Physical Properties of Collagen at Intra and Inter Molecular Levels

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K.H. Rajini

Supervisor

Dr. R. Sanjeevi

Bio-Physics Laboratory
Central Leather Research Institute
(Council of Scientific and Industrial Research)
Adyar, Madras 600 020
India

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1.1 INTRODUCTION

Collagen is nature's gift to mankind. Without collagen, our bones would be as brittle as China clay, a graze would rip open skin and our hearts could not pump blood without exploding. So, it is hardly surprising that this is the most common protein in the animal kingdom.

Collagen is a structural fibrous protein of the connective tissues found in skin, tendons, ligaments, cartilage, dentin and bone. Once called the ''excelsior of the body'' it is present in mammals in higher concentration than any other protein and serves primarily a mechanical function. Its molecular structure is unique among proteins, readily accessible in large quantities from a great number of animals (1 - 4).

Collagen constitutes approximately one-third of the total body proteins of mammals by weight. It is also phylogenetically a very old protein, appearing in the oldest invertebrate species. The properties of this protein makes it suitable for medical application (5, 6) and intimately dependent on the characteristics of its aminoacid composition and sequence which determines its three dimensional structure and interaction among other molecules in the environment. One of the first serious research on collagen was undertaken by the leather and glue industries, and the principles of collagen tanning have contributed much to our understanding of collagen cross-linking. Correlations between the degree of cross-linking and tensile strength, are basic understanding to the mechanical properties of this fibrillar protein (7).

1.2 PROPERTIES OF COLLAGEN

Collagen fibres are generally, white, opaque and readily recognized in tissues. They being a viscoelastic material possess high tensile strength and low extensibility.

They tend to swell markedly when immersed in acid, alkaline or concentrated solution of neutral salt and non - electrolytes. An outstanding characteristic of collagen fibre is the sharp contraction to about one third of its length, on heating. The phenomenon is easily observed on heating a bundle of collagen fibres in water when at a certain temperature, which varies from one species of collagen to another, the fibre rapidly, shrinks. The shrinkage temperature - Ts of various mammalian collagens is constant and is around 62 - 65°C. Among the fish collagens considerable variation occurs and ranges from 38 - 54°C. After thermal contraction the fibres lose most of the characteristic properties of native collagen. Collagen fibres are insoluble in water but can be made soluble by dissolving it in dilute solutions of acetic acid, formic acid etc., without altering the structure of collagen. The soluble collagen when heated melts at a temperature called denaturation temperature Tm which is less by 25 - 30° C than Ts. Type I collagen is easily available and for experimental purpose rat tail tendon (RTT) has been used.

1.2.1 Denaturation of collagen fibres

The three dimensional conformation (the primary, secondary, tertiary and quaternary structure) is characteristic of native collagen. This conformation can be disrupted and disorganized without breakage of any peptide linkage, only by the rupture of linkages, which enabled the structure to maintain its conformation in space, this is called denaturation. It can be triggered by diverse physical or chemical agents such as heat, pH variations,ultraviolet and ionizing radiations, detergents, organic solvents, urea and guanidine solutions. Denaturation is sometimes irreversible and sometimes reversible. In native state, collagen has the most stable conformation in intracellular

conditions, and if these conditions are changed, the conformation of collagen will be altered. Consequently, there will be rupture of hydrogen bonds and other secondary bonds, resulting in disorganization of secondary and tertiary structure (8).

1.2.2 Strength of collagen fibres

Collagen fibres in biological tissues are strengthened by the formation of native crosslinks. Native crosslinks within a tropocollagen molecule (intramolecular crosslink) and between different molecules (intermolecular crosslink) are formed by lysine and hydroxylysine residues. Intramolecular crosslinks in tropocollagen are formed between lysine residues locates in the nonhelical region near the amino terminus. Intermolecular crosslinks are formed by the joining of two hydroxylysine residues and one lysine residue. These crosslinks are formed between residues near the amino terminus of one tropocollagen and the carboxyl terminus of another (9). The strength of collagen fibres is determined by cross - links between the molecules making up the fibrils and between the fibrils making up the fibres. Collagen is relatively inert to chemical and enzymatic attack under physiological conditions but to increase its resistance to wear, both the mechanical strength and resistance to deterioration can be increased by both cross - linking and reduction of water content. The process of stabilization involves the chemical agent or the physical process initiating, ideally, irreversible and stable intra- and inter molecular chemical bonds between collagen molecules. Preferably, the agent promotes bonds between the functional groups of amino acids. Chemical methods typically utilize bi - functional chemicals that interact with collagen at different sites. The functional groups of the chemical agent react with those on the amino acid residues of collagen such as the amino function on lysine and hydroxylysine or the carboxyl

function on asparatic and glumatic acids, to give rise to cross - link between the collagen molecules.

Considerable variation in the stiffness can be achieved by the type of intermolecular cross - link introduced. The most commonly used are metal salts such as chrome, organic aldehydes such as glutaraldehyde and vegetable tannins (10,11). The mineral tanning agents cross - link through co - ordination bonds with the carboxyl side chains of glumatic and aspartic acids. These complexes are stable and increase the Ts by as much as 30 - 40 ° C which reflects on the extent to which the collagen molecules are stabilized.

1.3 PURPOSE AND SCOPE OF PRESENT INVESTIGATION

Collagen is a ubiquitous protein in the animal kingdom that evolved to provide support and framework for cells, and to give strength and resiliency to skin, bone cartilage and tendon. The enormous rate at which the knowledge on the structure, chemistry, bio - chemistry and the biology of collagen has been accumulated during the past forty years testifies the universal importance of this protein. The properties of this protein which makes it suitable for fabrication into medical products, are dependent on characteristics of aminoacid composition and sequence which determine the three dimensional structure and interaction among other macromolecules in the environment. As the chief structural protein of the body, collagen is uniquely designed to transmit tensile and compressive forces of great magnitude. Furthermore, its presence as a constituent of basement membrane in extracellular spaces suggest that it also contributes to the partitioning of solutes in the body and may be of some use in diffusion. The purpose of this research is to study the physical properties of collagen at

the intra and inter molecular level. These results are expected to be very useful for the study of collagen as biomaterial. The properties of collagen studied are

A. the solution properties - Part - I -

using Ultrasonic Technique

B. the mechanical properties - Part - II

using 1. Instron Testing Machine and

2. Scanning Electron Microscopy

PART - I

1.4 SOLUTION PROPERTIES

During the last decades, ultrasonic study of liquid mixtures has gained much importance in assessing the nature of molecular interaction and in investigating the physico - chemical behaviour of biological systems (12 - 14). Ultrasonic velocity and related data of liquid mixtures are found to be very useful in testing the theories of liquid state. In addition, ultrasonic velocity data along with some basic physical properties can be utilized to deduce useful properties of liquid mixtures, which are not accessible, by other means of study. The study of propagation of ultrasonic waves in liquid systems provides an effective means of examining certain physical properties of the mixtures. It is used to examine the changes in physical properties at the macrolevel. The ultrasonic parameters and their variation with concentration and temperature in liquid mixtures and solutions are helpful in understanding the nature of intra and inter molecular interactions in the liquid mixtures.

In dealing with macromolecules such as collagen, deoxyribonucleiacid and polypeptides in the presence of a solvent, the following situations are often encountered

- a solution of randomly coiled molecules, a solution of molecules having a helical conformation and a crystalline precipitate (15). At a given polymer concentration, occurrence of these depend on pH, temperature and the nature of dilutent. So, in the present study ultrasonic parameters of collagen solutions under different experimental conditions are investigated to study the physical properties of collagen molecule at intra molecular level. However, some of the findings are useful in getting an idea of the molecular interaction at the intermolecular level also. The experimental conditions are:

1) **Effect of temperature** - a) of collagen solution at two concentrations

b) of collagen + aqueous basic chromium sulphate,

2) **Effect of concentration** - a) crosslinking agents - Basic Chromium Sulphate, Glutaraldehyde and Formaldehyde

b) denaturants - Methanol, Ethanol and n- propanol

The ultrasonic velocity is determined along with density and viscosity of solutions by varying the temperature and concentration. From ultrasonic velocity, density and viscosity various ultrasonic parameters like adiabatic compressibility, internal pressure, free volume, classical absorption coefficient, viscous relaxation time, Rao and Wada constant are calculated to explain molecular interaction.

PART - II

1.5 MECHANICAL PROPERTIES

The mechanical strength of connective tissues is commonly associated with the content, organization and physical properties of collagen. The amount and orientation of the collagen varies with the function of the tissue. The ability of the fibres to confer stability to the tissues depends on its mechanical properties, its insolubility in body

fluids and the fact that it is metabolically inert. Collagen is a basic structural element of soft and hard tissues and gives mechanical integrity and strength to the body.

The mechanical properties of rat tail tendon are characterized by stress - strain curve, stress - relaxation and creep behavior and, have been examined with respect to the parameters strain, rate of strain, relative humidity and temperature by many researchers (16-18). The mechanical properties of tendons have been interpreted by using models.

Using the model proposed by Naresh et al (19) for mechanism of failure, 1. influence of length 2. role of hydrophobic interactions and 3.effect of cross - linking agents on mechanism of failure are studied (Part - II).

The experimental studies on collagen fibres has been divided into

i. computation of stress - strain curves,

ii. determination of stress - relaxation

iii. by subjecting the fibre to hysteresis

iv. calculation of viscous energy, elastic energy and plastic energy

v. evaluation of the relative contributions of energy components i.e.,

contribution of viscous energy = viscous energy / total energy

contribution of elastic energy = elastic energy / total energy

contribution of plastic energy = plastic energy / total energy

vi. determination of cross - over point between relative contribution of viscous + elastic energy and plastic energy.

vii. morphology of fibres (SEM)

1.5.1 Mechanism of failure

The model proposed by Naresh *et al* (19) gives a clear insight into the tensile behaviour by quantifying various viscoelastic components. By modifying the procedure suggested by Naresh *et al* (19) various components like elastic energy, viscous energy and plastic energy are determined. The method is briefly described below.

In an experimental stress strain curve, the stress registered at a given strain level is time-dependent as it will increase with increasing strain rate. For a given strain level, if elastic energy is determined by the conventional method-i.e., by conducting the hysteresis experiment and measuring the area outside the loop-it would vary with the rate of testing (Fig 1). This would make the elastic component time-dependent which is not true. Hence extending the a sample to a given strain level (irrespective of the strain rate) and allowing the stress developed to decay at that point, until the equilibrium is reached and then completing the hysteresis cycle would not only give a better quantification of elastic and plastic energies, but, also in the process, give a measure of the viscous component (Fig 2). Repeating the above methodology at various strain levels would help in reconstructing a time - independent stress-strain curve within experimental limits (Fig 3). Thus in the present study the stress-strain characteristics of collagen fibres were determined and experiments were done using the modified methodology. The enclosed areas under different regions were measured and the various energy components i.e., viscous, elastic and plastic energy are calculated in MJ/m^3. From the total energy at each strain level the relative energy contribution of viscous energy, elastic energy and plastic energy are calculated. .

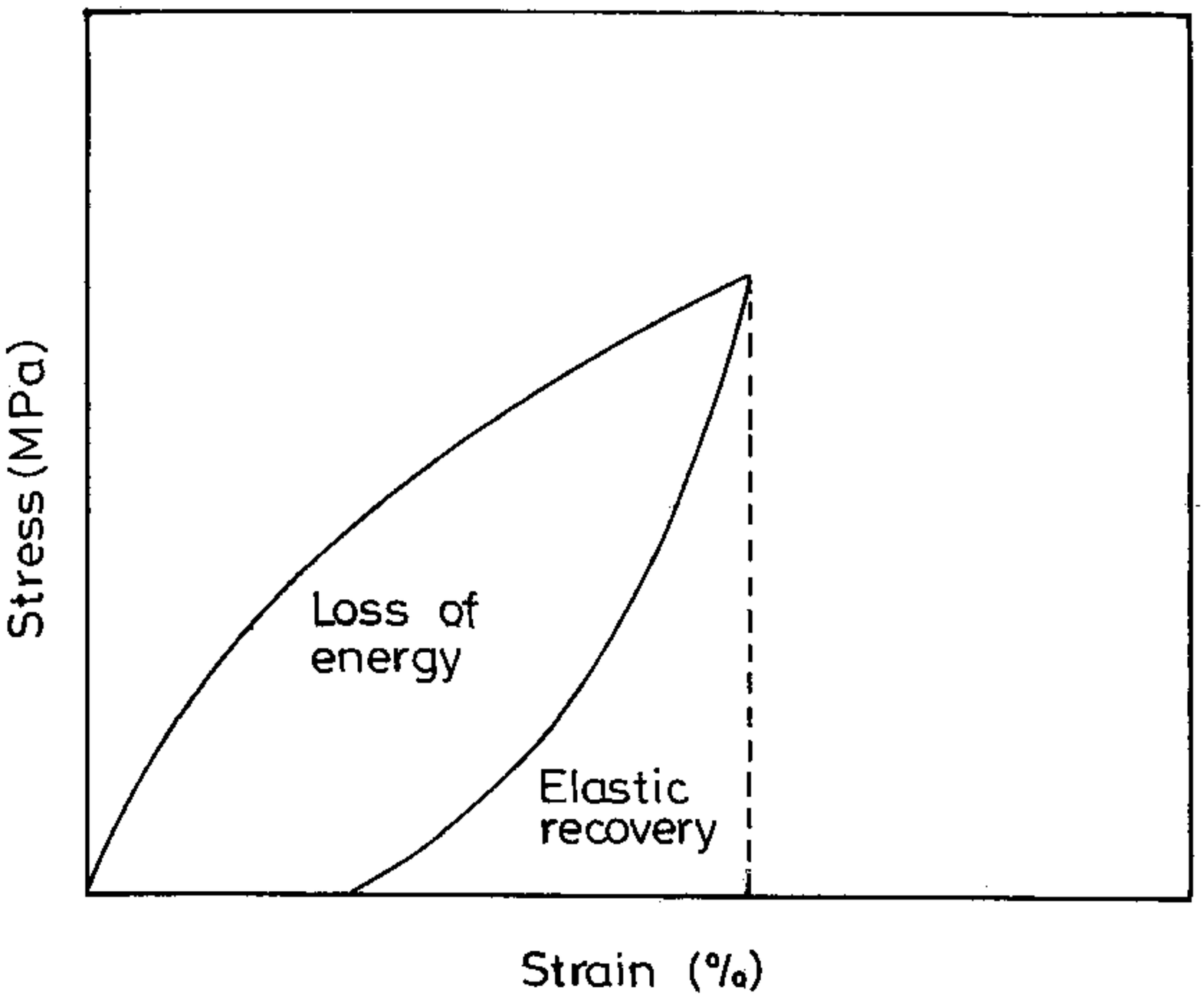

Fig.1. Conventional method of determining elastic plastic energies from the stress-strain curve

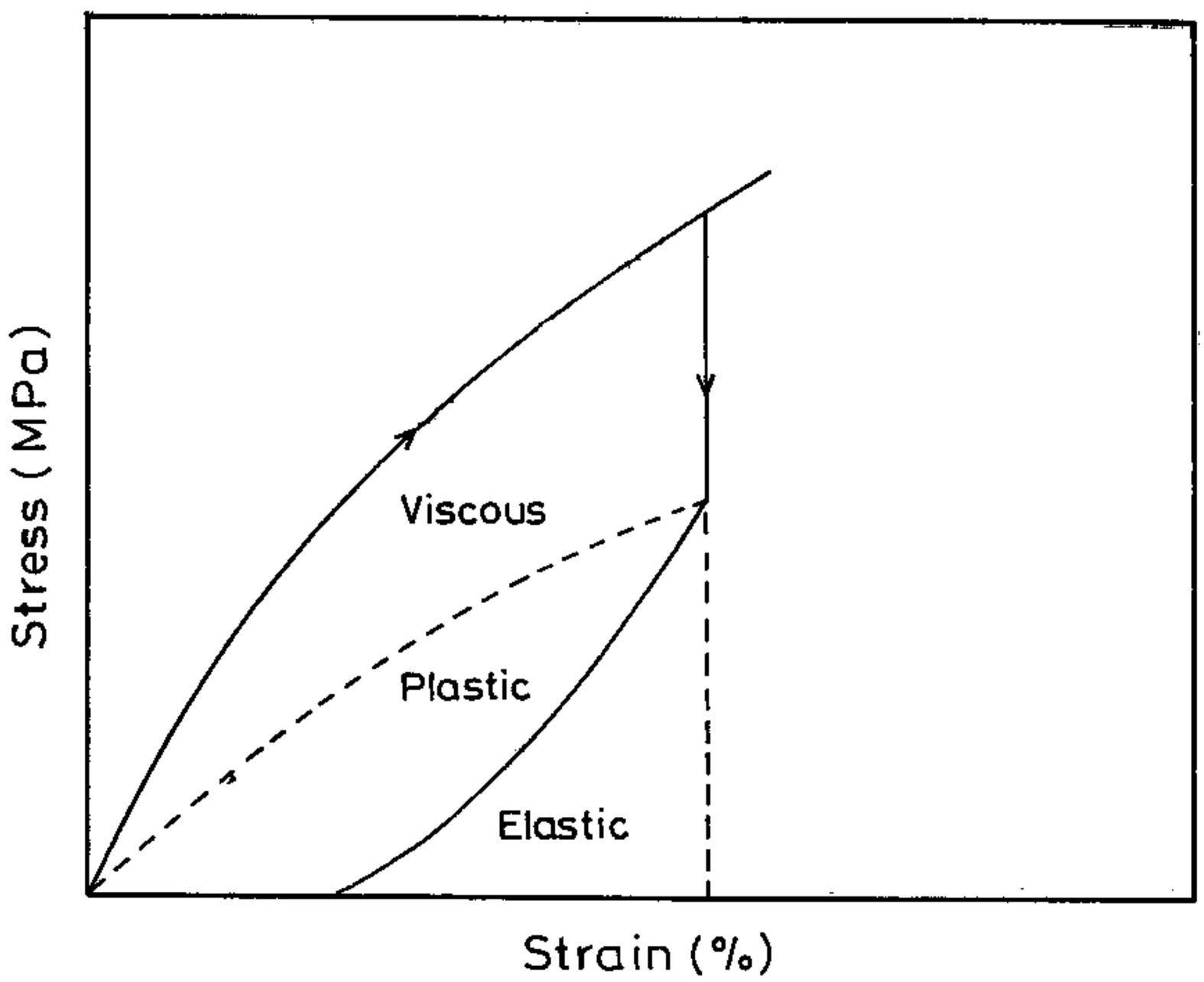

Fig.2. Method of determining the elastic, plastic and viscous energies from the stress-strain curve

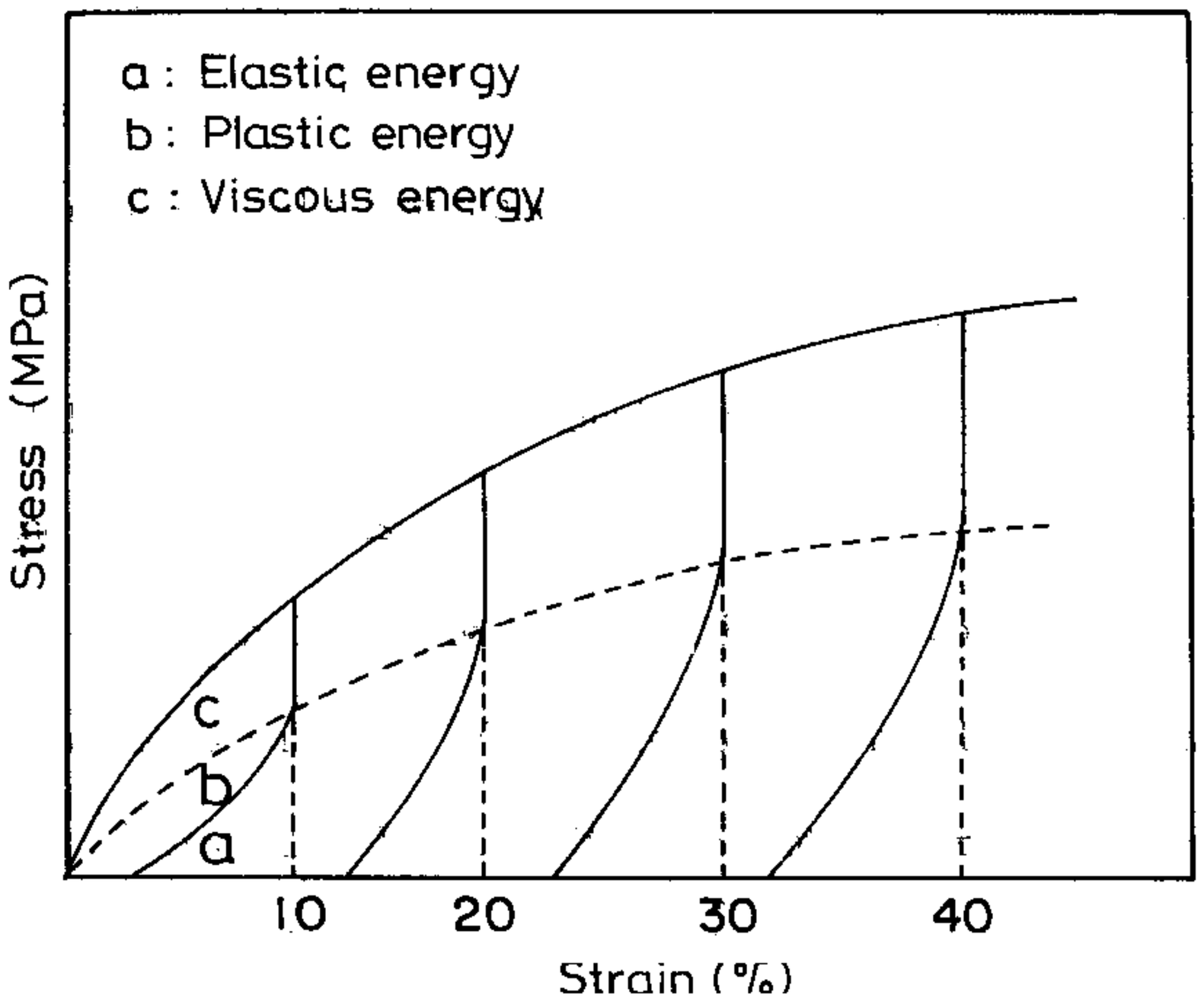

Fig.3. Determination of elastic viscous and plastic energies at various strain levels from stress-strain curve

When the collagen fibre is subjected to tensile load any one of these deformation is possible-i) slippage between fibrils ii) extension of collagen molecule and iii) extension of collagen fibre as a whole (20). According to Betsch and Baer (21), the collagen fibres are made of fibrils which are quarter staggered. These fibrils in turn are made up of three micro - fibrils or sub fibrils which have helical structure. There will be relative motion between the fibrils (viscous) and also there is a possibility of the micro fibrils getting elongated (elastic) and deformed (plastic) when the fibre is subjected to stress. Dunn and Silver (22) in their study on the energy components of tendons suggested the use of energy components in comparing the energy components between normal and diseased tendons.

Depending on the testing conditions the energy contribution from viscous and elastic components will vary. Viscous component may be predominant over elastic component and vice-versa. In view of this the relative energy contribution of viscous + elastic is compared with the relative contribution of plastic. When compared graphically this results in cross - over point. The cross-over point is significant, as it is able to reveal the strain below which the fibre can be subjected to reversible deformation and above which it results in irreversible deformation. This leads to mechanism of irreversible failure. This mechanism of failure is studied in collagen fibres under varying testing conditions like

i) influence of length

ii) role of hydrophobic interactions

ii) effect of cross - linking.

1.5.2 Scanning Electron Microscopy

The fractured samples were dried before testing. The samples were mounted on aluminium stubs with silver dag. Then they were coated with gold in Edwards SC 500 sputter coater at a current of 30A, operating at a voltage of 230V at a pressure of 0.1 Torr. The stubs along with samples were placed in the scanning electron microscope (Stereoscan 440). The fractured surface of the samples were scanned and photographed at different magnifications. The scanning electron micrographs revealed interesting features, from which the properties of the collagen fibres were inferred.

An extensive study carried out on the mode of fracture of fibres using SEM has led to the classification of the mode of fracture into three principal patterns. They are: (1) smooth fracture, fracture occurring in a single plane perpendicular to the fibre axis: (2) step fracture, with fracture initiating in a plane perpendicular to the fibre axis and propagating along the fibre axis, resulting in splitting of the fibre along its axis: (3) fibrillation, with the fractured split open into smaller fibrils (23).

However, different types of morphological characterisation and groupings have been reported in the literature. They are (i) deformed fibres, (ii) short peeling, (iii) peeling and fibrillation, (iv) fibril pills, (v) axial splitting, (vi) crushed fibres, (vii) bushy ends and (viii) surface debris (24, 25, 26). It is of importance to note that the morphological characterisation are extremely useful in understanding the various types of failures that might occur in the system due to the tensile forces.

- In **Chapter 1** the hierarchical structure of collagen, the different factors which stabilise collagen and its properties are discussed

- **Chapter 2** deals with the solution properties of collagen using Ultrasonic Technique, experimental conditions, results and discussion
- **Chapter 3** deals with the Mechanical Property of collagen, the methodology used, the experimental conditions, results and discussion
- **Chapter 4** gives the overall summary of the results obtained

A thorough understanding of the physical properties of collagen at the intra and inter molecular levels will give a very wide scope for the application of collagen as a very useful biomaterial.

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