmentation; slight acute inflammation.	Moderate fragmentation; slight granulation tissue.	Moderate fragmentation; slight acute and chronic inflammation; loose granulation tissue.
(5) DS-coated Collagen	Slight fragmentation; acute and chronic inflammation.	Moderate fragmentation; moderate granulation tissue.	Moderate fragmentation; moderate chronic and slight acute inflammation; Moderate granulation tissue.
(6) Ch 6-Scoated Collagen	Some fragmentation; acute and chronic inflammation.	Marked fragmentation; mild inflammation; slight granulation tissue.	Severe fragmentation; moderate granulation tissue.

greater extent than the non-sulfated HA. This is consistent with the results obtained in Section 4.4 where a similar contrast in behavior was demonstrated for *in vitro* degradation of the MPS-coated collagen. Since the interaction of MPS with collagen is known to involve the sulfate groups of the MPS (Podrazky *et al.*, 1971), it appears that this interaction is the major source of inhibition of the *in vivo* degradation of collagen by MPS. The monotonous decrease in weight and the relatively invariant weight percentage of collagen in the HA-coated collagen further suggest that the sulfate group is necessary for bonding to occur between the MPS and collagen synthesized by the cells in the tissue.

The difference in ability of the sulfated and non-sulfated MPS to interact with collagen and retard enzymatic degradation of the collagen may have some biological significance. In wound healing, it was observed (Dunphy and Udupa, 1955; Dunphy *et al.*, 1956; White *et al.*, 1961) that the MPS produced at the early stages was predominantly hyaluronic acid. This coincided with the phase when the collagen fibers in the tissue were disaggregated and apparently degraded. At the later stages of the wound healing when the collagen fibers were reorganized and degradation had apparently subsided, the hyaluronic acid was found to be mostly replaced by chondroitin sulfate. Tissue culture studies also indicate that young connective tissue cells from many sources growing in free conditions during their exponential growth phase produce hyaluronic acid (Grossfeld *et al.*, 1955; Morris and Godman, 1960), but later revert to chondroitin sulfate and collagen

production when their growth becomes confluent (Green and Goldberg, 1963, 1964), if confined in a diffusion chamber (Priest and Priest, 1964), or external stresses are applied (Prockop et al., 1964). These observations, coupled with the results of our present study suggest that variations in the type and quantity of MPS production by connective tissue cells may be implicated in the regulation of the state of their collagenous environment.

5.4. Implantation of Collagen-mucopolysaccharide Composite Materials.

5.4.1. Introduction. In Section 4.2.6, composite materials, containing collagen fibers dispersed within a muocopolysaccharide (MPS) matrix were prepared and characterized. It was demonstrated that the presence of a limited amount of MPS (< 11%) both strengthens the collagen (Section 4.3) and renders it resistant to in vitro degradation by bacterial collagenase (Section 4.4). In view of the correlation between in vitro and in vivo degradation (Section 5.2), it would appear that the collagen-MPS composite materials may be suitable as materials for skin replacement since two of the important requirements for the latter are good mechanical strength and resistance to in vivo enzymatic degradation (Section 2.6.2). In this Section, implantation of these materials and their subsequent characterization on explanation were used to assess their potential use as materials for skin replacement.

5.4.2. Experimental. Collagen-Ch6-S composite materials containing 0, 1.8, 4.8, and 11.2 wt-% Ch6-S were prepared and characterized as

TABLE 5.4

CHARACTERIZATION OF COLLAGEN/CHONDROITIN 6-SULFATE COMPOSITE MATERIALS

BEFORE AND AFTER IMPLANTATION

Properties	Preimplantation	In vivo, residence time, days 4 days	10 days	20 days
(1) Collagen Control ($1/\tau = 0.255 \times 10^{-2}$ min^{-1})				
ΔW	0.00±0.02	-0.16±0.02	-0.52±0.02	-0.60±0.02
R	9.3±0.8	10.6±0.8	12.4±0.8	13.4±0.8
E x 10^{-8} (dynes cm^{-2})	1.8±0.2	1.3±0.2	1.4±0.2	0.7±0.2
M_c x 10^{-4}	1,5±0,1	2.4±0.1	3.5±0.1	3.8±0.1
%-H	100 ± 7	87 ± 1	82 ± 7	78 ± 7
wt-% Collagen	100 ± 0.2	97.7±0.2	99.3±0.2	99.8±0.2
(2) 1.8 wt-% Ch6-S ($1/\tau = 0.149 \times 10^{-2}$ min^{-1})				
ΔW	0.00±0.02	-0.20±0.02	-0.28±0.02	-0.39±0.2
R	8.9±0.8	9.5±0.8	10.2±0.8	7.4±0.8
E x 10^{-8} (dynes cm^{-2})	1.9±0.2	1.5±0.2	1.0±0.2	1.0±0.2
M_c x 10^{-4}	1.4±0.1	1.8±0.2	2.9±0.2	3.0±0.2
%-H	98 ± 7	99 ± 7	89 ± 7	92 ± 7
wt-% Collagen	98.2±0.2	98.8±0.2	99.4±0.2	99.4±0.2

TABLE 5.4 (CONTINUED)

CHARACTERIZATION OF COLLAGEN/CHONDROITIN 6-SULFATE COMPOSITE MATERIALS BEFORE AND AFTER IMPLANTATION

Properties	Preimplantation	In vivo, residence time, days 4 days	10 days	20 days
(3) 4.8 wt-% Ch6-S ($1/\tau = 0.093 \times 10^{-2} min^{-1}$)				
ΔW	0.00±0.02	-0.04±0.02	-0.08±0.02	+0.33±0.2
R	5.4±0.8	6.2±0.8	7.4±0.8	5.7±0.8
$E \times 10^{-8}$ (dynes cm^{-2})	1.8±0.2	1.5±0.2	1.2±0.2	1.3±0.2
$M_c \times 10^{-4}$	1.3±0.1	1.5±0.2	2.0±0.2	2.4±0.2
%-H	100 ± 7	98 ± 7	85 ± 7	85 ± 7
wt-% Collagen	95.2±0.2	95.8±0.2	97.7±0.2	98.4±0.2
(4) 11.2 wt-% Ch6-S ($1/\tau = 0.052 \times 10^{-2} min^{-1}$)				
ΔW	0.00±0.02	-0.04±0.02	+0.18±0.2	+0.65±0.2
R	4.1±0.8	4.5±0.8	4.8±0.8	4.3±0.8
$E \times 10^{-8}$ (dynes cm^{-2})	1.9±0.2	1.6±0.2	1.6±0.2	1.7±0.2
$M_c \times 10^{-4}$	1.0±0.1	1.4±0.1	1.3±0.1	1.6±0.1
%-H	97 ± 7	94 ± 7	101 ± 7	98 ± 7
wt-% Collagen	88.8±0.2	90.5±0.2	90.2±0.2	97.2±0.2

described in Section 4.2.6. The materials were sterilized (as in Section 5.1.1) and implanted for 4, 10, and 20 days in the guinea pig, as described in Section 5.1.2. On explanation, the materials were characterized as in Section 5.1.4 and the implantation sites were subjected to histological studies, as in Section 5.1.5.

5.4.3. Results and discussion. The result of characterization of the collagen-Ch6-S composite materials prior to and after implantation in the experimental animals are tabulated in Table 5.4. The fate of the materials, following implantation, are further illustrated in Fig. 5.7 which compares the collagen control with the collagen-Ch6-S composite material containing 11.2% Ch6-S. Microscopic descriptions of the implantation sites are tabulated in Table 5.5

The most characteristic comparative results illustrated in Fig. 5.7 are (a) the negative fractional change in implant weight, ΔW with the control collagen contrasting with the positive change with the collagen-Ch6-S composite material; (b) the monotonous increase in the swelling ratio, R with the control collagen compared to the invariance in R of the composite material; (c) the somewhat greater decrease in tensile modulus, E of the control collagen; (d) the sharper increase in molecular weight between crosslinks, M_c, observed with the control collagen; (e) the drop in helical content, %-H of the control collagen, contrasting with the relatively invariant %-H of the composite material; and (f) the increase in collagen content observed with the composite material, contrasting with the invariant composition of the control collagen.

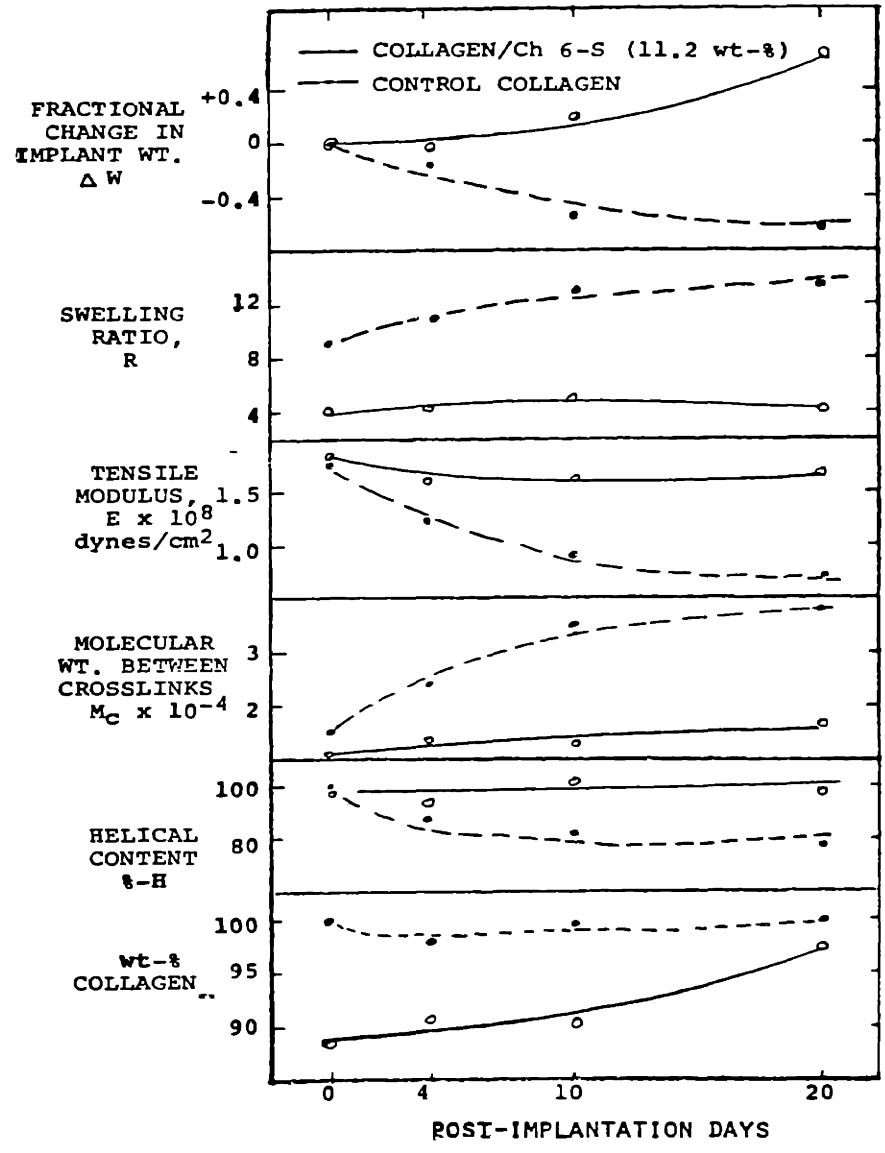


Fig. 5.7. The variation in physicochemical properties with post-implantation time of a collagen/Ch 6-S composite material (ca.11.2 wt-% Ch 6-S) compared to that of a pure collagen.

TABLE 5.5

REPORT FROM HISTOLOGICAL STUDIES ON THE IMPLANTATION OF COLLAGEN/CHONDROITIN 6-SULFATE COMPOSITE MATERIALS

wt-% Ch6-S	In vivo residence time, days: 4 days	10 days	20 days
Collagen Control	Extensive fragmentation; extensive ingrowth of histiocytes; Moderate acute inflammation; Extensive epithelium and appendages.	Marked fragmentation; Ingrowth of histiocytes; slight granulation tissue; Activiated fibroblast.	Total fragmentation; Extensive chronic inflammation; Extensive granulation tissue formation.
1.8 wt-% Ch6-S	Extensive fragmentation; Extensive ingrowth of histiocytes; Moderate acute inflammation; Severe chronic inflammation.	Moderate fragmentation; Ingrowth of histiocytes; Slight granulation tissue; Activated fibroblast.	Moderate fragmentation; Healing dermis and epithelium; Severe chronic inflammation; Moderate dense granulation tissue.
4.8 wt-% Ch6-S	Moderate fragmentation; Slight acute inflammation; Slight chronic inflammation; Slight granulation tissue reaction.	Marked fragmentation; chronic inflammatory reaction; Slight epithelilization; Acute inflammatory response.	Marked fragmentation; slight chronic inflammation. Moderate loose granulation tissue; Healing of epithelial and dermal elements.
11.2 wt-% Ch6-S	Moderate fragmentation; Slight chronic inflammatory response; V. slight granulation tissue.	Moderate fragmentation; Chronic inflammatory reaction; Healing of epidermal and dermal lesions.	Animal died. (Cause unknown)

These comparative results show that the control collagen was degraded to a much greater extent than the collagen-Ch6-S composite material, as was expected. This was further confirmed by the histological studies (Table 5.5) which showed total fragmentation of the collagen control compared to moderate fragmentation of the composite materials on the 20th post-implantation day. The monotonous increase in weight of the composite material, coupled with the increase in wt-% of collagen in the material suggests the attachment of newly synthesized collagen to the composite material. This was further confirmed by direct observation of the implantation site with a low-powered microscope. The composite material was found to be embedded in the tissue by the 10th post-implantation day (Fig. 5.6D) and evidence for tissue attachment was apparent (Fig. 5.6E) when attempts were made to remove the sample. The tissue attachement was so strong in some cases (especially with the composite materials containing higher content of Ch6-S) that considerable force was necessary to detach the samples from the tissue. A view of the site of attachment (Fig. 5.6F) reveals the presence of fibers which are apparently collagenous, based on the results of chemical analyses (Fig. 5.7). By contrast, the control collagen did not show any signs of tissue attachment to it (Fig. 5.6C) and was apparently surrounded by an inflammed tissue.

This latter observation was confirmed by the histological studies (Table 5.5) which revealed that the extent of chronic inflammation in the surrounding tissue increased as the amount of

Ch6-S in the composite material decreased. This suggests that while the collagen used in the composite material was recognized as a foreign material, the presence of Ch6-S could apparently suppress this recognition. This observation may have some biological significance since it has been observed that in certain cases the incidence of inflammatory arthritis is accompanied by the loss of MPS from the cartilage (Schubert and Hamerman, 1965). It may be speculated that the exposure of the underlying collagen fibers, caused by the loss of MPS from the cartilage, may result in an inflammatory response from the surrounding cells. This response may result in the release of lysosomal enzymes which can cause further degradation of the protein-polysaccharides (see Section 2.4.4) and the collagen fibers (see Section 2.4.1).

The results obtained in this Section, coupled with those obtained from studies of mechanical behavior of the composite materials (Section 4.3) strongly favor the use of the composite materials (as opposed to the pure collagen) as materials for skin replacement. The greater mechanical strength and resistance to *in vivo* degradation of these materials, their ability to form bonds with the surrounding tissue and the lower extent of inflammatory response induced by them are features which are favorable to their use as skin replacement.

CONCLUSIONS AND SUMMARY

A survey of the structure and function of skin was made in order to understand the problems involved in designing materials suitable for skin replacement. It was concluded that a suitable material is one with properties approaching those of the connective tissue matrix which forms the bulk of the dermal and subcutaneous layers of skin. A review was made of the nature of the connective tissue matrix, the mechanisms by which it is synthesized and degraded and the cells involved in these processes. The requirements for a suitable material for skin replacement were considered and it was concluded that two materials which may be likely candidates were collagen and collagen-mucopolysaccharide composite materials.

A number of methods were developed for the characterization of collagen in the solid phase. The properties studied include:

(1) Ultrastructural studies by means of scanning electron microscope (SEM). It was concluded from these studies that the SEM can serve as a useful tool for revealing a number of ultrastructural features of the connective tissue (see Figs. 3.1 to 3.8).

(2) Extent of crosslinking of collagen. This was studied by analyzing the stress-strain curve of the denatured collagen. The latter was shown to behave like an ideal rubber under appropriate conditions and its stress-strain curve was thus amendable to analysis by the theory of rubber elasticity (Treloar, 1958) to yield estimates of crosslinking density. For samples which were too small for stress-strain curve to be determined in tension, a method was developed

whereby estimates of the crosslinking density could be made from the swelling characteristics of the denatured collagen sample. This method was based on the theory of Flory and Rehner (1943). The applicability of this theory to denatured collagen was demonstrated.

(3) Helical content of collagen. This was studied by X-ray diffraction, infrared (IR) spectroscopy and optical rotation (OR) studies. Estimates of helical content of partially denatured collagen by the IR and OR methods were found to be in excellent agreement. The OR method was found to be the most sensitive method for determining the helical content of collagen. However, the method is suitable only for samples which are non-birefringent and which are thinner than 0.003". IR spectroscopy can be used with birefringent samples but is limited to samples of thicknesses less than 0.002". For samples which are both thick and birefringent, the X-ray diffraction method (or an alternative method) must be used to estimate the helical content of collagen.

(4) Susceptibility of collagen to <u>in vitro</u> enzymatic degradation by bacterial collagenase. This was studied by monitoring the force induced on a collagen fiber (held at fixed extension) in the presence of a solution of collagenase of known concentration. The rate at which the force relaxes was taken as a measure of the rate at which the collagen was degraded by collagenase. This rate was found to depend on a number of factors which include the temperature, pH and concentration of collagenase solution, the presence of inhibitors in the solution, the helical content and extent of crosslinking of the collagen and the strain

imposed on it. The biological significance of these influencing factors in the *in vivo* degradation of collagen was discussed.

Two forms of collagen-mucopolysaccharide composite materials were prepared. The first form consists of a collagenous core coated with MPS on the surface (called MPS-coated collagen). The second form consists of collagen fibers dispersed randomly within a MPS matrix. Both forms of materials were characterized for a number of properties which include: (a) helical content of the collagen component, (b) extent of crosslinking of the composite material, (c) swelling ratio; (d) tensile properties, (e) compressive properties, (f) wt-% of collagen (by hydroxyproline analysis) and MPS (by hexosamine analysis), and (g) the susceptibility of the composite materials to *in vitro* enzymatic degradation.

From these studies, it was concluded that presence of a limited amount (< 11 wt-%) of MPS can strengthen mechanically the collagen fibers as well as render them more resistant to *in vitro* enzymatic degradation. However, the presence of an excess amount of MPS (> 11 wt-%) has a deleterious effect on the mechanical strength of the collagen fibers while rendering them more susceptible to *in vitro* enzymatic degradation. These effects can be explained by considering models of the composite materials where the collagen molecules are envisaged to contribute positive charges while the MPS contribute negative charges (see Fig. 4.10). The presence of an excessive amount of either positive charges (i.e. very low MPS content) or negative charges (i.e. excessive MPS content) leads to a highly swollen structure which has poor mechanical strength and is easily

penetrable by collagenase molecules. The point where maximum mechanical strength and resistance to collagenase occurs thus corresponds to the complete cancellation of the charges of the collagen and MPS. The biological significance of these observations was discussed.

A number of implantation studies were made in order to assess the usefulness of the collagen and collagen-mucopolysaccharide composite materials as skin replacement. Materials with known varying degree of resistance to in vitro enzymatic degradation were implanted in guinea pigs for a fixed period of time (10 days) and the extent of in vivo degradation (as measured by the fractional weight loses in the explanted materials) were compared with the in vitro degradation. A direct correlation between the in vitro and in vivo degradation was found. This correlation is useful since it enables one to predict the extent of in vivo degradation of a material by subjecting it to the relatively simpler and less time-consuming in vitro test.

The characterization of collagen-MPS composite materials prior to and after their implantation in experimental animals revealed a number of results which suggest that they were more resistant to in vivo enzymatic degradation than the pure collagen. Fractional weight change of the samples and chemical analyses of their compositions revealed their ability to form attachments with newly synthesized collagen from the tissue. These were confirmed by direct microscopic observations of the samples as they were removed from the tissue (Fig. 5.6F). The pure collagen, on the other hand, was incapable of forming strong attachments with the surrounding tissue.

Furthermore, histological studies of the implantation sites revealed that the collagen-MPS composite materials induced a lesser inflammatory response than the pure collagen. All these observations, coupled with the observation that the MPS can strengthen the collagen fibers, suggest that the collagen-MPS composite materials are far superior as materials for skin replacement than is pure collagen. However, this conclusion has been based on studies involving only subcutaneous implantation of the materials in experimental animals. It must be confirmed by direct clinical application.

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