AN INVESTIGATION INTO SOME OF THE ORGANIC CONSTITUENTS OF SOFT AND HARD TISSUES OF THE BODY.

BY

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Chapter I.

Introduction.

The roles played by fibrous proteins in nature are at present being intensively studied and some of the advances in our knowledge of these proteins are the subject of several recent symposia.[1a,b]

The powerful techniques of X-ray diffraction and electron-optic analysis as well as an unprecendented improvement in the specificity and accuracy of chemical and chromatographic methods of analysis has led to ever increasing efforts to interpret biological phenomena within a framework of physico-chemical principles. In many fields, particularly those of virus research and muscle contraction these efforts have already been rewarded by a large measure of success.

In the present work, which forms part of a program of research into the chemistry of connective tissues of man and other vertebrates, the chemical properties and structural features of the extracellular collagenous proteins in some calcified and uncalcified connective tissues are compared. 'In vivo' calcification of connective tissue is generally thought to be the result of a physico-chemical combination between lime salts and the organic matrix mediated by the ionic and structural properties of the matrix and the activity of various cells and enzymes.

It/

It has long been recognised that the factors which determine whether a tissue will calcify or not probably reside in its organic matrix and much attention has been paid to the function of acid mucopolysaccharides in this respect. Thus chondroitin sulphate is thought to be a 'local factor' partly responsilbe for the calcification of cartilage.[2] However, mature bone and dentin which contain about 80% of their weight as lime salts possess only very small amounts of acid mucopolysaccharides[3], and in general, attempts to apply results obtained using rachitic cartilage directly to bone have not been successful.

The main purpose of the writer is to show that the collagens obtained from hard and soft mammalian tissues exhibit marked differences in chemical reactivity and structure, in spite of their very similar amino acid composition. These differences indicate that the properties of collagen may play an active role in the calcification of hard tissues as well as being primarily responsible for the lack of mineralisation characteristic of soft tissues.

As this subject is one in which new discoveries are continually being made no final mechanisms are proposed, and it should be borne in mind that all the results to be presented were obtained 'in vitro'.

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Chapter II.

Some histological features of hard and soft tissues.

This chapter is devoted to a very brief outline of some of the histological features of the tissues that were used in this study. Certain aspects of the growth and development of these tissues will be more fully discussed in a later chapter, when the chemical and physical properties of the intercellular substances have been described.

All vertebrate connective tissues are composed of cells, fibres and amorphous ground substance, and can be divided into 7 main varieties[1]:-

1. Dense fibrous
2. Cartilage
3. Bone
4. Dentin
5. Areolar
6. Adipose
7. Haemopoietic.

(5,6, & 7 were not investigated in this work.)

1. Dense Fibrous Tissue.

This type of tissue is formed from mesenchymal cells which, after developing into fibroblasts, produce abundant quantities of collagen, thus giving the tissue great tensile strength. The collagen fibres are embedded in an amorphous mucopolysaccharide ground substance and may be regularly arranged in parallel bundles, separated from each other by

rows/

rows of fibroblasts, as in tendon, or the fibres may be disposed in several planes and int rwoven with one another to form a dense cohesive tissue, eg. deeper part of the skin (XXXXX). Connective tissues contain, in addition, varying amounts of elastic and reticular fibres, but there are no connective tissue cells which specifically produce elastin or reticulin.

Elastin has been classed as a non collagen because of its amino acid composition, X-ray diffraction pattern, ext nsibility and low tendency to swell in weak acids(2). It is thought that elastin, which can be degraded by the pancreatic enzyme elastase(3), more nearly resembles the globular proteins. "elastin-like" fibres have recently been obtained by chemical treatment of collagen fibres(4). The elastic fibre networks present in arterial walls give these organs their characteristic elasticity.

Reticular fibres occur wherever connective tissue forms a boundary eg. all basement membranes, sarcolemma etc. The amino acid composition, X-ray diffraction pattern and electronoptical morphology show that reticulin is a collagen(5). Histologically, however, reticular fibres are readily differentiated from collagen and elastic fibres by their marked argyrophilia, which is thought to be due to association with polysaccharides.(6) The presence of carbohydrates may also explain the immunological activity of reticulin and its

resistance/

resistance to heat. There are also differences in carbohydrate and lipid contents associated with reticulin in developing connective tissue, and that forming the basement membranes of parenchymatous organs.(7)

2. Cartilage.

There are 3 main types of cartilage - hyaline, fibrous and elastic.

Hyaline cartilage, found at the epiphysis and articular surfaces of bones, consists of cells - chondrocytes, which reside in little spaces called lacunae. The lacunae are embedded in a gel of intercellular substance containing collagen fibres and large quantities of amorphous sulphated mucopolysaccharides. In some sites eg. external ear and epiglottis, elastic fibres are scattered throughout the extracellular matrix. Fibro-cartilage, which occurs in the intervertebral disks and at tendenous attachments to bone, is noted for its excessive amount of collagen fibres in the intercellular substance.

3. Bone.

There are two types of bone formation - intramembranous and endochondral. In the intramembranous mode of ossification, by which the mandible and flat bones of the scull are formed, connective tissue fibroblasts differentiate into osteoblasts and lay down an organic matrix which subsequently calcifies.

All the long bones are formed by the endochondral

type/

type of ossification in which epiphyseal cartilage calcifies and acts as a scaffold for bone formation by osteoblasts. Osteoblasts and osteoclasts then remodel the bone in accordance with the physical stresses and strains imposed on it. These bones grow by the process of endochondral ossification and the shaft thus formed is remodelled by intramembranous ossification.

The changes that take place in epiphyseal cartilage during endochondral bone formation have recently been studied by means of the electronmicroscope(8). The extracellular cartilage matrix was seen to consist of widely spaced unbanded fibres, embedded in large amounts of an amorphous ground substance composed of chondroitin sulphate. Calcification of the ground substance is first seen near the third dead chondrocyte capsule beyond the furthest penetration of capillaries. The deposition of crystals of calcium phosphates is at first haphazard and bears no relation to the collagenous fibres. Osteoblasts appear and deposit typical banded collagen fibres, as a loose network of osteoid, in a narrow zone between the osteoblasts and the calcified cartilage. The organic intercellular substances consist chiefly of collagen together with small amounts of a cement substance (probably chondroitin sulphate). Calcification of the osteoid is normally immediate, crystals being laid down close to or on the fibres. As they

increase/

increase in number, the crystals are arranged so that they emphasize the periodicity of the underlying collagen fibres. Neighbouring ossified fibres are often aligned so that their banded regions are in register over large areas. There is no change in the calcified cartilage at this stage and a double membrane exists between the cartilage and the bone matrices. Bone is similar to cartilage in that it also consists of cells in lacunae surrounded by intercellular substances. Unlike avascular cartilage, which dies on calcification, bone is permeated by the canaliculi and Haversian systems which serve as connecting channels between the lacunae and the blood capillaries at the bone surface. Oxygen and other metabolites brought by capillaries diffuse through the tissue fluid contained in the canaliculi and nourish the cells in the lacunae, waste products being removed by the same mechanism.

4. Dentin.

In addition to the pulp, the teeth consist of 3 different calcified tissues - enamel (ectodermal), dentin and cementum (mesodermal)

Dentin is a tubular structure formed by pulpal cells which differentiate into odontoblasts. Korff fibres are formed in the pulp and pass in between the odontoblasts into the dentin, where they are transformed into a collagenous calcifiable matrix called predentin. Calcification of the intertubular matrix consisting of collagen fibres and small amounts of

chondroitin/

chondroitin sulphate, is normally delayed for a definite period (24 hours in the rat incisor)

Cementum, which covers most of the root of the tooth, is chemically and physiologically similar to bone, but Haversian systems and blood vessels are normally absent.

The enamel of teeth is epithelial in origin and is formed by the calcification of an organic matrix containing eukeratin. In the process of calcification almost all the protein is removed. The mature enamel consists of thin prisms or rods which stand on the surface of the dentin, and are separated from each other by an organic cementing substance.

In addition to their collagen content, the organic fraction of connective tissues contain 1-2% of a protein-carbohydrate fraction which is more resistant than collagen to solution in hot water. The amino acid composition of this component does not closely resemble that of collagen or elastin. Small amounts of glucosamine, galactose, glucose, mannose, glucuronic acid, mucoprotein as well as lipid material are also present. The properties of these components were not studied in the present work. (9)

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Table a: Gross Composition of some Connective Tissues:

Tissue	Total Protein	Total muco-polysaccharide	Lime Salts.
Ox-hide	99.5	0.2 - 0.5	nil
Ox-bone	22-23	0.2	77
Ox-cartilage	50 - 80	20 - 40	nil
Human dentin	19-20	0.6	78
Fish scales	44	-	56

Chapter III.

Chemical and Physical Properties of the Intercellular Substances in Connective Tissue.

The intercellular substances of vertebrate connective tissues contain

A) protein (mainly collagen with some reticulin and elastin)

B) acid mucopolysaccharides (chondroitin sulphates)

C) mineral material, in the form of basic calcium phosphates, is present in hard tissues.

Table a shows the relative amounts of these components in each of the tissues studied[1].

The living tissue contains, in addition, various enzymes, salts in solution and metabolytes of cellular activity. The properties of these substances were not studied.

A. Intercellular Proteins.

The fibrous proteins of supporting tissues are divided into two groups on the basis of their molecular shape as determined by X-ray diffraction measurements[2] - the k-m-e-f group (keratin-myosin-epidermin-fibrinogen), and the mesodermal collagen group. The collagenous type of 'fibrous' protein, with which the author is mainly concerned, is further distinguished from the 'globular' proteins (eg. albumin) by its relative inertness, high molecular weight, distinctive amino acid

composition/

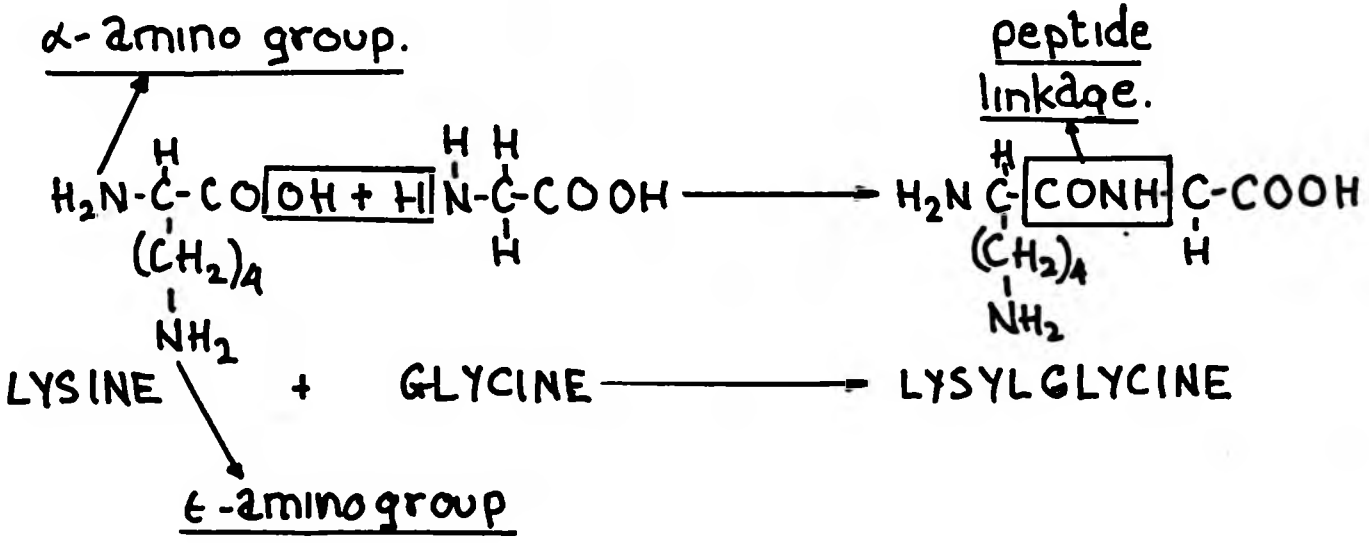


FIG.1. CONDENSATION OF AMINO ACIDS.

composition, electron-optic appearance, and high resistance to chemical, physical and some types of enzymatic attack. Reticulin is thus classed with the collagens, but elastin is not.

Collagen is widely distributed as a protein which supports the cellular structures of animal tissues. In vertebrates the collagenous system is mesodermal (cf. annelids and nematodes where the ectodermal cuticular system is of the secreted collagenous type.)[3] Bovine and human collagens resemble each other closely, but they differ in certain respects from the marine collagens[4]. The following description of the chemical and physical properties of the intercellular protein applies mainly to bovine skin collagen.

Chemical and Physical properties of collagen:

According to the peptide theory of protein structure[5], protein molecules are made up of amino-acid residues covalently linked together by the condensation of the NH_2 group of one amino acid with the -COOH group of another. (Fig. 1) On hydrolysis, (with acid or alkali), the polypeptide can be converted into a mixture of free amino acids. The ketoimide (CONH) links which are most easily split by strong acid, are those involving the amino groups of serine and threonine residues.[6] Dilute solutions of strong acids and concentrated solutions of weak acids preferentially split the CONH group adjacent to the end residue of aspartic acid,[7] whilst leucine, lysine and valine

tend/

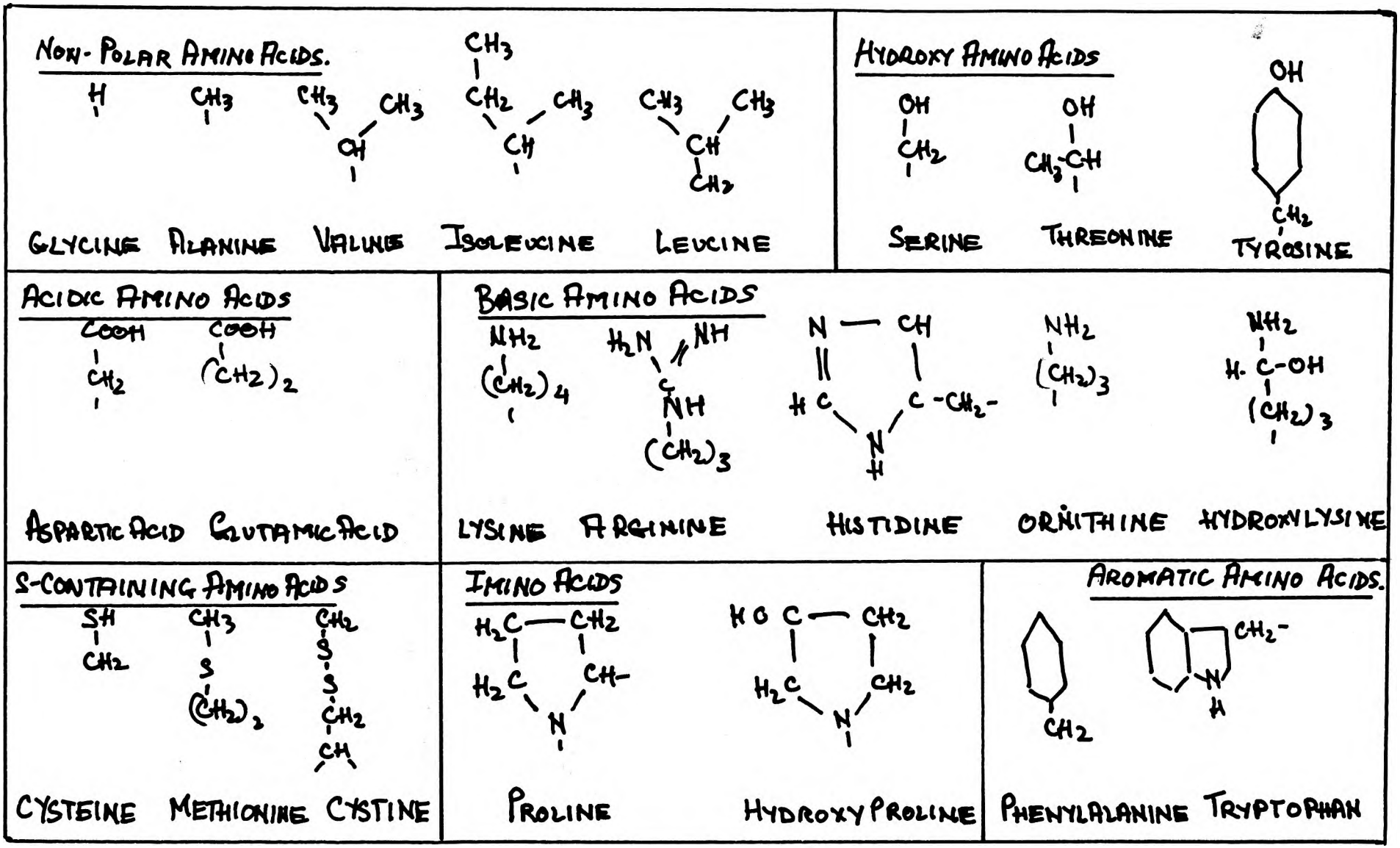


FIG.2. STRUCTURE OF THE 'R' GROUPS OF COMMON AMINO ACIDS.

tend to form relatively stable peptides.(6) Tryptophan is usually destroyed by acids, and, therefore, alkaline hydrolysis must be used for the estimation of this amino acid in proteins(7).

As all proteins are assumed to have this backbone of peptide linkages, the vast differences that exist between proteins may be due to variations in

a) the amino acid composition

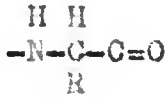
b) the order in which the amino acid residues are connected to each other in the polypeptide chain.

c) the size, shape and mode of folding of the protein chains

Note: c) may be influenced by a) and b).

a) <u>The amino acid composition of collagen and its effect on the stability of the protein.</u>

The general formula for the amino acid residue is



There are about 22 amino acids, each differing from the other by the composition of the 'R' group (Fig. 2). There are four types of R groups:

i) non-polar, - as in glycine and valine
ii) cationic, - as in lysine and arginine
iii) anionic, - As in glutamic and aspartic acids
iv) polar but nonionic - as in serine and threonine

The older methods of estimating amino acids in proteins(8) involved tedious chemical fractionation of the hydrolysate and repeated recrystallisation of amino acid derivatives. Most of these methods involved using large correction factors, as well

as large amounts of protein. More accurate colorimetric and paper chromatographic methods have superceded the gravimetric procedures, and with the development by Moore & Stein of a chromatographic column elution method, the complete amino acid composition of a protein hydrolysate can now be accurately performed in one week using only 10 mg. of material (9). An aliquot of the protein hydrolysate is placed on a column of ion exchange resin (Dowex 50) and by the addition of appropriate buffer solutions each amino acid is eluted from the column at a different rate. 1 ml. fractions of eluate are collected, treated with ninhydrin, to give a color which is spectrophotometrically recorded (Fig.2.p. 102a) This procedure is rapidly becoming the method of choice.

Another general method of amino acid assay is the microbiological one, (10) in which use is made of the fact that the rate of growth of certain bacteria is limited by the availability of an essential amino acid. Thus the rate of growth,

turbidometrically/

Table 1: Amino acid composition of Ox-hide collagen(98) expressed as g.amino acid/100g. dry ash free protein:

Amino acid	%
Alanine	11
Glycine	27.5
Valine	2.59
Leucine	3.33
Isoleucine	1.72
Proline	16.35
Phenylalanine	2.23
Tyrosine	0.29
Serine	4.31
Threonine	2.22
Methionine	0.89
Arginine	8.8
Histidine	0.78
Lysine	4.5
Aspartic acid	6.7
Glutamic acid	11.4
Hydroxyproline	14.1
Hydroxylysine	0.97

Note: Ox-hide collagen, human bone collagen, and ox-bone collagen also have similar amino acid compositions(98) (98).

turbidometrically measured, is an indication of the concentration of this amino acid.

On hydrolysis collagen yields a 'spectrum' of amino acids which differs sharply from that found with any other class of proteins[14]. It is seen from the data in Table1 that collagen contains a well balanced proportion of positively and negatively charged side groups, thus conferring a fair degree of reactivity on the protein. Collagen has a large content of non-polar amino acids particularly glycine and alanine. In addition, the prominence of proline and hydroxyproline, and the paucity of aromatic amino acids, is characteristic of collagen. Analysis of many types of protein has shown that the presence of hydroxylysine is, as far as is known, confined to collagen alone.[12]

The side chains of the anionic and cationic side groups in protein chains give the molecule amphoteric properties. Thus collagen will react with both acid and base (Fig.3). In strong acid solution the ϵ - amino groups of the basic amino acids (lysine, arginine, hydroxylysine) accept protons and become charged $-NH_3^+$ ions. At the same time the ionisation of side chain carboxyl groups (from glutamic and aspartic acids) is suppressed: The combination of acid and protein is virtually independent of the temperature (for equilibrium states)[13]. It has been shown[13] that the reaction of protein with alkali, however, is greatly increased by a rise in temperature, and

involves/

involves energies of the order of 10 K.cal./mol. This suggests that the titration of protein with alkali consists in the hydrolysis and discharge of the ionised basic groups and not in a simple combination of OH^- ions. These reactions are represented by the following simplified equations in which collagen in the isoelectric state is represented as $COO^-.R.NH_3^+$. On the addition of acid, protons are taken up by the free COO^- groups as follows:-

$$\underset{\text{collagen}}{COO^-.R.NH_3^+} + \underset{\text{proton}}{H^+} \longrightarrow COOH.R.NH_3^+$$

In the presence of alkali, collagen protons are discharged according to the equation:-

$$COO^-.R.NH_3^+ - H^+ \longrightarrow COO^-.R.NH_2 + H^+$$

followed by

$$H^+ + OH^- \longrightarrow H_2O$$

The transference of the proton from the one position of binding to another depends on the pH of the solution. xx. xxxxx xxxxxx. . All the electrovalent reactions of proteins take place either in acid or alkaline solutions. In the isoelectric range of pH where the protein is electrically neutral, due to its equal number of positive and negative charges, collagen shows ionic inertness. In contrast to soluble proteins, collagen binds very small amounts of acid or alkali

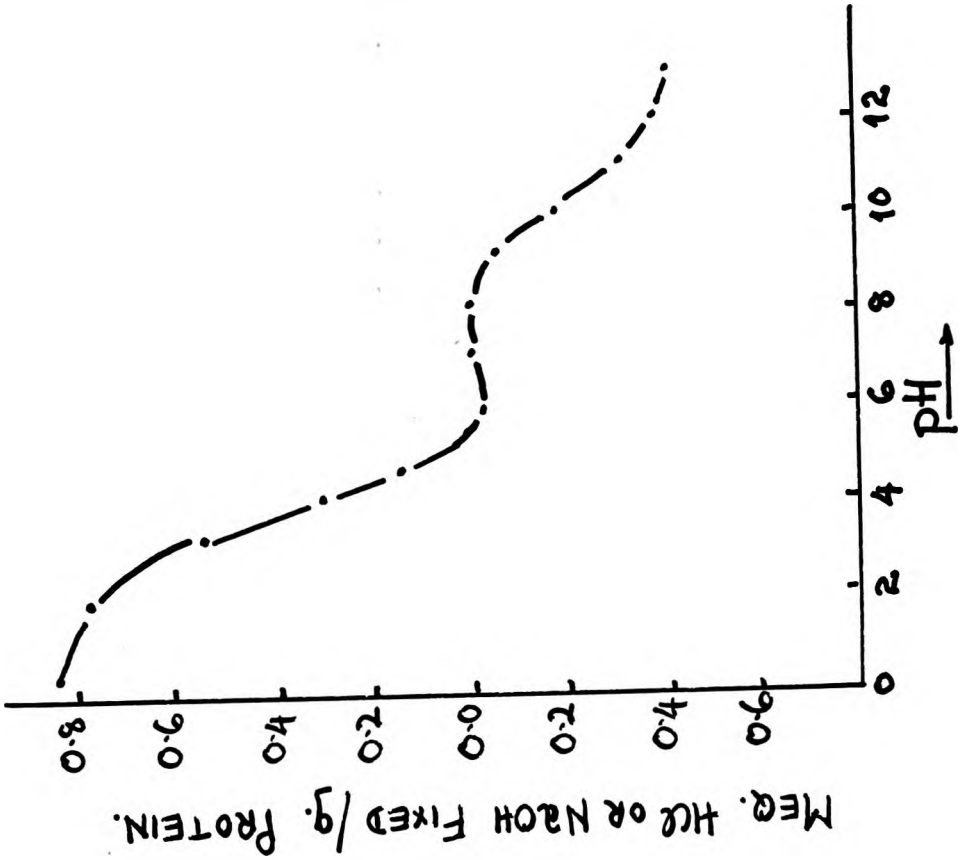
with-/

Table 2: Ionisation constants (pK) of protein groups(14):

Group.	pK	Group.	pK
COOH	3.0 - 3.2	Imidazole Histidine	5.6 - 7.0
Carboxyl (aspartyl)	3.0 - 4.7	α amino	7.6 - 8.4
Carboxyl (glutamyl)	4.4	ϵ amino	9.4 - 10.6
Phenolic -OH	9.8 - 10.4	Guanidine	11.6 - 12.6

Note: The influence of one group on another can extend over a chain of several carbon atoms (99).

Fig. 3: Titration Curve of Collagen.



within a wide range of pH on both sides of the iso-electric point, if the solutions are devoid of neutral salts. This may be due to the formation of salt-like crosslinks between anionic and cationic side chains, and suggests that a certain minimum potential of H^+ or OH^- ions is required to break these links[14]. However, this zone of inactivity may be eliminated if a neutral salt is added especially if the anion of the salt is the same as that of the acid used for titration. The large number of anions eliminates the Donnan effect, and overcomes the potential barrier set up against the anions of the acid by the cationic protein groups. In alkaline solution, however, sodium sulphate and not sodium chloride is effective[15]. From the titration curves the pK values of the various reacting groups of the protein can be ascertained[14] (Table 2 Fig. 3). It must be remembered that the pK value of any group can be influenced by neighbouring groups in the protein chain. The maximum binding capacity for hydrogen ions is 0.96 meq./g.protein (equilibrium value) reached at pH of about 2. However, the final base reacting figure is not reached even at pH of 13 and no definite figure can be given for this property as the basic guanidyl ion requires still higher pH values for its complete discharge.

The titration curve of native collagen has been interpreted in terms of titratable groups and the values obtained compare favourably with those obtained by chemical

analysis/

analysis of the protein[14]. (Table 1).

The stabilisation of collagen: X-ray diffraction studies on collagen[16] show that the polypeptide chain is almost fully extended and thus it is unlikely that any intra-chain bonding exists as it does in the globular proteins. The stability of collagen is due to a number of different types of lateral interchain links:-

i) The electrovalent salt link:[17] An example is the lysine-glutamic acid crosslink:

$$\dot{C}H(CH_2)_4NH_3^+ \cdot {}^-OOC(CH_2)_2 - \dot{C}H$$

$$\longleftarrow 10 \text{ to } 16\mathring{A} \longrightarrow$$

Since there is no sharing of electrons in this link, the distance between oppositely charged groups may be altered within certain limits without rupture of the crosslinking. This type of linkage is of major importance in the stabilisation of the marine collagens. The strength of this link depends on the dielectric constant of the environment[‡ ‡], and the distance between the charged groups. Thus water, acids and bases will tend to open this crosslink. By the action of solvents of low dielectric constant (eg. alcohol) the groups may be discharged by the transfer of a proton from the cationic to the anionic group,

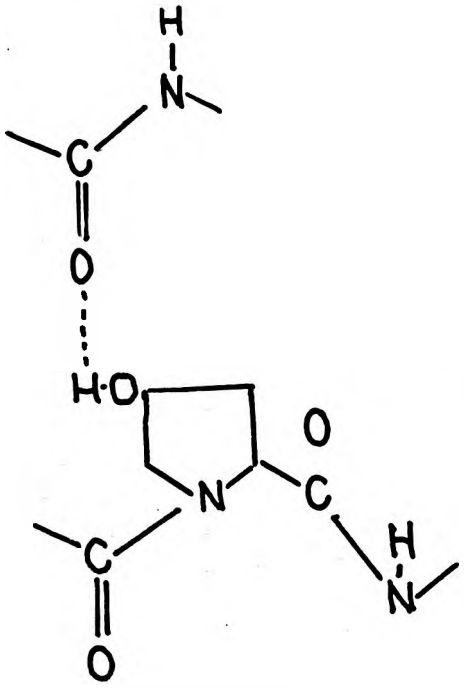


FIG. 4.

resulting in the formation of a hydrogen bond which confers a greater stability on the collagen fibre[17].

ii) Co-ordinate links[18] (hydrogen bonding):- The interchain carbamino link

although having a bond energy of only a few kilocalories can stabilise large protein molecules due to the large number of sites for hydrogen bonding.

Because of the large number of proline residues in the peptide chains there is a replacement of the .CO.NH- links by -CON= which results in fewer sites for interchain hydrogen bonding. However, the hydroxyl group of hydroxyproline, which is present in large amounts in mammalian collagen, can form hydrogen bonds with the -CO-NH- linkages of neighbouring chains (Fig.4). This concept is supported by the fact that the hydrothermal stability of marine collagen, which has a lower hydroxyproline content, is much less than that of mammalian collagen[4]. Furthermore, O-acetylation lowers the shrinkage temperature, but N-acetylation has no effect[19]. Marine collagens have slightly higher amounts of the hydroxy-amino acids, serine and threonine.

iii) Another type of force conducive to protein stability is gravitational attraction of neighbouring chains.

In/

In collagen this type of force is of small import as the appreciable amounts of bulky side groups tend to keep the chains at a relatively large distance apart.

iv) The fact that collagen is insoluble in concentrated solutions of lithium bromide which dissolves straight hydrogen bonded fibrous proteins (eg. silk) suggests the presence of an additional strong interchain crosslinking probably an ester bond -O-CO- [20].

v) Another stabilising bond which occurs in many types of protein especially the keratins, as well as certain of the marine collagens such as elastoidin[21] and ichthylepidin[22], is the covalent disulphide crosslink due to the presence of cystine (Fig. 2). This powerful interchain link is absent in bovine and human collagens.

It is thus concluded that the great mechanical strength and insolubility of collagen is due to the extended configuration of the protein chain and the high degree of interchain bonding due to hydrogen bonds and salt links.

The Hydration of Collagen: [17]

Collagen is capable of binding water molecules through electrostatic attraction at polar side chains, and by hydrogen bonding at the oxygen and nitrogen atoms of the

peptid/

peptide linkage[23] (Xxxx. p.20), particularly at the uncompensated carbonyl group which is opposite the frequently occurring CO=N- group due to the prolines. A certain minimum amount of water, about 20%, is normally bound as water of hydration[24] of collagen, both 'in vivo' and 'in vitro'. The average distance between adjacent polypeptide chains of dry collagen is about 10Å [16]. This distance is increased by the uptake of water to about 16Å. As the water of hydration is virtually unaffected by the pH value (in the range 1-10)[25] of the medium in which the collagen is equilibrated, all the bound water is probably not held by ionic groups as these vary considerably in strength with the pH value. The bound water is considered to be incapable of acting as a solvent for electrolytes and non electrolytes[X X].

The swelling of collagen:

The molecular cohesive forces of collagen may be weakened by reagents which can break hydrogen bonds or salt links. Treatment of collagen with such reagents produces swelling of the fibres and in some cases dissolution may occur. These changes can take place without appreciable hydrolysis of the peptide linkage[26]. Reagents such as HCl and NaOH whose dissociated ions affect the ionic side groups of the protein give rise to osmotic swelling[X 27 X] in which the fibres decrease

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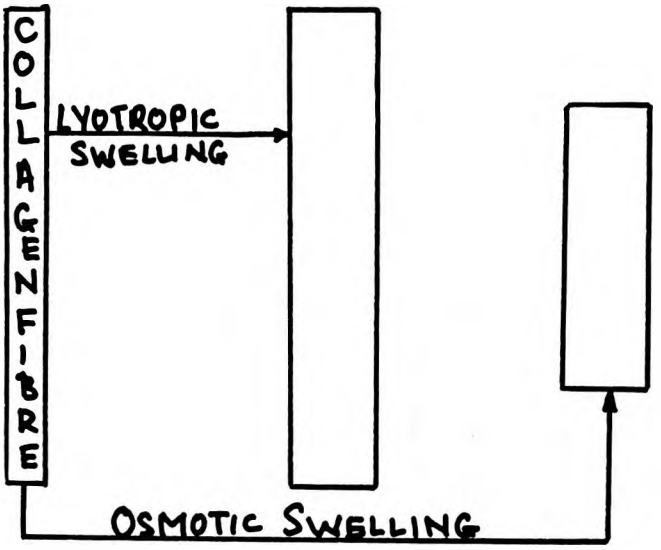

FIG.5.

in length and increase in diameter. Another type of swelling called lyotropic[27] in which only the width of the fibres increases, is produced by neutral hydrogen-bond breakers such as urea and guanidine. Both types of swelling can be concurrently produced by solutions of weak acids (eg. acetic) at pH=2 or by divalent bases (eg. calcium hydroxide) at pH values greater than 10. The effects produced by mild osmotic swelling are usually reversible, but the alterations in the co-ordinate cross-links resulting from lyotropic swelling are partly irreversible, and the protein is then said to be 'denatured'.

The osmotic swelling phenomenon (Fig.5) may be considered a result of the establishment of a Donnan membrane potential inside the fibres due to the discharge of some of the ionic groups of the protein by the reagent[*] [*]. This is accompanied by electrostatic repulsion of the remaining similarly charged groups. Water is thus able to flow into the partly disorganised structure. Osmotic swelling in acid solution may be considerably reduced and water removed from collagen by the addition of neutral salt such as sodium chloride[14]. This is explained, by the electrostatic theory,[*] [*] as a discharge of the positively charged protein by the combination of chloride ions with the cationic protein groups. This theory, however, does not explain the failure of sodium chloride to prevent swelling in alkaline media but the salts i of dibasic acids

such/

such as sodium sulphate markedly depress this swelling.[28]

The lyotropic swelling which is produced by acetic acid, urea and guanidine, is explained by the tendency of these unionised molecules to become hydrogen bonded to collagen thus forcing adjacent chains apart[27]. (Fig. 5). The total acid binding power of the protein is unaffected by lyotropic swelling[x]. Other lyotropic reagents include phenols, aromatic carboxylic or sulphonic acids particularly those which carry a hydroxy group, and detergents of the alcohol-sulphonate type[30].

The Contraction and Hydro thermal shrinkage of Collagen:

If mammalian collagen fibres are heated in water at the isoelectric pH, a sharp contraction to ¼ or ⅛ of the initial length occurs at Ts of about 62°C. This hydro-thermal shrinkage imparts a rubber-like elasticity to the protein, and the tensile strength of the fibres is reduced[31]. The actual range of temperature at which rapid contraction occurs is increased by subjecting the fibres to tensile stress, and varies according to the source of the collagen, and its pretreatment. Marine collagens in general, have much lower (about 20°C) Ts values than mammalian collagen[32], and degradation of both types of collagen will lower the absolute value of Ts[x x]. The maximum acid binding remains the same but the reactivity of the carboxyl groups in the pH range of 2.5 to 5 is increased due probably to weakening of salt links between some of the oppositely charged ionic groups induced by unfavourable

steric/

steric conditions in the contracted protein chains[33]. Marked hydrolysis or dissolution does not occur. However, the resistance of collagen to trypsin is lowered by shrinkage[K X]. X-ray diffraction studies show that the shrunken fibres have been transformed into an amorphous phase and have irreversibly lost their long range orientation but the short range crystallinity still remains[16]. The original high angle X-ray pattern can be obtained by re-elongation of the fibres[34]. By incorporation of crosslinking tanning agents, eg. formaldehyde, almost complete reversal of the shrinkage is attained when the fibre is cooled. This is known as the Ewald reaction[35]. Kinetic studies have shown that the process of shrinkage is a rate phenomenon[36](Kixx). For example, if a sample of collagen is kept in water at a few degrees below the instantaneous shrinkage temperature, a slow 'incipient contraction' takes place. The process of shrinkage has been postulated to occur in two steps, the first of which is completely reversible[K X]:-

	heat 25K.cals ⇌ cool		heat →	
Collagen I		Collagen II		CollagenIII
(native crystalline state)		(activated melted state)		(heat shrunk Δs increased

Collagen II represents the melted state and collagen III the coiled globular state. The treatment of heat shrunk skin collagen with ionised and unionised tanning agents show that the bonds broken in the formation of the randomised Collagen III are

mainly/

mainly the interchain hydrogen bonds[33]. The salt links are little affected as removal of the ε-amino groups of lysine does not lower the Ts of bovine collagen.

Fish skins in general, have a much lower Ts (30-53°C) xxxxxxxxxxxxxxxxx . This is probably due to their low total content of the hydroxyamino acids, particularly hydroxyproline, with a resultant lower degree of interchain hydrogen bond formation compared to bovine collagen[I p.19a]. This is supported by the fact that O- and N-acetylation of bovine collagen reduces the Ts by about 25°C which is close to the Ts of fish-collagen, whereas N-acetylation alone has no appreciable effect[34]. Elastoidin, however, which contains disulphide crosslinks gives the Ewald reaction[11].

Denaturation:

By denaturation of a protein is meant a change in the internal structure of the protein molecule involving a rearrangement of peptide chains due to the breaking of interchain bonds, with a concomitant alteration in solubility, biological activity and the characteristic physico-chemical properties of the protein[35].

The main types of denaturing agents for collagen are those which cause swelling or contraction and include urea, guanidine, acids and alkalis, and heat. The mode of action of some of these reagents will be described.[I I]

The rate of thermal denaturation is noted for its

high/

high temperature coefficient compared to chemical processes where Q_{10} is about 2-3 over a temperature range of 70-100°C Q_{10} for heat denaturation is about 600 over an interval of 2-3°C[35]. At any temperature the rate of denaturation is a function of pH. The minimum rate is at a pH between the isoelectric point and neutrality whilst the apparent activation energy for denaturation in acid media is at a minimum[36]. The presence of water facilitates thermal denaturation[41]. It is thought that, kinetically, denaturation occurs as a series of steps and is not an 'all or none' phenomenon[36]. Extrapolating the results of metal alloy systems and organic high polymers to protein molecules, the unusual features of denaturation, such as high temperature coefficient, effect of pH value, and amount of water present, as well as the progressive nature of the process, can be explained by regarding thermal denaturation as an order-disorder transition in a high polymer[42]. It is concluded that in the shrinkage of collagen the polypeptide chains abandon the ordered arrangement for the greater configurational freedom offered by the random coiled state[X X].

Prolonged treatment with boiling water results in dissolution and splitting of peptide bonds of collagen and the formation of gelatin (M.W. ranges from 60.10^3 to 250.10^3)[43]. Solutions of gelatin are amorphous and do not exhibit the long range structure of collagen[16]. They set to a solid gel on cooling, and unlike collagen, are not resistant to the action of trypsin. Gel formation is inhibited by small amounts of

urea/

urea or arginine[44]. The effect of these substances is eliminated by the addition of potassium iodide.

Reactivity of the side and terminal groups of skin Collagen:

It is possible to inactivate or remove side chain protein groups of bovine collagen without a concomitant general breakdown of the protein[45]. Application of these methods to marine collagens, however, usually causes extensive degradation of the protein[33].

Much of what is known of the ionic reactivity of collagen has been obtained by noting the effect that blocking or removing the ionic and polar side chain groups has had on the properties of the protein. Several attempts to detect N-terminal amino groups and C-terminal carboxyl groups in the native collagen have given negative results.[46,47] These groups are rendered undetectable, presumably by being:

a) blocked by esterification or reaction with other tissue components
b) sterically unavailable to the reagent used for their estimation
c) present in extremely minute amounts.

Cationic groups:

The ε - amino groups of lysine and hydroxylysine, and terminal amino groups in the intact protein can be blocked by treatment with dinitrofluorobenzene, DNFB, in the presence

of/

of 1% sodium bicarbonate[48] to form an acid stable bond.

The mild and non degradative conditions required for the reaction of DNFB with both soluble and insoluble proteins has made this reagent very suitable for investigating the structure of collagen.

The amino groups of lysine and hydroxylysine in collagen can be effectively blocked by treatment with acetic anhydride in the presence of sodium acetate (pH=8.0)[49]. Other well known acetylating reagents are ketene ($CH_2=C=O$)[50] and carbon suboxide (C_3O_2)[51].

About 85% of the ϵ-amino groups of lysine can be converted to -OH groups by diazotization with nitrous acid[52], under special conditions. However, the interference by some of the guanidyl groups of arginine in this reaction often makes interpretation of the analytical data difficult[53]:-

$$P^{\bullet}NH_2 + HONO \longrightarrow P^{\bullet}OH + N_2 + H_2O$$

$$-H_2C-NH-C(=NH)-NH_2 \xrightarrow{HONO} -CH_2{}^{\bullet}NH{}^{\bullet}CN + NH_3$$

The strongly basic guanidyl group of collagen is rather inert, the probable reasons being its function as the site of strong crosslinks, its interlinking with other protein groups and its resonance[54] (Kxxx). This is borne out by the fact that extremely high pH values are required for the titration of the guanidyl group. Furthermore, in the Sakaguchi reaction[55],

which/

which involves treatment with hypochlorite (Fixxx) in alkaline solution, less than half of the arginine residues in collagen are attacked (54).

The above methods for the estimation of cationic groups in proteins entail the use of rather severe and often degradative treatments in order to ensure complete reaction. As the author is primarily interested in the properties of collagen as near the native state as possible, the mild conditions of the DNFB method made this technique the most suitable for the study of collagen.

Anionic groups:

Inactivation of the carboxyl groups, belonging to glutamic and aspartic acids, is carried out by esterification using dimethyl sulphate, diazomethane, methyl iodide and bromide and 1,2-epoxides(45). These reagents, however, require a large number of consecutive treatments for complete reaction which results in considerable degradation of the collagen(53). These reagents also react with guanidyl and lysyl residues. A more selective method of esterification is by methylation of the carboxyl groups using methanol made 0.1N with respect to hydrochloric acid(45). 80-90% of the carboxyl groups were methylated with negligible effects on other groups. Methylated collagen has a great tendency to osmotic or lyotropic swelling thus indicating that a large proportion of the carboxyl groups contribute towards hydrogen bonding.

Reaction/

Reaction with polyacids, such as lignosulphonic acid, certain dye stuffs and polymetaphosphoric acids, indicates that only 10% of the carboxyl groups of collagen are available for the hydrogen ions of the polyacid, whereas the polyvalent anion is fixed by all the cationic protein groups[56]. Epoxides and bifunctional epoxides have been used to esterify and cross-link the carboxyl groups of collagen[57].

Polar groups (non ionic):

The free hydroxy groups of collagen belong to the residues of hydroxyproline, threonine, serine and hydroxylysine. The hydroxy group can be selectively esterified by treatment of the protein with concentrated sulphuric acid at low temperature.[58] All the amino groups and 80% of the hydroxy groups can be esterified by acetylation with a mixture of acetic anhydride and acetic acid[59].

The amide group:

This group is relatively inert and does not react with reagents for free amino groups[48]. It does, however, take part in the formation of crosslinks between amino and amide groups induced by formaldehyde[45]. The amide group is very easily hydrolysed to a free carboxy group, ammonia being evolved. Protein amide nitrogen is usually determined by distillation and titration of the ammonia liberated during a short period of hydrolysis (2 hours) with weak mineral acid (1N). In this method some peptide bonds are unavoidably split[6]

Table 3: Some peptides obtained from the partial hydrolysis of collagen (60, 100)

Dipeptides:	Val. Gly., Glu. Gly., Ala. Gly., Thr. Gly., Hypro. Gly., Gly. Gly., Gly. Asp., Gly. Glu., Gly. Pro., Gly. Ala., Glu. Ala., Leu. Ala., Thr. Ala., Glu. Glu.
Tripeptides:	Ala. Ala. Gly., Ala. Gly. Ala., Lys. Pro. Gly.

Note: The N-terminal residue of each peptide is written first.

b) The sequence of amino acids.

The cross banding of the fibrils suggests that repeating units coincide in the aligned polypeptide chains implying a certain sequential order in the types of residues.[1] From the data on the amino acid composition of mammalian collag the frequencies of the principal residues are:- glycine 2.95, proline 8.2, hydroxyproline 9.6, alamine 10.2, arginine 21.3, lysine 33.5 (i.e. every third residue is glycine, every 8th is proline, and so on.)

Some idea of the more important sequences can be obtained by isolation and analysis of peptides obtained by the partial hydrolysis of collagen. Some of the di and tripeptides that have been obtained in this way are listed in Table 3 [60]. Recent work shows that gly-pro-hypro is a common sequence in collagen and 'fits' into one of the molecular models proposed for collagen[61].

It seems permissable to conclude that the amino acid residues are apparently laid down according to some definite principle, although it is possible that glycine and valine can replace each other. Since collagens, from widely differing sources differ in amino acid composition, it is possible that some amino acids form a more permanent framework into which other members can be built according to the biological requirements. These hypotheses can only be supported or rejected when more information on partial hydrolysates becomes available.

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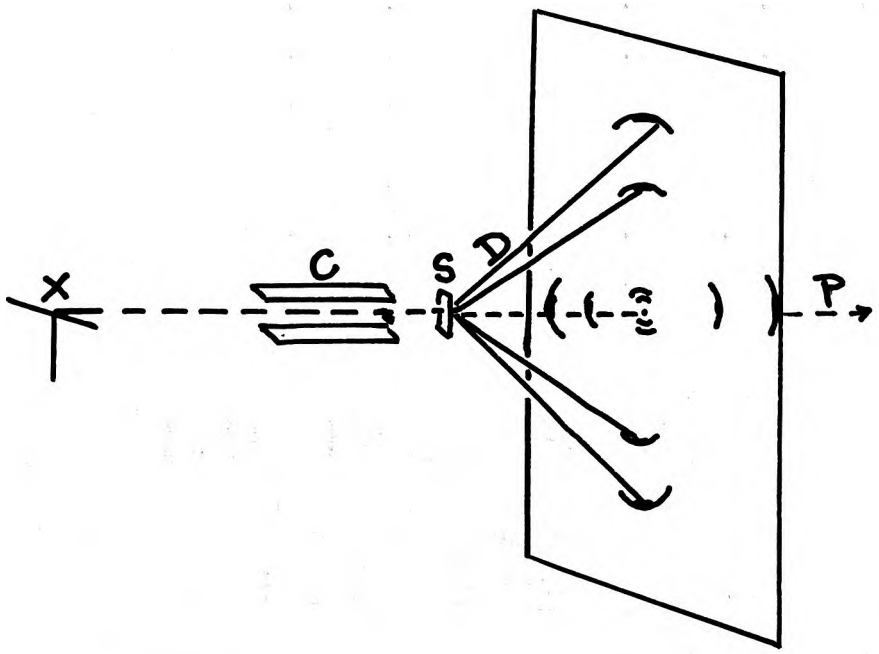

Fig. 6: Schematic diagram of X-ray Diffraction method.

X denotes X-ray source
C " Collimater
S " Specimen
D " Diffracted ray
P " Primary beam.

The molecular configuration of the intercellular proteins.

The use of the optical and electronmicroscope and the X-ray diffractometer, has led to a conception of the architecture of collagen molecules which is in accord with most of its chemical and physical properties. An outline of this work is given below.

If an X-ray beam is passed through a material in which the atoms are arranged in fixed positions, the emergent beam will produce a diffraction pattern on a suitably placed photographic plate[16] (Figs. 6). Materials of a high degree of crystallinity give a sharply defined pattern. The diagram for collagen has the form of a series of rings and arcs ( ). By stretching the fibre by about 10% of its length (β form) much sharper diagrams are obtained. By measuring wide angle diffraction, structural details up to 20Å are revealed. Small angle diffractograms cover the size range of 20-1000Å which overlaps that of the electronmicroscope.

The electron microscope, by making use of the tiny wavelength associated with an electron, can resolve particles whose size is well below the wavelength of visible light[1 2]. The electron beam, which is focussed by means of magnetic fields, passes through the dehydrated specimen in an evacuated chamber, and casts an image on a fluorescent screen or photographic plate placed below. To increase the scattering power of thin histological sections, they can be coated or 'shadowed' with a thin layer of a heavy metal such as gold or platinum.

Apart/

Fig. 7: Structural elements in Collagen fibres.

Apart from the advantage of the high magnification (upto 100,000 diameters) shadowed electronmicrographs have a 3 dimensional appearance, which is of considerable help in the visualisation of small structures.

Fig.7 indicates the major structural elements found in collagen. The '<u>fibres</u>' are microscopically visible aggregates having diameters of 100 to 200 μ in tendon, and 20 - 40 μ in skin. According to Bear[16] these may be divided into '<u>fibrils</u>' having diameters of a few hundred Angstroms and can be resolved only be the electronmicroscope. After special treatment, the fibrils may be frayed into smaller elements called '<u>filaments</u>' which consist of still finer hypothetical '<u>protofibrils</u>'. The protofibril (diameter = 12 - 17 Å) contains the essential chemical and configurational structure of collagen, and consists of one or two polypeptide chains.

The fibrils exhibit banded regions ( ) which are disc-like enlargemtnts of fibrillar diameter with greater than average power to deflect or absorb electrons and to take up electron stains. The presence of these periodically repeating cross striations, 640Å apart, was predicted from the low angle diffractograms[16] ( ) of collagen fibrils. By careful staining the bands can be shown to possess a fine polarised structure of up to 10 sub-bands. It is thought that the bands are regions of relative disorder and imperfect packing of the intrinsically more dense residues. The apparently continuous cross striations seen in most fibrils indicate that

the/

the protofibrils may be aligned with their corresponding chemical features normal to the fibril axis. The fibrils often occur in parallel bundles with their band levels in register. X-ray evidence of the uptake of basic and acidic stains at the banded regions, suggest that the bands contain the side chains of lysine, hydroxylysine, arginine, histidine and aspartic and glutamic acids, in relatively high concentration. The inaccessibility of the hydroxy polar group to chemical attack by stains and tanning reagents, tends to place this group in the resistant interband region, together with the non polar residues such as glycine, alanine and leucine. These bands are also of significance in the ossification of collagen fibrils, because the mineral material tends to aggregate around the banded regions with relatively small amounts present at the interbands. There is evidence which suggests that the fibrils in bone are hollow[62], and filled with mineral material.

In addition to the protofibrils of indefinite length, a kinetic unit building block of collagen called '<u>tropocollagen</u>' has been observed in acid solutions of ichthyocol[63]. The unit was a stiff rod 2000Å long and 14Å in width[64]. The particles were monodisperse, having a molecular weight of 340,000[X] [4]. It is suggested that these particles can be oriented 'in vitro' to give many different structural forms of collagen, some of which are not found in nature, eg. fibrous long spacing (FLS) and segment long spacing (SLS). Certain collagens may be rendered soluble at acid, neutral, and slightly

alkaline/

alkaline pH without s vere degradation of the molecule (63) and then reconstructed into either the normal 640Å fibril, or the other types of related fibres depending upon the conditions of precipitation such as pH value of the solution, the salt used and its molarity. For instance, an acetic acid solution of collagen may be precipitated as normal 640Å fibres by making the solution 0.02 to 0.2M with respect to NaCl. [illegible]. If the salt concentration is increased to 0.35M fibres having a 220Å periodicity are formed (resembling embryonic connective tissue fibrils). When the salt concentration is 0.5M, non-striated fibrils are precipitated [illegible].

Fibres with long spacings (FLS) of 2400Å having a symmetrical intraperiod structure can be obtained simply by dialysis of citrate extracts of collagen [illegible]. Similar fibrils are formed from acetic acid solution to which [illegible] glycoprotein is added followed by dialysis. If smaller amounts of glycoprotein are used, long spacing as well as normal 640Å spacing can be seen in a single fibril. Still smaller amounts of glycoprotein yield the 640Å fibrils only. The FLS fibril can be converted into the normal 640Å fibril by dissolving in acid and dialysing against saline. This formation of the FLS fibrils has been observed under the electronmicroscope:- During dialysis of acid solutions of ichthyocol, deeply stained areas in approximately hexagonal array were seen to extend [illegible] thin fibrous processes which condensed into clouds of dense spheriods and tactiods 3000Å x 100Å. The tactoids dissolved and the FLS

fibrils/

fibrils appeared after 30 minutes. Condensation from large clouds is not always observed. Sometimes spheroids of 100 to 400Å in diameter develop into thin segments (2400 x 100Å) which aggregate laterally and lengthwise to form FLS fibrils. Other nonspecific inducing agents for the formation of FLS fibrils include thrombin, tyrosinase, collagenase (inactivated), chondroitin sulphate, sulphated dextrin, gum arabic.

The third type of fibril, viz: segmented long spacing (SLS), can be obtained from neutral phosphate extracts or by the addition of adenosine triphosphate (ATP) to acid solutions of ichthyocol. In this type of fibril the tactoids aggregate laterally to form broad flat segments of characteristic polarised fine structures each containing 18 intraperiod bands with a total length of 2000Å. Inosine triphosphate is also active in the formation of these fibrils.

Since a host of seemingly unrelated substances are capable of producing the 3 types of precipitated collagen fibrils, it is likely that the specificity resides in the 'tropocollagen' particle, and not in the precipitating reagent. An hypothesis has been put forward (63) that 'tropocollagen' is synthesised by connective tissue cells and is subsequently converted into its characteristic fibrous form by the ground substance. Fig. 8 summarises these views.

Molecular structure of collagen.

The detailed structure and stereochemical configuration of the polypeptide chains of which the collagen fibres are composed has been investigated by means of wide angle X-ray diffractograms (16). The diffraction pattern of β - collagen is well-known, and is quite characteristic of this class of fibrous proteins (Xixx). The main features are a strong meridional arc at about 2,86Å, and weaker arcs on the meridian at 4.0Å and 9.5Å; an equatorial reflection at 11.4Å, the second order of this reflection, and a diffuse arc at about 4.4Å. Other reflections are also present. This pattern is usually considered to be due to an arrangement of polypeptide chains lying roughly parallel to the fibre axis. If the fibre is made to swell, there is an increase in the equatorial spacing from 10.4Å in the very dry state, to about 16Å with comparatively little effect on the rest of the pattern (65). The infra red spectrum of collagen is characterised by the perpendicular dichroism of the N-H and C=O stretching frequencies (66), and a high value for the π N-H stretching frequency. This higher frequency may be associated with the presence of weak hydrogen bonds. The dichroism shows that N-H and C=O bonds are roughly perpendicular to the fibre axis. The relatively large amount of prolines present in collagen (XXXXX(53) impose stereochemical restrictions on the configuration of the N atom and the adjacent α C atom. In all models so far proposed, the peptide

linkage/

linkage is made planar as found in most dipeptides. The various structures that have been proposed for collagen fall into two main groups:-

1) Sheet structures of parallel chains (67) about 4.5Å apart, hydrogen bonded together in layers with a space of 10.4Å between the layers in dry collagen. Lateral swelling causes separation of the layers to 16Å (Xxxx). However, the density calculated for this model is low (normal for dry gelatin = 1.35g./cm^3).

2) Helical structures (68) packed perpendicular to their length in hexagonal array with a=12.5Å. These models give better density values but account less well for the swelling properties of collagen.

The first configuration for the collagen protein chain was a sheet structure put forward by Astbury (67)(Xxx). In this, the proline and glycine residues adjoin one another on the same side of the chain. The model is not in accord with the more recent amino acid analysis of collagen and should give rise to a fibre period of 8.6Å, a spacing which is not observed on the meridian. Furthermore, the nitrogen atoms in the model are pyramidal and not planar.

A second sheet structure (Xxxx), proposed by Huggins (68), does not readily accomodate proline residues, and the peptide links are non planar.

Randall (69) has put forward a model which has planar peptide links and accounts for the infra red dichroism. The

prolines/

prolines, however, cannot be accomodated without distortion of the sheet.

A Y -helical structure (Xxxx) was proposed by Pauling and Corey in 1951 (70). It suffered from the disadvantage of having two cis and one trans peptide link, but there is no evidence for cis residues in collagen. Further, the 3 residue repeating unit of 8.6Å in axial length is absent from the X-ray diagram. The chains are held together by hydrogen bonds which are fairly perpendicular to the chain direction and thus in agreement with infra-red data (66). A modified form of this helix was put forward by Bear, in which the residue axial length is 0.95Å and a 3 residue repeat then corresponds to the 2.86Å reflection (16).

In 1952 Cochran, Crick and Vand worked out the Fourier transforms for helical structures. It then become recognised that the collagen diagram bears definite characteristics of a helical structure (71). According to Bear (16) the X-ray pattern is now interpreted as being due to ten 'scattering groups', each group being a total of 3 or sometimes 4 amino acid residues forming 3 turns of a genetic helix. The axial projection of this 3-turn unit is 28.6Å in relaxed material, and about 30Å in the stretched condition ((Xxxx).

However, while a helical polypeptide chain configuration is definitely indicated for collagen, no completely detailed model has been recommended as yet (72).

Extracted/

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Extracted collagens.

The skin collagen of young animals can be fractionated by extraction with phosphate and citrate buffers into alkali soluble and acid soluble fractions (73). When α ^{14}C glycine was administered to rabbits, it was found that the level of radioactivity after one day was highest in the alkali-soluble citrate extracted skin collagen (E.S. collagen) dropping sharply again at the end of 3 days. These findings suggest that the alkali-soluble collagen is a 'procollagen' which 'turns over' rapidly to form the relatively inert mature collagen. Light scattering studies show that these extracted collagens are in solution in the form of macromolecules about 150Å long with a diameter of 11Å (74). Investigations of the electrophoretic behaviour of rat tail tendon collagen dissolved in acetic acid, as well as that of E.S. collagen, suggests that both materials consist of a single component of isoelectric pH=5.8. In the presence of high salt concentrations, there is evidence that E.S. collagen is an inhomogeneous mixture, indicating that side to side aggregation of molecules has taken place. It is noted that the extracted skin collagens have virtually the same amino acid composition as the native material (75).

The effect of enzymes on collagen.

Native collagen is attacked by pepsin and the proteolytic enzymes of Cl.histolyticum and Cl.welchii. Unlike

gelatin/

Table 4: Classification of Proteolytic Enzymes:

Enzyme	Requisite Main chain Group	Side chain Specificity
Carboxypeptidase	-CONHCHRCOOH	$C_6H_5CH_2$- $OH-C_6H_4-CH_2$
Intestinal Aminopeptidase	$H_2NCHRCONH$	$CH_3 \diagdown \diagup CH-CH_2$ CH_3
Pepsin and Cathepsin I	-NHCHRCONHCHRCO	$C_6H_5-CH_2$- $OH-C_6H_4-CH_2$
Trypsin and Cathepsin II	-CONHCHRCONH-	$NH_2-(CH_2)_4$- $NH_2 \diagdown C-NH(CH_2)_2$ $NH \diagup\!\!/$
Chymotrypsin	-CONHCHRCONH	$C_6H_5-CH_2$ $HO-C_6H_4-CH_2$

gelatin, collagen, in its native state, is resistant to the action of trypsin, chymotrypsin or papain (76). Denaturation of the collagen, produced by heat or swelling agents, leads to a marked increase in susceptibility to all forms of enzymatic attack ~~(~~ ~~)~~. Bergman's classification of proteolytic enzymes in which both the main chain and side chain groups acted on by the enzyme is shown in Table 4 (77). Thus, for example, trypsin tends to degrade polypeptides into fragments having C-terminal lysine or arginine. Dentin is appreciably attacked by the bacterial collagenases only after some decalcification had taken place (78). In assessing the resistance of collagens to enzymatic attack it is essential to use purified enzymes, on collagen in as near the native state, as possible. There is some evidence which suggests that trypsin treatment of native collagen results in the hydrolytic solution of some small polypeptides rich in hydroxyproline (79).

The Birefringence of collagen fibres.

The study of the anisotropy of collagen preceded the modern methods of X-ray diffraction and electronoptics, and provided early evidence for the lttice structure of the fibres and the changes undergone during swelling and contraction (16).

The double refraction of collagen fibres is normally positive in the direction of the fibre axis, indicating the presence of rodlets or chains alligned in parallel in this direction (16). Thus, the intrinsic double refraction is due to

the/

the anisotropic property of the alligned units. By the incorporation of phenols, aromatic aldehydes and certain vegetable tannins, the sign of the double refraction in the dry state can be reversed, suggesting that the tanning agents are oriented with respect to the collagen fibril (80).

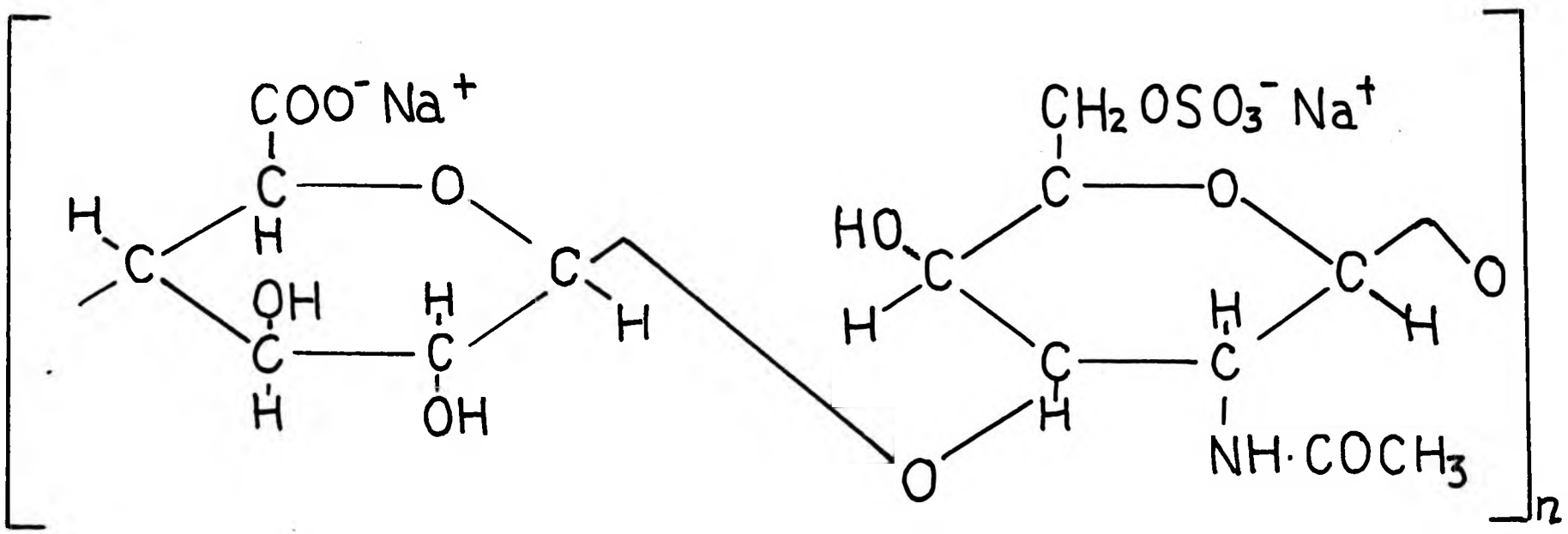

FIG. 9. CHONDROITIN SULPHATE.

B. Chemical and Physical Properties of the 'amorphous' ground substance.

A mucoid ground substance is present in all connective tissue (81). Large amounts,(up to 40%) are present in hyaline and elastic cartilage. Smaller amounts,(about 1%), are present in loose connective tissue, whilst calcified tissues contain quantities less than 1%. It has been shown (82) that there are at least 5 types of mucopolysaccharide ground substances in connective tissue, viz: hyaluronic acid and chondroitin which are both non sulphated, and chondroitin sulphates A, B, and C. Chondroitin sulphate B is absent in cartilage and bone but occurs in skin. All types of chondroitin sulphate are easily degraded to low molecular weight products by alkali, or enzymatically by treatment with testicular hyaluronidase.

The chondroitin sulphates are distinguished from each other on the basis of their composition, solubilities, optical rotation, digestion with hyaluronidase and their color reactions with carbinol and orcinol (82). Chondroitin sulphate from mammalian cartilage is an unbranched polysaccharide polymer with a 'mer' consisting of glucuronic acid and galactosamine (M.Ws. of 16 to 43.10^3 have been reported)(83). The amino group of galactosamine is acetylated and the 6 position is sulphated as indicated in Fig. 9 . The molecular configuration of the molecule of chondroitin sulphate is dependent upon the ionic environment (80). In pure water the specific viscosity does

not/

not decrease linearly to a limiting value with decrease in concentration as is the case with uncharged polymers. Instead, the specific viscosity increased sharply with dilution (84). This was explained by assuming that the hydrodynamic shape of the polyion was a tightly coiled sphere (or near sphere) which changed to the equivalent of a loose flexible coil following an increase in intramolecular coulomb repulsion due to increased ionisation on dilution. Viscosity studies (84) have shown that chondroitin sulphate can bind and exchange cations with a concomitant change in the configuration of the (CSA) molecule. Chondroitin sulphate reacts with both collagen (85) and calcium salts, and may thus play a specific role as a local factor (86) in the calcifying mechanism of connective tissue. The mucopolysaccharide pattern of growing bone differs from cartilage in that bone possesses hyaluronic acid and 'keratosulphate' which are both absent in hyaline cartilage (82). Chondroitin sulphate, as well as other high molecular weight anionic polyions, is a chromotrope which can induce a metachromatic color in a dye capable of undergoing this change eg. Toluidine blue O. This criterion is used for the histochemical 'identification' of the chondroitin sulphates (87).

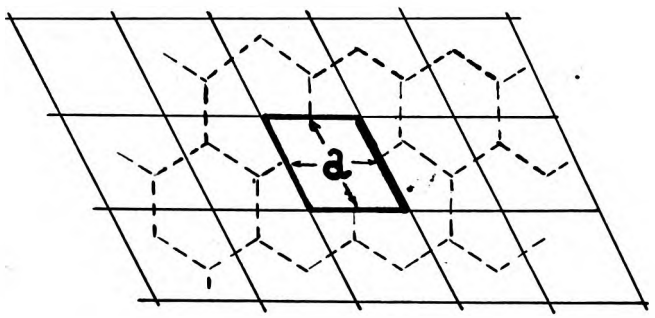

Fig. 10: Internal lattice structure of bone salt.

C. Properties of the mineral material of calcified tissues.

The hard tissues of the body are characterised by their high content of extracellular mineral material mainly in the form of lime salts (XXXXX(91) Appreciable amounts of citrate and sodium ions as well as smaller quantities of chloride ions are also present (88). X-ray diffraction methods show these lime salts to be crystalline members related to the apatite series of minerals (89), and their chemical composition may be represented by the formula $Ca_{10}(PO_4)_6(OH)_2$. An alternative structure,(not generally accepted), is that of hydrated tricalcium phosphate - $3Ca_3(PO_4)_2 \cdot H_2(OH)_2$.(90) The hydroxyapatite present in bone, dentin and enamel is in the form of minute hexagonal crystals (Xxxx) composed of hundreds of unit cells (91)(Fig.10).

The 'unit cell' of a crystal is the simplest two dimensional conceptual arrangement of all the atoms in the same ratios and spatial relationships in which they are present in the crystal. The unit cell structure can be extended throughout the structure to form the three-dimensional 'crystal lattice'. The crystal lattice of hydroxyapatite may be described in two ways (89, 91) :-

a) In a cross section perpendicular to the long axis it is seen to consist of a series of contiguous hexagons (Fig.10). A Ca^{++} ion, surrounded by $PO_4^{\equiv}$ ions is present at each

intersection/

intersection. At the centre of the hexagon there is an OH^- ion shared by 3 Ca^{++} ions.

b) In the same cross-section, the points in space occupied by the OH^- ions are joined to form a series of diamond shaped parallelograms (unit cells) with angles of 120° and 60° at the intersections. The four equal sides of the parallelogram are 9.4Å in length (the 'a' axes). When extended in a third dimension ('c' axis) at right angles to the a-axes, the unit cell forms a parallelpipidon or 6-sided right prism, four of whose faces are rectangles with 2 faces as parallelograms. The c-axis measures 6.9Å.

The crystals of bone salt are roughly hexagonal in shape with the c-axis of the unit cells oriented parallel to the long direction of the fibre. In mature bone, the crystals are 25-30Å thick, approximately 400Å long and nearly as wide (89). The growth of the crystals from particles less than 50Å, in the infant, to crystals of 1500Å by 500Å by about 100Å, in senile bone, has been observed (62). The crystals in enamel are larger and more perfectly formed than those in bone and dentin (92).

From electronoptic studies it is reported (62) that the crystals form a periodic pattern around the major bands of

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the collagen fibres with relatively small amounts in the inter-band regions. X-ray diffraction studies show that collagen fibres 'in vitro' can orientate hydroxyapatite crystals. Chemical evidence for the association of certain side chains of some of the basic amino acids of collagen with the mineral material will be presented later (P. 110). As the crystals of bone salt are only a few unit cells thick, its surface area is enormous, being about $100m^2/g$. One half to two thirds of the unit cells are located on the surface of the crystal and large numbers of Ca^{++}, $PO_4^{\equiv}$ and OH^- ions are free to take part in adsorption and ion exchange reactions with the surrounding fluids (85). Exchange studies indicate that 20% of the P and 33% of the Ca in bone is on the surface of crystals.

The bone-crystal can be pictured (89) as consisting of "a surface hydration shell containing non specific boundary anions in rapid equilibrium with the surrounding medium". The next interior unit cells contain more specific cations and anions, also in equilibrium with the solution or the hydration shell. The innermost core consists of hydroxyapatite, whose ions are slowly 'recrystallising' in order to preserve an equilibrium with the outer layers. Recrystallisation occurs much faster in newly deposited bone than in old bone.

Isionic exchange has been demonstrated 'in vitro' in bone, enamel and dentin by using radioactive isotopes (93). The final distribution of the isotope was dependant on crystal size, temperature, pH and other factors. It should be borne in

mind/

mind that procedures for the removal of the organic phase, which involve heating or ashing of the tissue, usually cause an increase in the size of the crystals, resulting in less surface area per unit weight of the mineral fraction being available for exchange reactions. In heterionic exchange, an ion in the crystal lattice is reversibly displaced by a different ion from solution. Isomorphic substitution of H_3O^+ for Ca^{++} explains the observed inconstancy of the Ca/P ratio in hard tissues which ranges from 1.4 - 1.8 (89), the internal lattice structure being unaffected. Thus strontium sodium and hydronium ions will displace surface Ca^{++}, and carbonate can displace surface phosphate. Whilst flouride ions can take the place of OH^-. The reaction of fluoride is not completely reversible (94), and at low pH is not limited to the surface. Bone 'deorganicised' by glycol ashing, exhibited a greater percentage of exchangable phosphorus than did powdered fresh bone under identical conditions (95). The citrate and carbonate present is generally thought to exist partly adsorbed on free or entrapped surfaces and in the hydration layer (89).

The relative amounts of Ca, PO_4 and CO_3 in bone is dependent (within limits) on the Ca/P ratio of the diet consumed (86), and the P/CO_2 ratio of the blood serum during osteogenesis. High Ca/P ratio diets caused higher P/CO_2 ratios in serum and higher CO_3/PO_4 bone ratios than in animals on low Ca/P ratio diets (86). It has also been postulated that the low Ca/P ratios in young bone may be due to $PO_4^{\equiv}$ ions

attaching/

attaching themselves to the preosseous organic matrix (96).

Due to isomorphic substitutions, hydroxyapatite is a crystal lattice in which the composition varies from part to part of the crystal, and thus its equilibrium solubility cannot be described by a single classical solubility product (97). Attempts to determine such a solubility product have resulted in many numbers called 'constants' but which vary by 11 log units or more (89).

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Chapter IV.

Combination of Mineral Material with the Organic Matrix of Hard Tissues.

'In vivo' calcification of connective tissue is generally thought to be the result of a physical-chemical combination between lime salts and the organic matrix, mediated by the ionic and structural properties of the matrix and the activity of various tissue cells and enzymes.

The equilibrium that exists between the mineral phase and the calcium and phosphate ions in the extracellular fluid has been empirically described by Howland and Kramer (1) who showed that the presence or absence of rickets could be correlated with the product of total $[Ca] \times [P]$ each expressed as mg. per 100 ml. of plasma. When the product was less than 30, rickets was present. When the product was 40 or more, rickets was absent. These findings, which have been related to the ion product $[Ca^{++}]\,[HPO_4^{=}]$ indicate a relationship between calcification and the solubility product of $CaHPO_4$. Neuman has pointed out (2) that the normal ion product of serum (about 50) is far less than the solubility product of $CaHPO_4$, and that the process of calcification is, therefore, not one of precipitation. Furthermore, all attempts to detect $CaHPO_4$ in osseous tissues have been unsuccessful. The Ksp of $CaHPO_4$

is/

is now recognised (3) as an upper limit below which crystallisation occurs and above which precipitation will take place. Neuman & Neuman (2) have suggested that "the organic phase of osteoid or endochondral cartilage may bind either calcium or phosphate ions in the proper space relationships of the apatite lattice," thus initiating crystallisation by a 'seeding process' in which the organic phase acts as a template.

In early work (4) it was thought that the enzyme alkaline phosphatase found in bone and other tissues, acted on a suitable substrate of organic phosphate esters and caused the solubility product of calcium phosphate to be exceeded, with resultant precipitation of this salt during the process of calcification. This theory was found to be unsatisfactory as calcification of bone does not occur by a process of precipitation, but rather by one of crystallisation (2). Suitable substrates for the enzyme are not plentiful, and those in blood are in the corpuscles. Furthermore, phosphatase is found in many other tissues which do not normally calcify. In relation to the action of alkaline phosphatase, the author has adopted the opinion expressed by Irving (5) that "phosphatase is concerned, certainly in bone and probably elsewhere in the body, with matrix formation, and its association with calcification in bone is probably incidental".

Other roles have been assigned to phosphate. Thus it was found (6) that calcification 'in vitro' was inhibited

by/

by phosphoric esters and that this inhibitroy action could be destroyed by phosphatase. After the enzymes of bones have been destroyed, 'in vitro' calcification will not take place in media containing organic sources of P, but only if the P is inorganic. (7).

The fact that certain procedures stopped calcification of hypertrophic cartilage 'in vitro', but had no effect upon phosphatase activity, (7) led to the postulation of a second mechanism. The researches into this mechanism have taken two courses, one into the enzyme aspect, and the other, a search for a specific calcifiable substance in epiphyseal cartilage.

It was found (8) that cartilage cells showing the most rapid formation of calcifiable matrix contained large amounts of glycogen and phosphorylase. However, calcification 'in vitro' can take place in the absence of all enzymes (7), thus if the glycolytic enzyme system does play a part in calcification, it may be concerned with moving phosphate ions against concentration gradients or through cell membranes.

The combination of Ca with a specific calcifiable substance in cartilage, has been postulated. There is evidence (9) which shows that PO_4 is taken up by/a mucopolysaccharide, probably chondroitin sulphate, which already contained some Ca. The uptake of calcium from inorganic sources can be inhibited by the exposure of cartilage to metachromatic and other basic

dyes/

dyes, but these dyes did not stop calcification from organic sources if Ca and phosphate were both present.(10) Chondroitin sulphate cannot be the only factor responsible for the formation of a normal calcifiable matrix, because articular cartilage, which contains up to 20% mucopolysaccharides, should then be highly mineralised. The fact that treatment with dyes does not inhibit calcification from organic media, suggests that "there are differences between calcification from inorganic and organic sources, or that calcification caused by the glycolytic cycle does not involve chondroitin sulphate." (5).

The observations and theories cited above pertain only to the 'in vitro' calcification of rachitic cartilage. It has not proved possible to cause rachitic osteoid to calcify 'in vitro' (11) although a complex of collagen and chondroitin sulphate (12) took up 30% by weight of lime salts which had an hydroxyapatite X-ray diffraction pattern (13).

The part played by collagen in calcification.

In bone and dentin strong evidence exists (14) for the participation of collagen fibres in the combination of lime salts to the protein matrix. Unlike cartilage, the amount of mucopolysaccharides in bone and teeth is so small (15) as to be completely overshadowed by the collagen fibres. Mineral material in bone is deposited, not only in the scanty ground substances in between collagen fibres, but within and on the fibres (16), being particularly dense at the banded regions of

the/

the submicroscopic fibrils (16).

X-ray diffraction studies 'in vitro' show rat tail tendon which was dipped into solutions from which hydroxyapatite crystals were forming, oriented the crystallites which were deposited on the fibres so that their C-axes were more or less parallel to the long axis of the collagen fibrils (17). Orientation of mineral crystals with respect to the collagen fibres has also been observed in bone and dentin (17). These results combined with electronoptic studies of ossifying osteoid (14) indicate beyond reasonable doubt that the protein phase regulates the deposition of some of the mineral material. In particular, the ionically reactive banded regions of the collagen fibril accumulate large amounts of mineral material with relatively small amounts in the interband regions, thus preserving the periodicity of the fibril (16). As both calcified and non calcified collagens exhibit similar electronoptic morphology, it is postulated that the calcifiability of hard tissues may be due to differences in the chemical properties of the collagen.

The remainder of this work concerns the experimental demonstration by the writer of chemical differences between mammalian collagens obtained from soft and hard tissues, and a relationship between the chemical properties of bone and dentin collagen and the mineralisation of these tissues. An attempt will be made to correlate these findings with the mechanism of ossification at the molecular level. In addition fish scale proteins obtained from the South African pilchard (occelata Jenyns) were investigated.

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Chapter V.

Experimental

This section deals, firstly with a description of the basis of some of the methods available for investigating the composition, structure and chemical reactivity of proteins. This is followed by the actual procedures used by the writer for the preparation, and subsequent investigation of various soft and hard tissues.

Note: DNFB denotes dinitrofluorobenzene.
DNP denotes dinitrophenyl.

1. The amino acid composition of proteins.

a) Chromatographic methods:

A wide variety of methods have been proposed for the qualitative and quantitative analysis of protein hydrolysates (1). For qualitative and semiquantitative work, the writer has used one dimentional and two dimentional paper chromatographic procedures for the analysis of acid hydrolysates of ox-hide and human dentin collagen. After separation, the amino acids were eluted from the paper and colorimetrically determined with ninhydrin.

Another method, which gave more accurate results, was to convert the free amino acids to their yellow DNP derivatives which were then separated on 'celite' columns, or paper

chromatograms/

chromatograms and colorimetrically determined. These methods, which are rapid in operation and require no special apparatus, gave results for some of the amino acids which compared favourably with those recorded in the literature.

The ion-exchange chromatographic elution method of Moore & Stein[2] was used in this work to determine the composition of the fish-scale proteins. By this method, a highly accurate and complete amino acid analysis (with the exception of tryptophan) can be performed in about one week, using 5 to 10 mg. protein. An aliquot of the protein hydrolysate is applied to a 0.9 x 100 cm. column of 'Dowex 50'. By the addition of appropiate buffer solutions at specified temperatures, each amino acid is eluted from the column at a different rate and its concentration colorimetrically determined by treating 1 ml. fractions of the eluate with ninhydrin.

b) Colorimetric and spectrophotometric methods:

Colorimetric methods were used for the estimation of cystine and cysteine (3). Tyrosine and tryptophan were determined in alkaline hydrolysates by ultra-violet absorption measurements (4).

2. Protein structure.

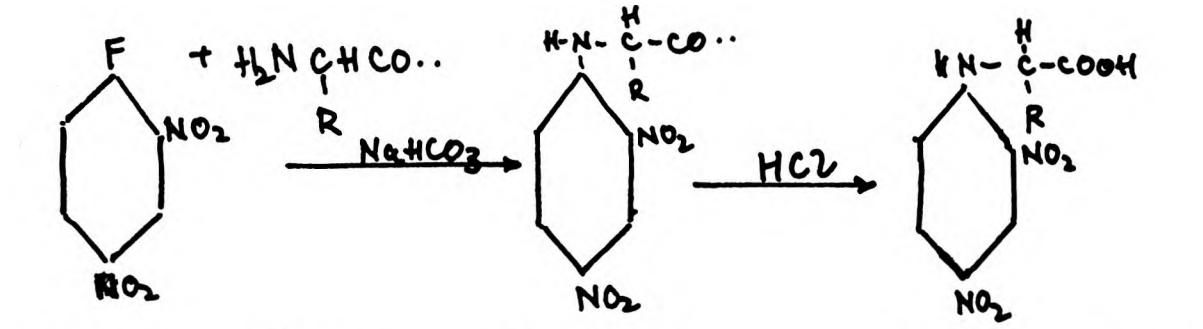
Numerous techniques are available for the purification and structural characterisation of soluble proteins and peptides eg. counter-current distribution, ultra centrifugation, light

scattering/

scattering measurements, osmotic pressure determination, electrophoresis and chromatography. Most of these methods are not directly applicable to the normally insoluble bovine collagen which requires fairly drastic conditions of temperature and pH to bring it into solution. Thus X-ray diffraction and electron-optic methods have been used to elucidate the structure of collagen in as near the native state as possible. In addition chemical methods are available for the determination of structural features of both soluble as well as insoluble proteins. These methods have been used to determine the type and concentration of N-terminal and C-terminal amino acid residues present at the ends of protein chains. By combining end-group analysis with partial hydrolysis, the complete structures of many peptides X X and that of the protein, insulin, as well as terminal sequences of many other proteins, have recently been determined (5). From the quantitative measurement of the terminal groups of a protein, its degree of homogeneity and molecular weight can also be deduced. Thus a protein, which is found to have only one N-terminal and one C-terminal amino acid present in equal amounts, is probably a homogenous, unbranched, polypeptide chain. If the concentration of either terminal residue is, say, one micromol. per g. of protein, then the molecular weight of the protein is 10^6 (provided cyclic side chains are absent.

Some of these methods for investigating the structure and chemical reactivity of proteins are described below:-

a)/



DNFB + PROTEIN → DNP PROTEIN → DNP AMINO ACID.

FIG. 1.

a) N-terminal methods:

i) Sanger's dinitrofluorobenzene (DNFB) method (Fig.1) has been successfully used to elucidate the structural features of numerous proteins and peptides both soluble and insoluble (5). The ease of quantitative reaction ~~X~~ ~~X~~ and the mild conditions employed, make DNFB a very useful reagent for the investigation of the structure of proteins in as near the native state as possible. The DNFB reagent also reacts with non-terminal free amino groups such as the ϵ-N-amino groups of lysine and hydroxylysine (but not those of arginine or the amide group) in the intact protein to form an acid-stable bond, and the extent of this reaction can be used to indicate the availability of these reactive, side-chain amino groups ~~XXXX~~.) to the reagent. DNP glycine and DNP proline are very unstable in acid, but methods of minimising errors due to this have been devised (6). Most of the DNP amino acids are not completely stable to acid, and correction factors have to be applied. The correction curve is determined by measuring the destruction of known amounts of the DNP amino acid, in the presence of DNP protein and HCl, kept at the temperature of hydrolysis for various lengths of time. Most of the DNP amino acids are bright yellow in color, having an absorption maximum at 360 mu. DNP proline has a maximum absorption at 390 mu. Many of the DNP amino acids can easily be separated by methods of paper and column chromatography (1). By this method quantitative amino

acid/

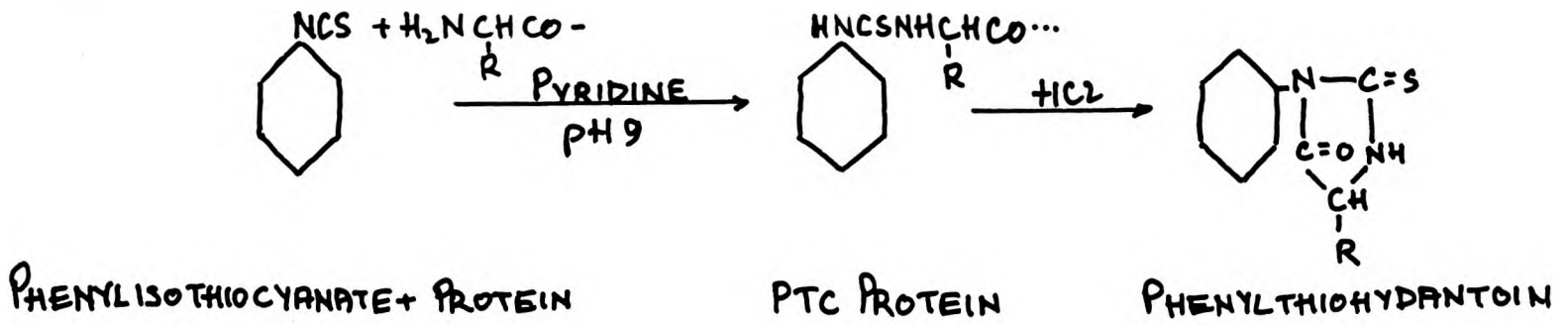
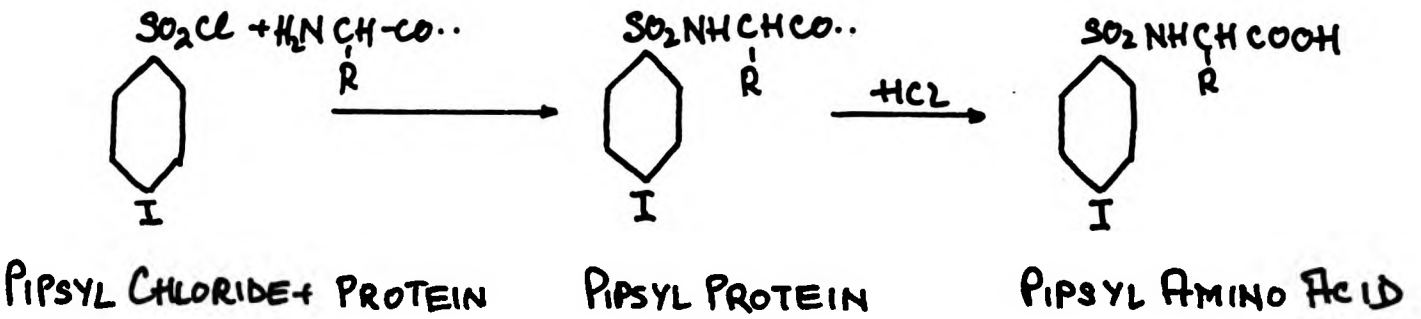

NCS + H2N CH CO -
R
PYRIDINE
pH 9
HNCSNHCHCO···
R
+HCl
N—C=S
C=O NH
CH
R
PHENYLISOTHIOCYANATE+ PROTEIN
PTC PROTEIN
PHENYLTHIOHYDANTOIN

FIG. 2.


SO2Cl + H2N CH-CO··
R
SO2NHCHCO··
R
+HCl
SO2 NHCH COOH
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I
I
PIPSYL CHLORIDE+ PROTEIN
PIPSYL PROTEIN
PIPSYL AMINO ACID

FIG. 3.

acid analysis of protein hydrolysates can also be performed (7). Dinitrophenol and dinitroaniline, which often appear as artefacts associated with the reaction, have distinctive solubility and chromatographic properties which permit of their easy identification.

The DNP method has been successfully applied to many different proteins including fibrous proteins, muscle proteins, protamines, hormones, enzymes, blood proteins, as well as egg, milk, and virus proteins.

ii) Another method for the determination of N-terminal amino acids in the phenylthiocarbamyl (PTC) method of Edman (8)(Fig.2). The initial coupling to protein takes place under more drastic conditions than in the DNFB method, but the hydantoins, which are finally formed, are very stable to acid and only mild conditions are required to liberate them from the protein chains. This method is thus suitable for the stepwise degradation of peptide chains.

iii) A third method, which has only been used on a few proteins, is the p-iodophenylsulphonyl method (pipsyl) X X of Undenfriend and Velick (9) (Fig.3). The use of radioactive iodine in the 'pipsyl' molecule makes possible the accurate estimation of the acid stable pipsyl derivatives.

The writer has chosen the DNFB technique for investigating the properties of ox-hide collagen, and has applied this method to fish scale proteins and to collagens derived from mineralised tissues.

b)/

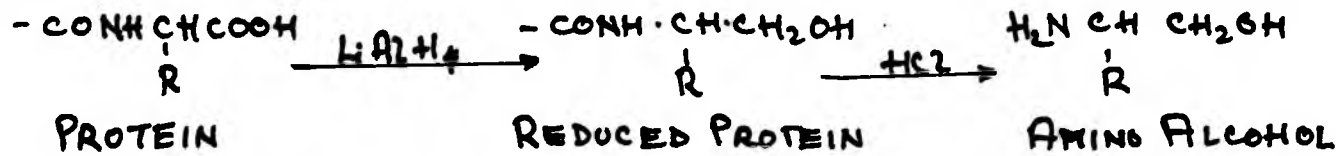

- CONH CH COOH
R
LiAlH4
- CONH · CH · CH2OH
R
HCl
H2N CH CH2OH
R
PROTEIN
REDUCED PROTEIN
AMINO ALCOHOL

FIG 4.

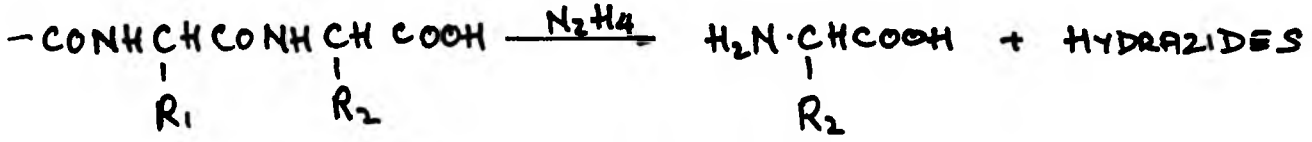

- CONH CH CONH CH COOH
R1
R2
N2H4
H2N · CHCOOH
R2
+ HYDRAZIDES

FIG. 5

b) C-terminal methods.

i) Carboxypeptidase: This enzyme splits those amino acids having a free carboxyl group and can be used for stepwise degradation from the C-terminal end. In most cases the results cannot be interpreted uniquely, but the probable C-terminal sequences of tropomyosin, actin and ribonuclease and other proteins have been demonstrated. The action of the enzyme may be blocked by a terminal or penultimate proline residue or may be very slow in the case of a terminal glycine group (10). It is difficult to decide whether the second amino acid to appear is the penultimate residue of a single polypeptide chain, or a second more slowly liberated end group. Serine, for example, is liberated more slowly than isoleucine.

ii) Reduction: By reduction with $LiAlH_4$ the free carboxyl groups are converted to hydroxyl groups, and the amino alcohols produced may be identified and estimated (11)(Fig.4). In the case of ovomucoid and lysozyme, the results agreed with those obtained by other methods. With insulin, however, non specific reductive cleavage of peptide bonds occurred, particularly that of gly-ser.

iii) Hydrazinolysis: The terminal carboxyl groups are liberated as free amino acids when the protein or peptide is treated with anhydrous hydrazine (12), the peptide bound carboxyl groups being converted to hydrazides (Fig. 5). The free amino acids liberated are then identified and estimated

by/

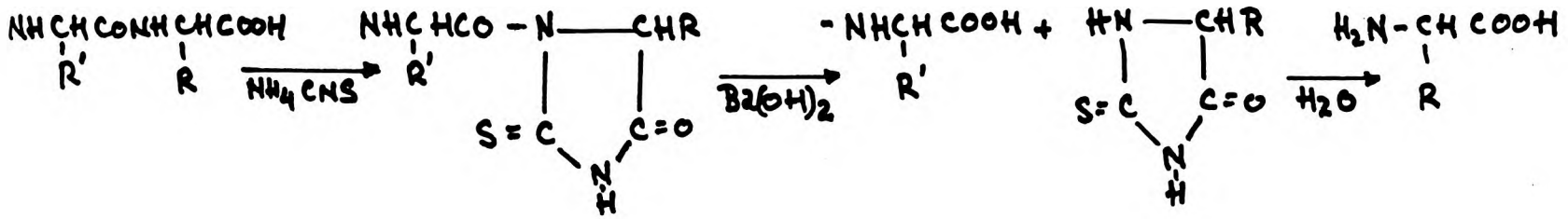

NHCHCONHCHCOOH
R'
R
NH4CNS
NHCHCO-N—CHR
R'
S=C
C=O
N
H
Ba(OH)2
-NHCHCOOH +
R'
HN—CHR
S=C
C=O
N
H
H2O
H2N-CHCOOH
R

FIG. 6.

by chromatographic procedures. The method fails when the C-terminal residue is arginine, cystine, asparagine or glutamine, and may fail for a terminal aspartic acid group (10). The yields of lysine, histidine and tryptophan are very low. A chromatographic 'background' of glutamic acid, serine and alanine spots, sets a limit to the use of the method for high molecular weight proteins, which have small concentrations of terminal residues. The advantages of the hydrazine method are that it does not fail for proline or glycine, and more recently, clear cut results have been obtained with slightly modified techniques for lysozyme (33) and some muscle proteins.

iv) Thiohydantoin method (30): The protein or peptide is treated with ammonium thiocyanate in the presence of acetic anhydride (Fig. 6), to form a thiohydantoin of the C-terminal residue which can be chromatographically identified. Aspartic and glutamic acids do not form thiohydantoins and the method is not reliable for terminal serine or proline (30). The present writer has applied this method to simple peptides with satisfactory results, but on attempting to use this technique on fish scale proteins, clear cut results were not obtained. Using this method, free terminal carboxyl groups were not detected in bovine skin collagen (34).

A study of the various sequences, present in several proteins, reveals no simple general law or principle which conditions the order in which amino acids are laid down in

protein/

protein chains. Each protein has its unique arrangement, which is probably determined by its mechanism of synthesis, as well as the nature of the biological role that it later performs.

Although many peptides (13) have been isolated from partial hydrolysates of collagen, no definite and detailed sequences of any considerable length have, as yet, been put forward.

3. Reactivity of some of the protein side chains.

i) One of the milder methods available for estimating the availability of the side chains of lysine and hydroxylysine, is the DNFB method, described above (P.61), which was used in this work.

ii) Another method, used in conjunction with the DNFB technique, makes use of the ability of acid and basic dyes to combine with protein groups of opposite ionic charge (14). Thus Orange G, in acid solution, combines with N-terminal groups and basic side chains, and Safranine O, at alkaline pH, reacts with C-terminal groups and acidic amino acid side chains (Fixy). These techniques are based on the photoelectric determination of the uncombined dye remaining after reaction with protein, and are applicable to both soluble and insoluble proteins. Due to their high molecular weights, the number of N and C-terminal residues present in a given weight of protein, constitutes an extremely minute proportion of the total number

of/

of side chain amino and carboxyl groups. Thus, although the dye-binding method does not differentiate between terminal and side chain ionic groups. from a quantitative point of view, the results of this method can be used as an indication of the number of ionised side chains present under the experimental conditions, provided that extensive hydrolysis of peptide links has not taken place during reaction with the dye.

The dye binding method suffers from the fact that combination takes place at pH values far removed from the isoelectric range of most proteins, whose internal structures are probably disorganised thereby. In addition, blocking the ϵ - amino groups, by the introduction of alkoxy groups or by reaction with formaldehyde, did not cause a decrease in base binding power (14). Furthermore, the link between protein and dye is not stable to changes in the pH value or ionic strength of the surrounding medium. In view of these restrictions, the results of dye binding experiments must be interpreted with caution, and preferably in conjunction with those obtained using other methods.

Evidence presented earlier (P.28) showed that most of the side chain carboxyl, guanidyl and hydroxyl groups of collagen are mainly concerned in hydrogen bond formation, and most of these side chains are, therefore, not easily available for reaction. The treatment required to make these groups more

reactive/

reactive (15), usually result in some degree of denaturation of the protein. The ε amino groups of lysine and hydroxylysine, however, do not seem to be greatly involved in any of the powerful stabilising links of collagen, as they can be blocked by various reagents (16) under relatively mild experimental conditions, and their inactivation does not cause pronounced swelling of the protein (15), or other indications of the rupture of hydrogen bonds.

It was, therefore, considered possible that those ε amino groups which are fairly free, or involved at most in interchain electrovalent salt links, might play a part in the combination of lime salts with the protein matrix in hard connective tissues such as bone and dentin. This possibility formed the basis for the research to be described.

A. Preparation of Materials.

The following tissues were investigated:-

a) Ox-hide

b) Ox articular cartilage

c) Ox-bone

d) Human dentin

e) Pilchard scales (Sardina ocellata Jenyns)

a)/

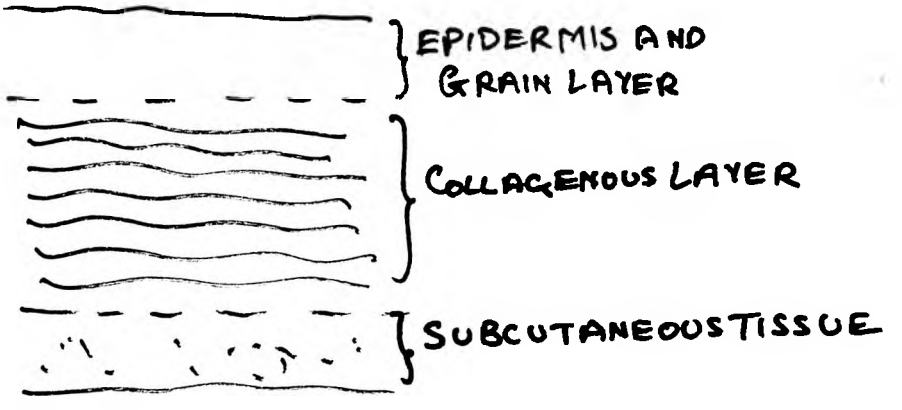

EPIDERMIS AND
GRAIN LAYER
COLLAGENOUS LAYER
SUBCUTANEOUS TISSUE

FIG. 7.

a) Ox-hide collagen:

Samples of collagen were prepared with the minimum chemical treatment, as it was thought that the small amounts of reticular and elastic fibres present, would lead to less error than the drastic treatments which are necessary for their complete removal.

Hide from the thigh region of a freshly killed ox was washed first with water to remove blood etc., left to stand overnight in 0.9% saline at 4°C, and then rewashed with water. The grain layer (Fig. 7), which contains the greater part of the muscle and elastic tissue as well as hair roots, was split off and the remaining material, excluding adipose tissue, was cut into 0.5 cm. cubes (approx). The cubes were defatted with 3 changes of light petroleum ether (B.P. 40 - 60°C).

b) Articular cartilage:

Soft articular cartilage from the distal end of an ox tibia, was removed by means of a scalpel, and washed with water and then with 0.9% saline overnight at 4°C, followed by distilled water until free of chloride ions. A chondroitin sulphate protein complex, which forms about 20% by weight of the articular cartilage (17), was extracted by soaking 10 g. of cartilage in 200 ml. of 2% KOH for 2 days at 4°C. The alkaline solution was decanted and the mucoprotein precipitated by the addition of 2 volumes of ethanol containing 1% NaCl. After standing overnight at 4°C , the precipitate was filtered off and washed well with 80% aqueous ethanol.

c)/

c) Ox-bone:

The diaphysis of an ox tibia, soaked in 0.9% saline overnight and then washed with water, was cut into sections about 1" thich using a hacksaw. These sections were then split into 0.25 cm. cubes (approx.) by means of root-splitting dental forceps. Care was taken, here and in the preparation of dentin, to prevent the generation of heat as this can lead to denaturation as well as solubilisation of some of the protein fraction (18). Ox-bone collagen, obtained by removal of the mineral material by the treatment of ox-bone with sodium versenate (see p. 84) was defatted with 3 changes of petroleum ether.

d) Human dentin:

Freshly extracted, sound, permanent human teeth were filed free of cementum, and the crowns removed by means of root-splitting forceps. The roots were split into small pieces (roughly 0.25 cm. cubes). The pulpal material was scraped away and the dentin allowed to soak, first in 0.9% saline overnight at 4°C and then washed well with water and allowed to dry in air. Equal weights of all teeth, except the molars, were

pooled/

Table a: Composition of Fish Scales:

Gelatin	21%
Ichthylepidin	23%
Mineral Material	56%

pooled and no attempt was made to study the different types (eg. incisors or canines) separately. Dentin collagen, obtained by the removal of the mineral material by treatment with sodium versenate (see P.84), was defatted with 3 changes of petroleum ether.

e). Fish scales:

Pilchard scales were soxhlet extracted with hexane for 12 hours. The purified scales (0.2g.) were then demineralised by soaking in 1N trichloracetic acid (10 ml.) for 3 hours. The demineralised scales (0.2 g) were fractionated into the following two components by heating in 10 ml. of water for 2 hours at 80°C viz:

a) a water soluble gelatin and
b) an insoluble protein, called 'ichthylepidin'(82).

About 16% of gelatin was extracted without prior demineralisation (Table a).

Ichthylepidin was noted for its extreme insolubility in a large number of protein solvents and its appreciable cystine content (0.5%).(p.103)

Extraction of mucoprotein: Crude extracts of mucoprotei were obtained by treating 1 g. quantities of ox-hide collagen, human dentin collagen and ox bone collagen with alkali, and proceding as described under articular cartilage - section b) P.68.

The preparation of modified collagen: Samples of ox-

hide/

hide, bone and dentin collagens were modified or 'denatured' in the following ways:-

i) By heating in water at 70°C for 5 minutes (heat shrunk)

II) By soaking at room temperature in 8M urea for 24 hours

iii) By soaking at room temperature in 1N acetic acid for 24 hours.

iv) By soaking at room temperature in 1% sodium hydroxide for 24 hours.

v) By treatment with hyaluronidase for 16 hours at 37°C (pH=4.6).

'Deorganicised' bone and dentin: The mineral fraction of bone and dentin was isolated from the organic fraction by soxhlet extracting the tissues with boiling 85% aqueous ethylene diamine for 100 hours. (19). The organic fraction was degraded to soluble, low molecular weight, compounds by the strongly basic ethylene diamine. This treatment probably causes some growth of the mineral crystals (19).

'Synthetic' hydroxyapatite (20): 100 cc. of a saturated solution of $Ca_3(PO_4)_2$ in water was boiled in an open beaker until approximately 50 ml. had evaporated. 1 g. of sodium hydroxide was added and the mixture boiled under reflux for 100 hours. The resulting suspension was centrifuged at 2000 r.p.m. for 30 minutes. The supernatant was poured off, and the precipitate washed with distilled water, spun, and washed again. The solid material, dried at 60°C gave an X-ray diffraction pattern typical of the apatite series of minerals (Fig.).

A/

B. Methods.

a) The amino acid composition of the collagens:

The connective tissue proteins were broken down into their constituent amino acids by acid hydrolysis. Tryptophan, which is destroyed by this treatment (4), was determined separately in an alkaline hydrolysate (P. 81). Cystine and cysteine, when present, were determined by colorimetric methods (3). The proteins investigated included ox-hide collagen, human dentin collagen, ox bone collagen, demineralised fish-scales, fish-scale gelatin and ichthylepidin (21).

The conditions of hydrolysis were as follows:-

0.1 g. of protein was hydrolysed with 30 ml. of constant boiling hydrochloric acid, for 24 hours in a sealed tube at 110°C. The hydrolysate was concentrated to dryness 'in vacuo', a little water was added to the residue which was again concentrated to dryness. This was repeated until all the hydrochloric acid was removed, the temperature of distillation being kept below 60°C. The residue was dissolved in about 10 ml. of distilled water and transferred quantitatively to a 25 ml. standard flask, and diluted to volume. Aliquots of this solution were taken for amino acid assay. The following methods were used:

i) Paper chromatography of free amino acids.

ii) Paper and column chromatography of DNP amino acids.

iii) Moore & Stein's ion exchange column chromatography of the free amino acids.

iv) Chemical and spectrophotometrical methods.

For/

i) Paper chromatography:

For a rough indication of the relative amounts of the different amino acids present in protein hydrolysates, one-dimensional paper chromatography on Whatman No. 1 filter paper using butanol:acetic acid: water (4:1:5) as solvent was used. An aliquot of the hydrolysate, containing 100 microgram of nitrogen, was applied to the base line as a thin strip 1 cm. long and about 2 mm. wide, by means of a graduated capillary pipette. The chromatogram was developed by the descending solvent for 24 hours, and allowed to dry in air. The path taken by the amino acid mixture was cut into consecutively numbered strips 1 cm. long and 3 cm. wide. Each strip was placed in a centrifuge tube containing 2 ml. of 1N hydrochloric acid and thoroughly macerated by means of a glass rod. After centrifugation at 2000 r.p.m. for 5 minutes, 1 ml. of the clear supernatant was placed in a graduated test tube containing 4 ml. of 50 mg.% ninhydrin in 0.5M citrate buffer (pH=5.0), containing 0.1 ml. of 10 mg.% stannous chloride. The tubes were plugged loosely with cotton wool, placed in a boiling water-bath for 30 minutes, after which they were cooled and diluted to 5 ml. with 50% ethanol. The optical density of each tube was read at 570 mu. on the Beckman spectrophotometer against a water blank. Proline and hydroxy-proline, which both give a yellow color with ninhydrin, were read at 440 mu. By reference to a standard curve, the optical density of each cut-out strip was converted to a microgram of amino acid, and plotted against the distance run.

When/

When two dimentional chromatography was used, a mixture of phenol (70%) and water (30%) was used for the first run. The phenol was removed by drying the paper at 80°C and dipping it into ether. Butanol:acetic acid: water (4:1:5) as solvent, was used for the second run in a direction at right angles to the first.

ii) The DNP method:

The hydrolysate of free amino acids may either be chromatographically separated by 2-dimentional paper chromatography and then sprayed with DNFB (22), or the hydrolysate may first be quantitatively converted to a mixture of DNP amino acids, which is subsequently separated by methods of paper (23) and column (7) chromatography. The writer prefers the latter method of dinitrophenylation prior to separation as, the resolution of mixtures of the yellow DNP amino acids can then be observed visually. The experimental conditions, which were similar to those of Courts (24), were as follows:

An aliquot of the protein hydrolysate, freed from hydrochloric acid by evaporation 'in vacuo' and containing about 300 microgram of nitrogen, was shaken for 6 hours at room temperature with 0.1 g. DNFB in 5 ml. of 67% ethanol saturated with sodium bicarbonate.

The alcohol was removed by evacuation 'in vacuo' and the remaining mixture extracted with ether to remove excess DNFB

and/

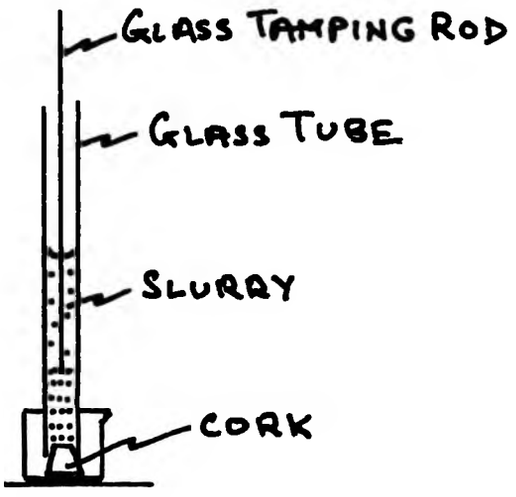

GLASS TAMPING ROD
GLASS TUBE
SLURRY
CORK

FIG. 8.

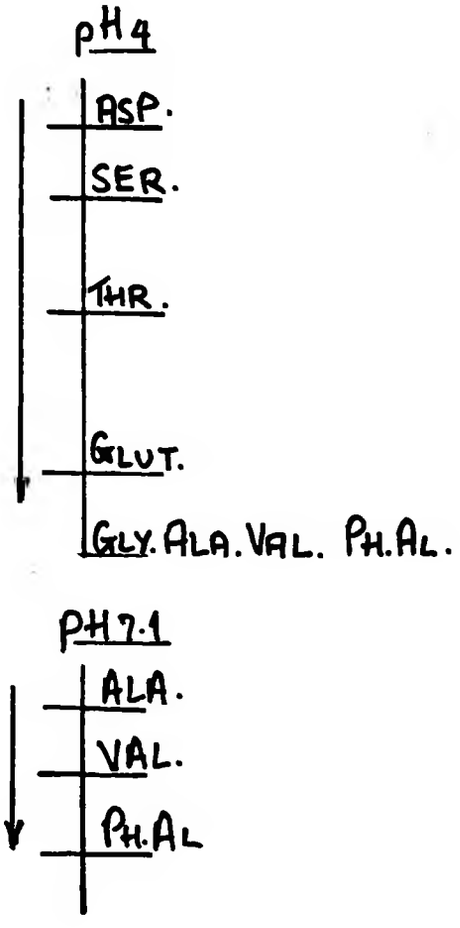

pH 4
ASP.
SER.
THR.
GLUT.
GLY. ALA. VAL. PH. AL.
PH 7.1
ALA.
VAL.
PH. AL

FIG. 9.

and most of the dinitrophenol. The ether-soluble α and di DNP amino acids were extracted by acidifying the mixture with 1N hydrochloric acid and shaking with ether. α DNP arginine remained in the watery layer. The dinitrophenol in the ether extract was removed by 'cold finger' sublimation (7a). The remaining material was dissolved in 1 ml. of water-washed chloroform, and quantitatively transferred to a 1 cm. diam. x 10 cm. long column of 'celite', buffered at pH 4 (24), by mixing with 60% of its weight of a phosphate-citrate buffer. The buffer solution was made up by adding 54 ml. of 0.2M Na_2HPO_4 to 46 ml. of 0.2M citric acid. The column was prepared by pouring a slurry of the buffered celite and chloroform into a glass tube (1 cm. diam. x 20 cm. long) one end of which is closed by means of a cork (FIG8). The celite is tamped down by means of a glass rod with a flattened end until a column 10 cm. long is obtained. The cork is then removed and the column is ready for use. The DNP mixture was eluted with 9:1 chloroform-ether mixture. The DNP amino acids separate as yellow bands in the order shown in Fig. 9. As solutions of DNP amino acids in chloroform are not stable to light (7a), the chromatographic columns should be wrapped in black paper. The fastest fraction containing glycine, alanine, valine and phenylalanine was collected and the elution continued until good separation of aspartic acid, serine, threonine and glutamic acid were obtained. Elution was stopped by pouring off the eluant. The celite containing each band was removed from the column by means of a glass spatula and placed in separate

15 ml./

15 ml. centrifuge tubes to which 5 ml. of 1% sodium bicarbonate was added. The mixture in each tube was shaken and centrifuged at 2000 r.p.m. for 5 minutes. The DNP amino acid was thus extracted into the clear aqueous layer, which was pipetted into a 25 ml. standard flask. The remaining celite and chloroform mixture was washed and spun twice with 1 ml. portions of 1% sodium bicarbonate solution, the washings being added to the volumetric flask which was then diluted to the mark with more of the bicarbonate solution. The optical density at 360 mu. was recorded on the Beckman spectrophotometer and the concentrations obtained from a standard curve. (see p. -88a- and -88b-)

The fast-moving band containing DNP glycine, alanine, valine and phenylalanine was evaporated to dryness and reapplied to a similar column and eluted with chloroform. DNP glycine (R_f=0.5) separates from the remaining amino acids which are eluted and applied to a celite column buffered at pH 7.1, (the buffer is made up of 83 ml. 0.2M Na_2HPO_4 and 17 ml. 0.2M citric acid). DNP alanine, valine and phenylalanine separate and are determined as described above.

Other similar systems proposed for the separation of DNP amino acids on columns of celite or kieselguhr (7), differ mainly in the nature and composition of the eluting solvent; eg. Mills (7a) has shown that nearly all the common DNP amino acids can be separately eluted from a single column of kieselguhr (1.5 x 12 cm.), by mixtures of methylethylketone and chloroform.

As the celite column can be rapidly repacked, it was found/

found to be easier and more accurate to remove the bands bodily from the column by means of a glass spatula, rather than to run the risk of decomposition and less sharp separation, which sometimes occurs, if the bands are eluted from the column by appropiate solutions. As a further check on the identity of the DNP amino acids, the bicarbonate solutions of the DNP amino acids, after measurement of their optical density, were acidified with 1N HCl, and extracted into ether. The ether extracts were concentrated to small volume and applied to paper chromatograms as described on P.91. Known DNP amino acids were run concurrently.

The lysine and hydroxylysine contents can be conveniently determined as their ε-N DNP derivatives, on a separate portion of the hydrolysate, by first blocking all the α amino groups with copper before dinitrophenylation with DNFB. This method has been described in detail on P. 88.

iii) The method of Moore & Stein (2):

Both the paper chromatographic method for the determination of free amino acids (P.73), and the DNP method just described, were mainly used on proteins of known composition for the purpose of assessing the order of the manipulative and inherent errors of these procedures. The fish scales, demineralised scales, gelatin and ichthylepidin, whose complete amino acid analysis is not known, were therefore, investigated by the more accurate and comprehensive ion exchange elution method of Moore and Stein (2):-

The/

Table 1: Buffers used in Moore & Stein's method of Amino Acid Analysis:

pH	Composition.
3.27 ± 0.01	0.1M Citrate
4.25 ± 0.05	0.1M Citrate
6.5 ± 0.03	0.1M Citrate
6.8 ± 0.03	0.1M Phosphate
5.0 ± 0.1	0.1M CITRATE

NINHYDRIN REAGENT :-

2 g. OF NINHYDRIN (EASTMAN-KODAK) DISSOLVED IN 55 ml. OF ETHYL CELLOSOLVE WERE MIXED WITH 55 ml. OF 0·5M CITRATE BUFFER, pH=5, CONTAINING 0·08 g. OF STANNOUS CHLORIDE.

The ion exchange resin, Dowex 50 (obtained from Dow Chem. Co. U.S.A.) which consists of styrene copolymerised with 8% divinyl benzene to produce 8% cross linking, was purified by washing 1 lb. of resin with 5 litres of 4N HCl, until the filtrate was colorless. After 2 washes with distilled water, the resin was washed with 2N NaOH until the filtrate was alkaline. The resulting sodium salt was heated on a steam bath, with 3 volumes of N NaOH for 3 hours. The supernatant was decanted and replaced with fresh N NaOH. Portions of the alkaline slurry were poured into the chromatographic tube (XXXX) and allowed to settle. Air bubbles were removed by stirring with a stainless steel wire. In this way a column of resin 100 cm. long was obtained. A separating funnel containing distilled water was attached to the top of the column and the resin washed free of alkali. 100 ml. of 0.1M citrate buffer pH = 3.27was then run through the column which is now ready for use. The composition of the buffer solutions and the ninhydrin reagent required are shown in Table 1 .

100 mg. of protein were hydrolysed for 24 hours at 110°C with 30 ml. of constant-boiling hydrochloric acid. The hydrolysate was evaporated 3 or 4 times 'in vacuo' to remove HCl, and made up to 25 ml. with 0.1M citrate buffer pH= to which 1 drop of thiodiglycol was added. 1 ml. of this solution was applied to the column, allowed to sink in, and was followed by 1 ml. of buffer solution which is also allowed to sink in before filling up the column with more buffer. Care

must/

must be taken not to disturb the surface of the resin during the additions of hydrolysate and buffer. 1 ml. fractions of eluate were collected by a circular mechanical fraction cutter carrying about 200 tubes. Every 15 minutes a separate tube was placed under the outlet of the column ( ). The eluting buffer was supplied at the rate of 1 ml/15 minutes, to the column by a forced-feed system, consisting of a motor driven piston in a glass cylinder containing the buffer solution. The temperature of the column was controlled by means of a surrounding water jacket through which a small centrifugal pump circulated water of the correct temperature.

The order of emergence of amino acids from the column, and the conditions of pH and temperature used are shown in p.103b . For the separation of the basic amino acids a 15 cm. column of Dowex 50 was used. The buffers used are shown in p. 103b

The 1 ml. effluent fractions were treated with 2.1 ml. ninhydrin reagent. The tubes were heated at 100°C for 20 minutes in a glycerine bath. Evaporation was minimised by inserting loose-fitting glass cones into the mouths of the tubes. After cooling in tap water, the contents of the tubes were diluted with 4 ml. of 70% isopropanol and their optical density at 580 mu r recorded on an 'Evelyn' colorimeter. Proline and hydroxyproline, which gave a yellow color with ninhydrin, were read at 440 mu. The optical density of each fraction was plotted against the ml. eluate (p.103b) A series of separate peaks each corresponding to one amino acid was obtained. The total optical density, of each peak corrected for the color given by the blank solutions,

Table 2: Conversion factors:
To convert optical density to mg. amino acid divide by F.

Amino acid	F.
Hypro.	37.3
Asp.	34.4
Thr.	44.1
Ser.	57.6
Glut.	38
Pro.	8.94
Gly.	76.7
Ala.	61.7
Val.	44.3
Meth.	33.6
Ileu.	38.4
Leu.	42
Tyr.	26.2
Phal.	29.3
Hist.	33.3
Lys.	39.7
Hylys.	31.7
Arg.	30.2

was converted to mg. of amino acid by means of factors obtained from analysis of a mixture of amino acids of known composition (see Table 2).

The columns can be regenerated by elution with 100 ml. of 0.2N NaOH, and can be used indefinitely.

iv) Colorimetric and spectrophotometric methods:

Cystine andcysteine were colorimetrically determined on a 4 hour acid hydrolysate of protein, by making use of the phospho-18 tungstic reagent of Folin and Dennis (3) as follows:-

0.5 g. of protein was refluxed with 5 ml. of constant boiling hydrochloric acid for 4 hours. The hydrolysate was filtered and the filtrate and washings diluted to 50 ml. 5 ml. aliquots were pipetted into three 25 ml. standard flasks marked A,B, and C. The hydrolysates were then neutralised by the addition of a predetermined quantity of 6N NaOH. 5 ml. of 2M acetate buffer, pH 5.7, was added to A, B, and C. 0.5 ml. of 0.001M $HgCl_2$ was added to B, and 1 ml. of saturated $NaHSO_3$ was added to C. 2.5 ml. of the Folin-Dennis reagent was added to each flask which was allowed to stand for 30 minutes to develop the blue color, before dilution to the mark and reading the optical density at 608 mu. The optical density was converted to mg. by a calibration curve.

Calculation:

mg. cystine = C-A

mg. cysteine = A-B

Determination/

Determination of -SH groups:

50 mg. of protein was shaken at 30°C with 10 ml. of a 2 mg.% solution of Bennetts reagent (25)(see equation) in 100 ml. neutral formamide, which was distilled under vacuum and contained ½ to 1% of water. The coloured supernatant was tested periodically for disappearance of the red color by recording the optical density of the supernatant solution on a Hilger colorimeter (Blue - green filter). A sample of protein whose -SH groups were blocked by treatment with iodoacetamide was used as a control.

The determination of tyrosine and tryptophan (4):

Alkaline solutions of the protein obtained by dissolving 100 mg. of protein in 50 ml. of 0.100N sodium hydroxide under reflux if necessary. The solution was diluted to 100 ml. with 0.100N sodium hydroxide and its absorption values at 280 mu, 294.4 mu, 340 mu and 370 mu were measured on the Beckman spectrophotometer. The absorption curves of tyrosine and tryptophan intersect at 294.4 mu.(4). The calculation is performed as follows:-

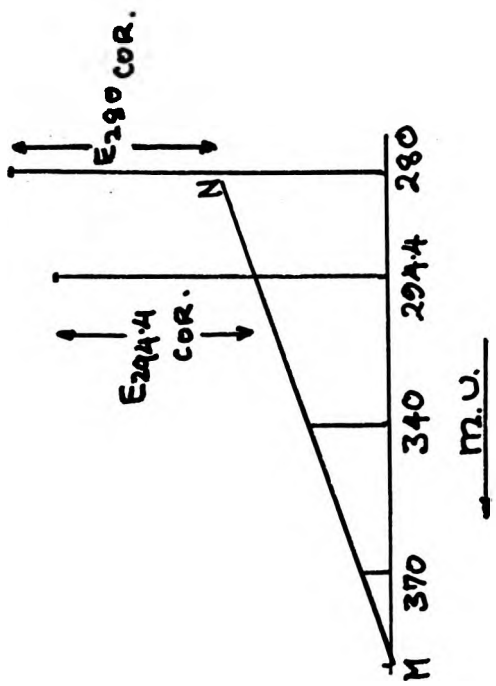
Let x = total number of g.mol. of tyrosine + tryptophan/L.

y = g.mol. of tyrosine only.

Let A = molar extinction coefficient of tyrosine at 280 mu i.e. at any wavelength other than the point of intersection of 294.4 mu = 1576

B = molar extinction coefficient of tryptophan at 280mu = 5225

Fig. 9a:



Let E_{cor} = the observed, corrected absorption at 280 mu.

The corrected values of E are shown in Fig. 9a and are obtained by calculation from similar triangles since the slope MN is known (Fig. 9a).

x is determined at the point of intersection (294.4 mu) from the equation:

$$x = \frac{E_{cor}\ 294.4}{2375} \quad X \quad X$$

Then $E_{cor}280\ mu = y.A + (x-y)A$

$$y = \frac{E_{cor}280 - xB}{A-B}$$

$$\text{i.e.} \quad y = \frac{E_{cor}280 - 5225\left(\frac{E_{cor}294.4}{2375}\right)}{-3649}$$

The method cannot be used if other substances such as nucleic acids which absorb in the ultra violet range, are present.

Protein nitrogen:

a) Total nitrogen was determined by a micro-kjeldahl procedure (26) in which 10 mg. of protein of known moisture content was digested for 12 hours with 3 ml. of a mixture of/g.

copper/

copper sulphate and 20 g. potassium sulphate in 50% sulphuric acid. After digestion, the mixture was carefully diluted with water and cooled in ice. 20 ml. of 30% aqueous caustic soda was added to lib rate the ammonia, which was steam distilled into 5 ml. of 5% boric acid containing bromo-cresol-green indicator, (XXX.) for ten minutes. The ammonium borate solution was titrated to its original green color by the addition of standard $\frac{N}{50}$ sulphuric acid.

Calculation:

$$\%N = (\text{ml. of N acid}) \times \frac{14}{(\text{wt. of protein in mg.})} \times 100$$

b) Amide nitrogen was determined by hydrolysing 100 mg. of protein with 2N HCl for 2 hours (26). The hydrolysate was made alkaline, and the liberated ammonia was collected and titrated as described for total nitrogen.

Moisture content:

The moisture content of all tissues was determined by noting the percentage weight loss sustained by 50 mg. of material after heating for 16 hours in an oven kept at 110°C.

b)/

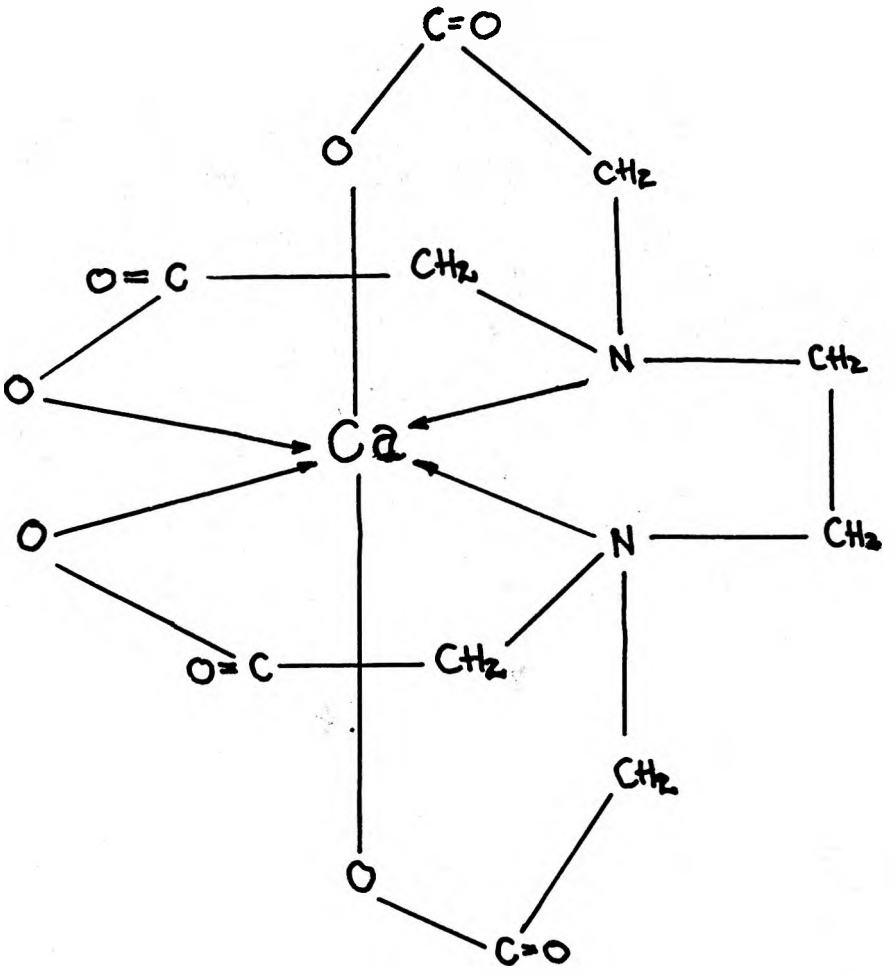


FIG. 10.

b) Demineralisation Studies.

i) The demineralisation of hard tissues by chelation and strong acids:

The removal of mineral material from hard tissues for the purpose of studying the structure of their organic fraction, should be rapidly carried out under conditions least likely to cause excessive 'denaturation' of the protein and mucopolysaccharide constituents. Thus, demineralisation should be carried out rapidly at low temperature and neutral pH.

Methods of demineralisation in general use, include treatment with mineral or organic acids such as hydrochloric acid, nitric acid, (xxxxxxxxxxxxx) phosphoric acid, and acetic and citric acids at pH values below 5. Citric acid acts on mineral material as both an acid and a chelation agent for calcium ions. Phosphate-citrate buffers have also been used (xxxx) As these reagents (with the possible exception of the citric acid and buffer solutions) are all powerful hydrogen bond breakers, they can cause a disruption of the molecular configuration of collagen, and are, therefore, unsuitable for the isolation of collagen in a state suitable for subsequent structural analysis. Decalcification of hard tissues may be effected, under mild conditions, by treatment at 4°C with 0.5M ethylenediamine tetra-acetic acid (versene) neutralised with sodium hydroxide to pH 7.3 (xx). The versene solution dissolves the lime salts by forming a soluble chelation complex with the calcium ions (Fig.10). Although this method is a relatively

slow/

slow one, compared to demineralisation by acids, it is the most suitable if structural damage to the protein is to be minimised. Even in versene decalcified bone, it will be shown (P.115)that availability to DNFB of the number of lysyl ε amino groups of collagen changes with time. In view of this, demineralisation of hard tissues with versene at pH 8 and dinitrophenylation with DNFB, were carried out simultaneously (P.116).

A kinetic study was made of the action of 1N hydrochloric acid, 1N trichloracetic acid, and 0.5M sodium versenate at pH values of 7.3 and 8.8, on bone and dentin. 0.10 g. samples of hard tissue, of known moisture content, were placed in 10 ml. of each demineralising reagent kept at 4°C. The sodium versenate solution was renewed daily. At regular intervals of time, the samples were separated by filtration, washed free of acid and reweighed, after drying in an oven at 105°C for 16 hours. The filtrate and washings were reserved. The percentage dissolution on a dry basis was plotted against the time of treatment. These values were checked by calcium and phosphate estimations on the supernatant. The percentage calcium and phosphorus was also determined on samples of bone and dentin ashed at 900°C in the presence of sodium acetate. Calcium was determined by precipitation, at pH 4.0, as oxalate, which was titrated with standard potassium permanganate (27). Phosphate was determined colorimetrically by conversion to phosphomolybdic acid, followed by reduction, in acid solution, to 'molybdenum blue' by means of 'elon' and sodium ~~ex~~ sulphite (3)(Xxxx). A portion of

the/

the supernatant solution was tested for the presence of free amino acids by boiling for 30 minutes with 5 parts of 0.05% ninhydrin in 0.5M citrate buffer (pH=5.0), to which 0.1 ml. of 0.01% stannous chloride was added. Ninhydrin could not be used directly on those supernatant solutions which contained versene, as a 'blank' solution of versene also gave a blue color with ninhydrin, presumably due to a small impurity of ethylene diamine. An aliquot of each of the versene containing supernatants was applied to a paper chromatogram which was irrigated with a mixture of butanol-acetic acid-water (4:1:5). On spraying with ninhydrin in butanol (1 mg./ml.) the amino acids, when present, could be distinguished from other fast moving ninhydrin positive contaminants.

ii) The dissolution of bone and dentin in buffer solutions:

The rate of dissolution of mineral material of bone and dentin was determined over the pH range of 4 to 8.

Two sets of buffer solutions were prepared:

1. By the addition of 1M sodium hydroxide to 1M acetic acid, a series of buffer solutions was prepared having pH values of 4.7, 5.2, 5.5, 5.8, 6.1, 6.4, 6.7, 7.0, 7.3, and 8.0. The pH values were checked using a 'Cambridge' pH meter.

2. A similar set of buffers was prepared by the addition of 1M sodium hydroxide to 1M phosphoric acid. Titration curves of these buffers were prepared by noting the increase in pH value

due/

due to the addition of a known volume of standard sodium hydroxide.

Procedure: 0.1 g. samples of bone and dentin were shaken with 100 ml. of each of the buffer solutions for definite periods of time, ranging from 2 to 24 hours. The percentage dissolution was calculated from the weight of dried solid remaining after treatment with buffer, and plotted against pH. (p.108a xix.). The supernatants of the acetate buffer solutions were analysed for amino acids, calcium and phosphate. The supernatant phosphate buffer solutions were analysed for calcium and free amino acids.

The percentage dissolution at 4 hours (equilibrium value) was plotted against the pH value of the buffer. These experiments were repeated using synthetic hydroxyapatite as well as deorganicised dentin and bone.

c) Determination of N-terminal and ε-N-amino groups of collagen using Sanger's DNFB method:

i) Preparation of standard DNP amino acids (29):

1. ∝ and di DNP amino acids:

The DNP derivatives of pure amino acids (obtained from B.D.H. London) containing one ∝ amino group, were prepared by shaking 0.3 millimol. of the amino acid in 14 ml. of 8% sodium bicarbonate aq., with 0.6 millimol. of DNFB (obtained from Light & Co., England) in 28 ml. ethanol, for 24 hours at room temperature. The ethanol was evaporated off under reduced

pressure/

Table 3: Melting Points of some DNP amino acids:

DNP amino acid	M.P. °C (uncorr.)
Ala.	176
Arg.	250
Asp.	192
Gly.	203
Leu.	202
Ileu.	166
Di DNP lys.	145
Ser.	199
Thr.	152

pressure, and the remaining mixture was extracted several times with peroxide-free ether to remove excess DNFB as well as some dinitrophenol which was formed during the reaction. The watery layer was then acidified by adding a few drops of concentrated hydrochloric acid, and the resulting precipitate of DNP amino acid was filtered off, recrystallised and its melting point determined (Table 3). The di-DNP derivatives of lysine and hydroxylysine were similarly prepared. Arginine yielded only the α-DNP compound. Spectral and calibration curves (Figs.11,12) were prepared of the α and di-DNP amino acids dissolved in 1% aqueous sodium bicarbonate.

2. ε N DNP lysine and δ N DNP hydroxylysine:

The α-amino group of these diamino acids was blocked with copper before reacting with DNFB. Solid copper carbonate was slowly added to a boiling solution of 0.5 g. of the amino acid in 10 ml. of water until no more dissolved. The mixture was filtered, and 1.5 g. of DNFB in 20 ml. of ethanol together with excess sodium bicarbonate was added to the filtrate. After shaking for 2 hours at room temperature, the mixture was filtered. The precipitate was washed with small amounts of water, ethanol and ether, and suspended in 5 ml. of water. Sufficient 1N hydrochloric acid was added to obtain a clear solution which was cooled in ice. Hydrogen sulphide gas was then bubbled through the solution for 2 minutes. A little charcoal was added, and the insoluble copper sulphide removed by filtration. The

filtrate/

Fig. 11: Spectral curve of α and ε DNP amino acids excluding proline.

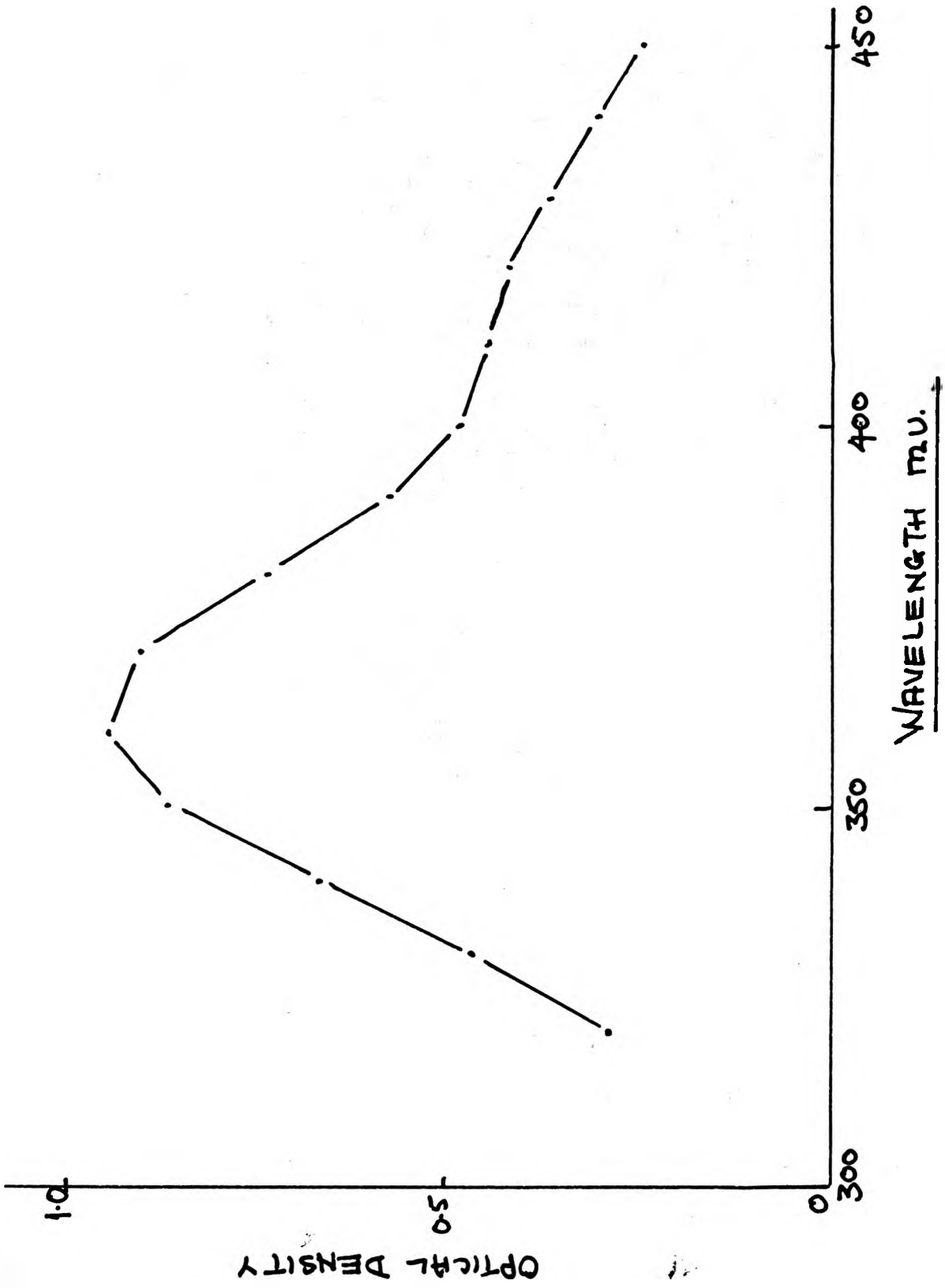
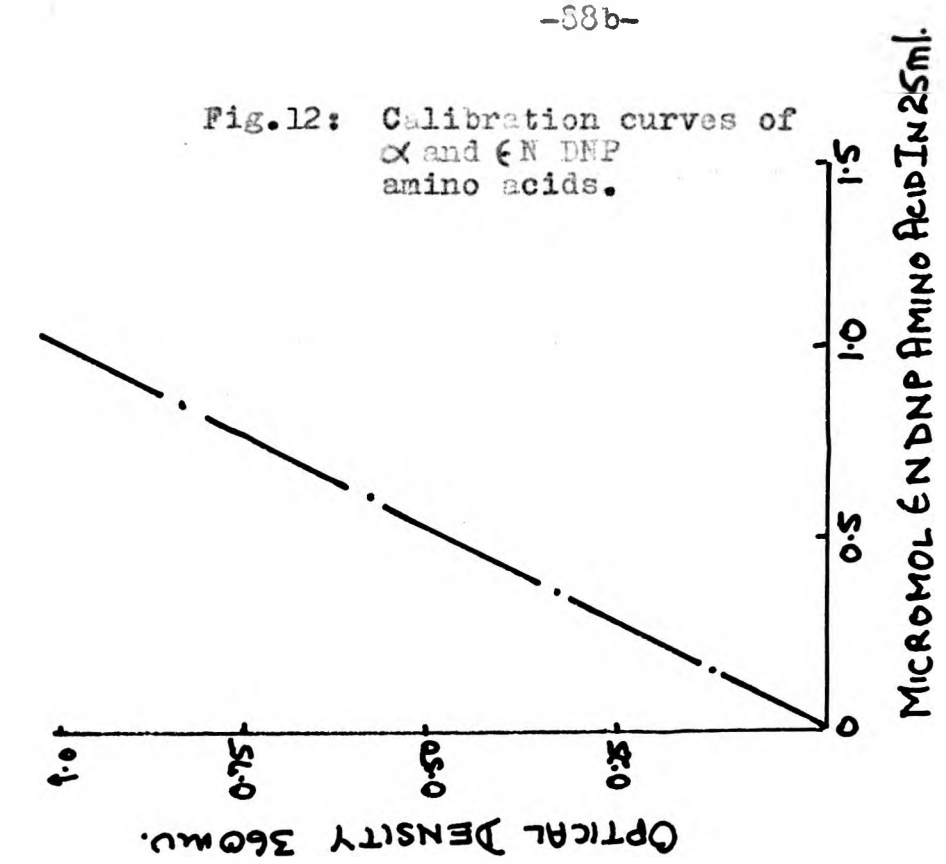
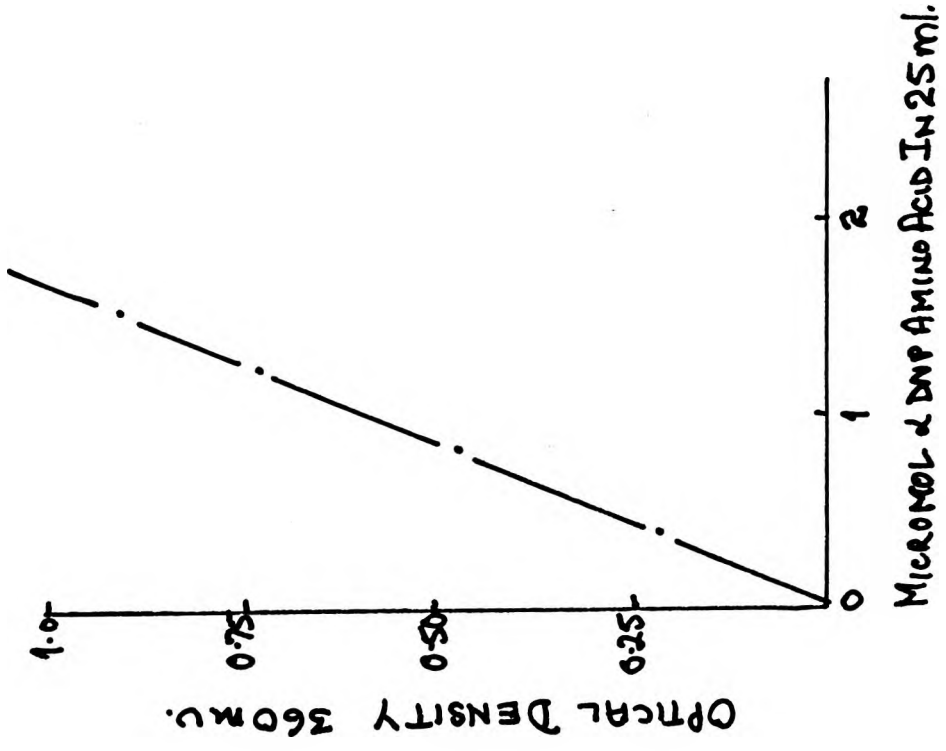


Fig.12: Calibration curves of α and ϵ N DNP amino acids.





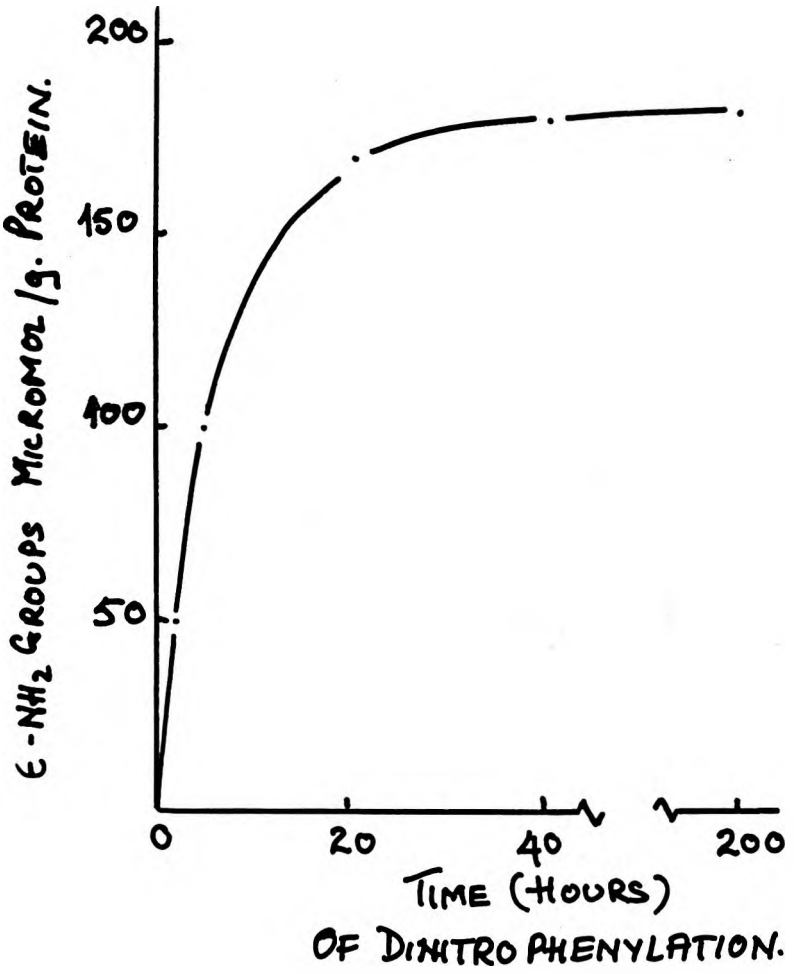


Fig. 13: Dinitrophenylation of ox-hide collagen.

filtrate was evaporated to dryness, 'in vacuo', and the solids crystallised from water. This was followed by recrystallisation from 20% hydrochloric acid. Spectral and calibration curves were prepared of the 4 DNP amino acids dissolved in 1N hydrochloric acid (Figs.11,12).

ii) Procedure: for the dinitrophenylation of hard and soft tissues:

0.1 g. of tissue and 0.5 g. DNFB (excess) were shaken for 36 hours at room temperature in 15 ml. of 66% aqueous ethanol, saturated with sodium bicarbonate (pH=8). The same results were obtained if ethanol was omitted from the mixture or if the pH was adjusted to 10.0. Increasing the time of dinitrophenylation to one week (28) was found to have no effect on the final concentration of α and ϵ amino groups detected (Fig.13). The yellow, insoluble DNP protein thus obtained, was removed from the dinitrophenylating mixture, washed with distilled water, and shaken with 10 ml. ethanol for 1 hour. The DNP derivatives of fish-scale gelatin were partially soluble in water and were therefore, precipitated by saturating the reaction mixture with ammonium sulphate (24). The gummy precipitate was filtered off, washed well with alcohol and ether and dried over P_2O_5 in a vacuum dessicator. The DNP protein was dried in air and hydrolysed for 16 hours with 20 ml. of glass-distilled hydrochloric acid (6N), at 110°C, in a sealed tube, in order to liberate free DNP amino acids (Fig.1). Hydrolysis times of 2 hours and 8 hours were also used in order to detect unstable DNP proline and DNP glycine, (29) if present.

The/

The ether soluble α DNP amino acids were separated from the water-soluble ε N DNP lysine and hydroxylysine, by repeated extraction of the acid hydrolysate with 5 ml. portions of peroxide-free ether. The watery layer was set aside for the determination of ε N DNP amino acids. The combined ether extracts were evaporated to dryness under suction, and dissolved in a small volume of water-washed chloroform. The chloroform solution was applied to the surface of a phosphate-buffered (pH 7.0) celite chromatographic column (5 cm. long, 1 cm. diam.) (7b). On eluting with chloroform, a stationary yellow band of α DNP amino acids was separated from small amounts of the fast moving artefacts, dinitrophenol and dinitroaniline (X). The celite containing the stationary yellow band, was transferred by means of a glass sputula, to 4 ml. of 1% sodium bicarbonate aq. in a centrifuge tube. After centrifugation for 2 minutes at 2000 r.p.m. the yellow supernatant was transferred to a 25 ml. volumetric flask. The remaining celite was washed twice with 1 ml. of 1% sodium bicarbonate solution, and the washings, after centrifugation, were added to the volumetric flask, which was diluted to the mark with 1% sodium bicarbonate solution. The optical density of the solution at 360 mu was recorded on the Beckman spectrophotometer, (model DU) and the total concentration of N-terminal amino acids read off a calibration curve. For identification of the N-terminal amino acids, the bicarbonate solution was acidified, extracted into ether, and aliquots applied to three paper chromatograms.

The/

The following systems were used (23):

1. Benzene saturated with 3% acetic acid and containing 2% butanol.
2. Teramyl alcohol saturated with phthalate buffer (pH=6).
3. Water-saturated butanol.

Known DNP amino acids were run concurrently.

The spots of DNP amino acids were cut out, eluted with 1% sodium bicarbonate and their concentrations colorimetrically determined. The total concentration of 5 ml, of the yellow, water soluble, ε N DNP lysine and ε N DNP hydroxylysine was obtained by measuring the optical density 'D' at 360 mu., of a standard volume of the acid hydrolysate of the DNP dentin after removal of the α DNP amino acids by ether extraction (supra). The optical density reading was converted to micromol. of DNP amino acid by reference to a calibration curve (Fig.12). By means of paper chromatography on Whatman No.1 filter paper using butanol : acetic acid : water (4:1:5) as solvent, ε N DNP hydroxylysine was separated from the faster moving ε N DNP lysine. Each spot was cut out, eluted with 5 ml. 1N hydrochloric acid, and the optical density at 360 mu. recorded. In this way the molar ratio (R) of ε N DNP lysine to ε N DNP hydroxylysine was obtained.

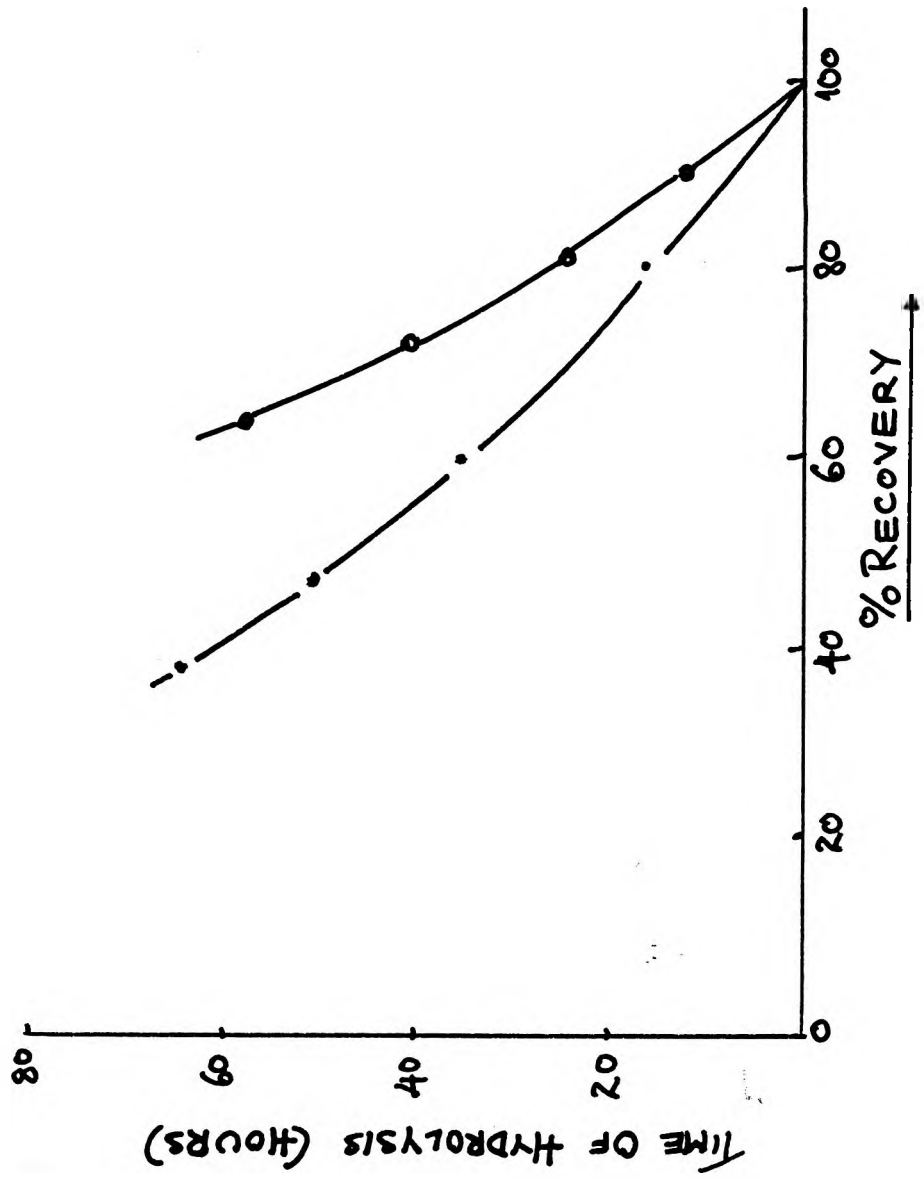
Correction curves for the destruction of DNP amino acids during the hydrolysis and subsequent chromatographic procedures, were constructed by hydrolysing known quantities of the DNP

amino/

Fig. 14: Decomposition of α DNP asp. and glut. acids —o—o—o—

and ε N DNP lysine and hydroxylysine —•—•—

in 6N HCl at 110°C (sealed tube)



amino acids for various lengths of time, in the presence of protein, and measuring the amounts remaining after chromatographic separation. The percentage loss in the hydrolysis step alone, was also determined. (Fig. 14)

The DNFB method as described above, was performed on uncalcified ox skin and cartilage, as well as fully calcified ox bone and human dentin.

In accordance with results of other workers (28) it was found that only 50-60% of the total lysyl and hydroxylysyl ε-amino groups in ox-hide collagen was recovered as ε N DNP amino acid from hydrolysates of the DNP collagen, even after prolonged treatment with DNFB. The DNP hydrolysate, was, therefore, analysed as follows for the presence of free lysine and hydroxylysine which had not reacted with DNFB when in the intact protein (Fig.).

An aliquot of the DNP hydrolysate was neutralised by the addition of solid sodium bicarbonate. The solution was brought to the boil and a slight excess of solid copper carbonate was added. The solution was filtered. The filtrate and washings were dinitrophenylated for 5 hours at room temperature, by reacting with 0.1 g. DNFB in two volumes of ethanol. The mixture was extracted with ether, to remove excess DNFB and dinitrophenol. The watery layer was acidified with 1N hydrochloric acid, and the copper precipitated by passing H_2S for two minutes. The solution was then filtered. The filtrate and washings were boiled to remove the H_2S and diluted to a

suitable/

suitable volume before reading at 360 mu. on the Beckman spectrophotometer. This method which was applied to ordinary acid hydrolysates of all the collagens, was used to determine the total concentration of lysine and hydroxylysine in the protein. The concentration of each amino acid was then determined by measuring the molar ratio (R) of ε N DNP lysine to ε N DNP hydroxylysine, and substituting this value in the following equations:-

$$\text{Micromol } \varepsilon \text{ N DNP lysine/g.} = \frac{R.T}{R+1}$$

$$\text{and micromol } \varepsilon \text{ NDNP hydroxylysine per g.} = \frac{T}{R+1}$$

where T = total concentration of ε N DNP lysine and ε N DNP hydroxylysine in micromol./g. protein.

and R = molar ratio of ε N DNP lysine to ε N DNP hydroxylysine, which was determined as described on Page 91.

In studying the change in the availability of the terminal and ε amino groups of hard tissue collagen during the process of demineralisation, the procedure was modified so that both demineralisation, by means of a chelating agent, and dinitrophenylation with DNFB were carried out simultaneously in one operation as follows:

0.2 g./

0.2 g. samples of hard tissue were treated with a mixture of 0.5 g. DNFB in 5 ml. ethanol, and 10 ml. of 0.5M neutralised ethylenediamine-tetra-acetic acid (versene) adjusted to a pH of 8.8. At regular intervals of time, usually about 24 hours apart, samples were removed, allowed to dry in air, and reweighed in order to calculate the percentage dissolution. The moisture content was determined on a separate sample by drying at 105°C for 16 hours. The partially demineralised samples of dinitrophenylated tissue were hydrolysed, and their concentration of DNP terminal and ε N DNP amino groups determined.

Samples of bone and dentin that had been decalcified with N hydrochloric acid or normal trichloracetic acid at 4°C, were allowed to dry in air, and then treated with DNFB as described above. The results were expressed as micromol. of terminal or ε amino group per gram of original protein used. Graphs were constructed showing the change in the availability of free amino groups of all the collagens with time of soaking in the various demineralising agents and with the percentage dissolution of mineral material. The DNFB method was also performed on samples of ox-hide collagen which were subjected to the same demineralisation procedures as the hard tissues.

d)/

d) Determination of C-terminal residues by the Thiohydantoin method (30):

15 mgs. of ammonium thiocyanate were added to 30 mg. of protein in 4 ml. of 90% acetic anhydride in acetic acid and mixture was heated for 30 minutes on a steam bath. After cooling, the acetic anhydride was removed by extraction with petrol-ether. The gummy residue was dissolved in 0.4N barium-hydroxide (pH = 12.5 or higher), and allowed to stand for 90 minutes. The pH was adjusted to 6.5 by the addition of sulphuric acid, and the barium sulphate formed was removed by centrifugation. The thiohydantoins were extracted with ethyl acetate. The extract was concentrated to dryness 'in vacuo' and redissolved in a small quantity of ethyl acetate. Aliquots were applied to a paper chromatogram which was irrigated with the organic phase of a mixture of butanol, acetic acid and water in the proportions 4:1:5. Known thiohydantoins were run concurrently. Other chromatographic systems have been used (31). On spraying with Folin-Dennis reagent followed by exposure to ammonia fumes, most of the thiohydantoins showed up as blue spots. Alternatively, the thiohydantoins may be hydrolysed to amino acids which are then separated and identified by paper chromatography.

e)/

Fig. 15: Spectral curves of Orange G. ——•——•——
and Safranine O ——o——o——

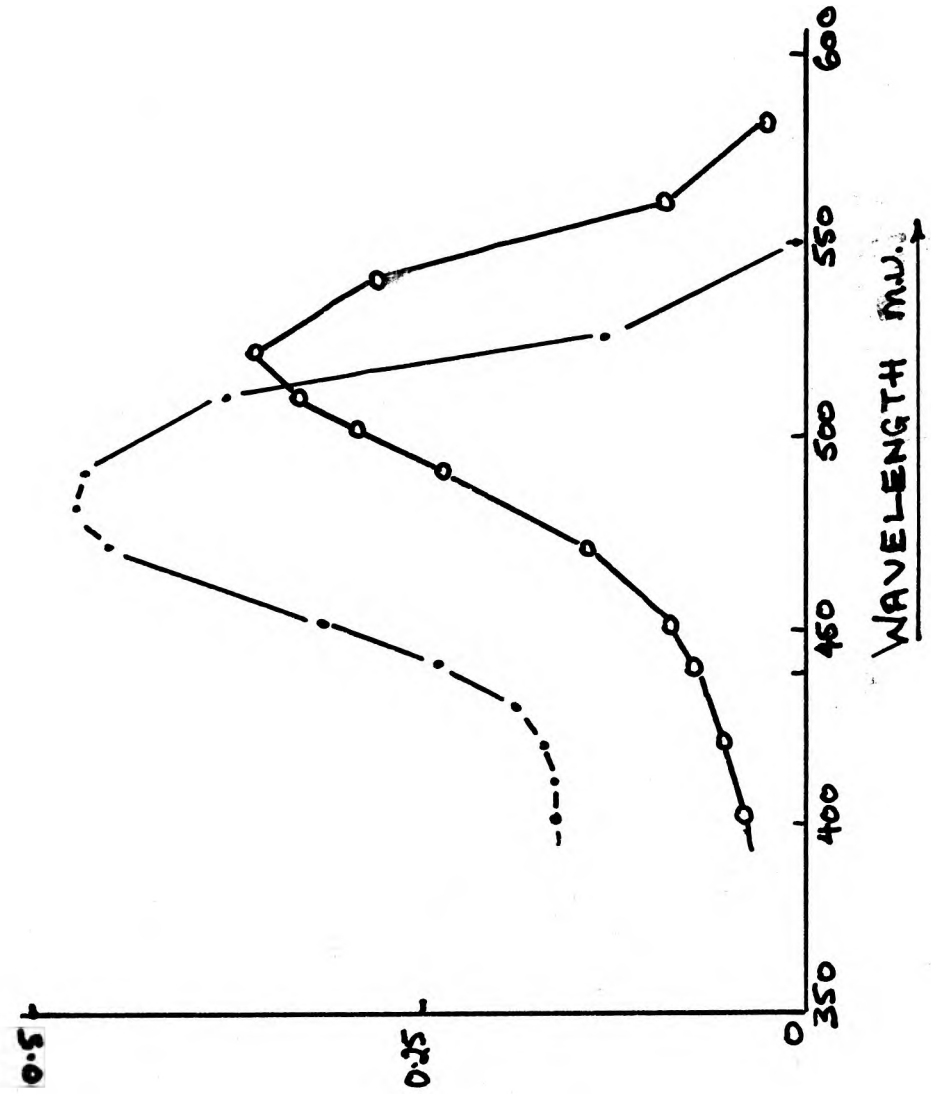
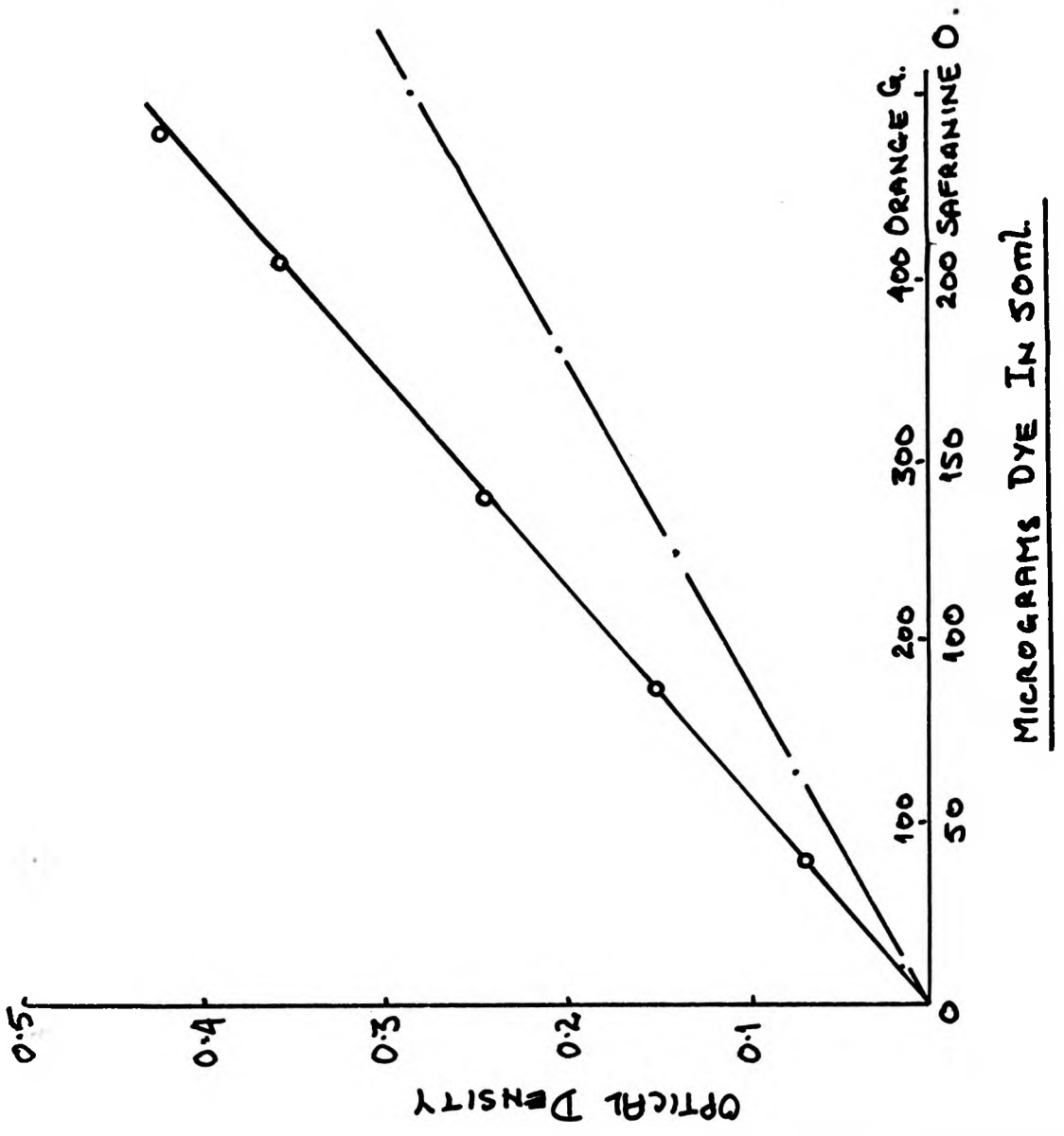


Fig. 16: Calibration curve.
Concentration of Orange G ——•——•——
and Safranine O ——o——o——
is plotted against the optical density
at 480 mu. and 520 mu. respectively.



e) Dye binding experiments:-

A modification of the method of Fraenkel-Conrat & Cooper (14) was used. Spectral and calibration curves of Orange G and Safranin O were prepared (Figs.15,16). Combination of dyes with the proteins of the hard tissues and demineralisation of these tissues were carried out simultaneously.

In the reaction with 'Orange G' (obtained from G.T. Gurr, England) which combines with the basic groups of proteins, known weights of tissue containing about 2 to 5 mg. of protein were placed in 2 ml. of 0.100% Orange G aq. in 50 ml. conical flasks containing 10 ml. of 20% citric acid (pH=1.9). The flasks were shaken for 8 hours and allowed to stand. At intervals of 24 hours the supernatant solutions were transferred to 25 ml. standard flasks and made up to volume with distilled water. An aliquot, (usually 5 ml.) was diluted to 50 ml. The amount of dye that had combined with the protein was measured by recording the optical density of the solution against a water blank at 480 mu. on the Backman spectrophotometer (Model DU) and comparing this value with that obtained from the smae dilution of the dye which had not been placed in contact with protein (~~Xxxx~~). The amount of dye bound to protein was expressed in microequivalents per gram of protein and plotted against the % dissolution (~~Xxxx~~). (p.121a)

The acidic groups were similarly measured by treating a known weight of tissue containing 2 to 5 mg. of protein with

2 ml/

2 ml. of 0.1% Safranin-O in 50 ml. conical flasks containing 10 ml. of 0.5M versene adjusted to pH 11.0. The flasks were shaken for 8 hours and allowed to stand. At intervals of 24 hours the supernatant solutions were transferred to 25 ml. standard flasks and diluted to the mark with distilled water. An aliquot, (usually 2 ml.) was diluted to 50 ml. before reading the optical density against a water blank at 520 mu. on the Beckman spectrophotometer. The amount of dye bound to the protein was expressed as microequivalents per gram of protein, and plotted against the percentage dissolution (Xxkgx).(p.121a)

The procedures, as described above, were also used for the investigation of the noncalcified collagens, both in the native state, and after modification by treatment with urea, acetic acid, alkali, heat and DNFB.

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Chapter VI.

Experimental Results.

1. Composition of the tissues.

(i) Gross Composition:

All the tissues studied contained collagen, together with a mucopolysaccharide ground substance. Except for cartilage, which contained about 20% of mucoprotein, the other connective tissues possessed very small quantities of mucopolysaccharides. Large amounts of lime salts were present in bone and dentin. The proportions in which the major constituents were found to be present in the dry, fat-free tissues are shown in Table 1.

Table 1 : Composition of the Connective Tissues

Tissue	% Protein	% acid mucopoly-saccharides	% Mineral Material	% Nitrogen
Ox-hide	99.5	0.20	-	18.0
Ox carti-lage	80(approx)	20	-	-
Ox-bone	23.0	0.10	77	3.70
Human dentin	20.0	0.60	80	3.40
Fish scales	44	-	56	7.01

(ii) The amino acid composition of some connective tissue proteins.

a) Distribution of amino acids: Fig. 1 shows the relative/

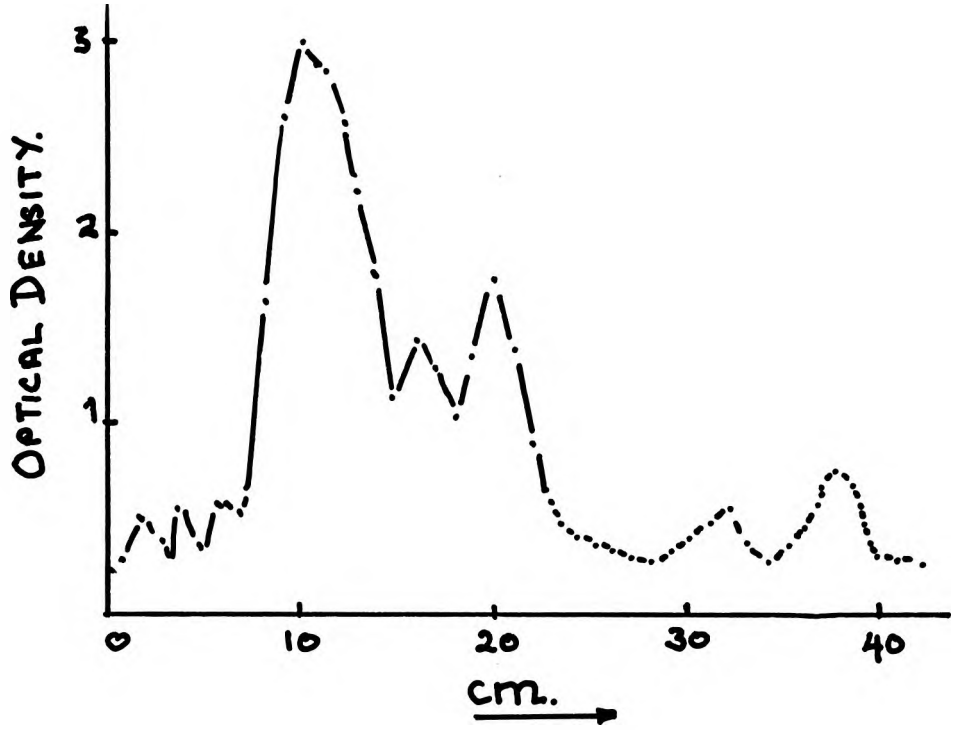


Fig. 1. Paper chromatogram of hydrolysate of dentin collagen developed with butanol: acetic acid:water, 4:1:5.

1 cm.x 3 cm. strips were eluted and treated with ninhydrin.

relative amounts of amino acids in dentin collagen as determined by paper chromatography.

The resolution was not sufficiently good for quantitative determination of the individual amino acids but semiquantitative analysis showed the presence of large amounts of glycine, moderate amounts of alanine, glutamic acid, the prolines and arginine, together with small amounts of the aromatic and basic amino acids. These results agree with similar analyses reported in the literature (1).

b) Determination of amino acids:

Table 2 shows the values obtained for some of the amino acids in various collagens expressed as g. amino acid per 100 g. of dry, fat-free protein. Most of the results were obtained by column and paper chromatographic fractionation of the DNP derivatives of the amino acids of the protein hydrolysates. These values also agree well with those reported in the literature (2).

Notes for Table 2: (on p. 102)

1. The ox-bone collagen and dentin collagen were obtained by demineralisation of the bone and dentin at 4°C with versene pH=7.3.

2. The fish scales were demineralised with 1N trichloracetic acid at 4°C.

Table 2/

Table 2: Partial amino acid composition of bovine, human and marine proteins: (Avg. of 4 Determinations)

Amino acid	Tissue				
	Ox-hide collagen	Ox-bone collagen	Human dentin	Fish scale gelatin	Fish scale ichthylepidin
Aspartic acid	6.7±0.40	6.6 ± 0.35	6.5 ± 0.40	7.0±0.38	6.5 ± 0.35
Serine	4.3±0.43	4.4 ± 0.37	3.2 ± 0.40	1.6 ± 0.45	4.2 ± 0.41
Threonine	2.2±0.30	2.0 ± 0.35	2.6 ± 0.30	1.6 ± 0.35	3.0 ± 0.30
Glutamic acid	10.5±0.25	10.8 ± 0.20	10.8 ± 0.28	10.5 ± 0.30	9.5 ± 0.35
Glycine	25.8±0.20	26.0 ± 0.20	24.0 ± 0.22	25.8 ± 0.25	24.0 ± 0.28
Alanine	10.1±0.40	10.0 ± 0.38	9.0 ± 0.50	12.6 ± 0.45	10.0 ± 0.50
Valine	2.3±0.21	2.5 ± 0.25	2.5 ± 0.23	2.2 ± 0.30	2.2 ± 0.30
Phenyl-alanine	2.1±0.30	2.2 ± 0.26	2.1 ± 0.26	Not determined	
Lysine	1.0±0.10	1.04 ± 0.18	1.02 ± 0.12	2.6 ± 0.25	1.6 ± 0.30

c) Determination of the amino acids by the method of Moore & Stein:

The amino acid compositions of gelatin and ichthylepidin, both extracted from fish scales, were determined by this method. The results, xr expressed as g. amino acid/100 g. of dry protein, are shown in Table 3. The elution curves obtained with ichthylepidin, are shown in Figs. 2 (i), (ii), and (iii).

Table 3/

Fig. 2 (i): Elution curve of the hydrolysate of diethylaminoethyl cellulose chromatographed on 100 cm x 0.9 cm column of Dowex 50 using citrate buffer, pH 3.27 at 37°C

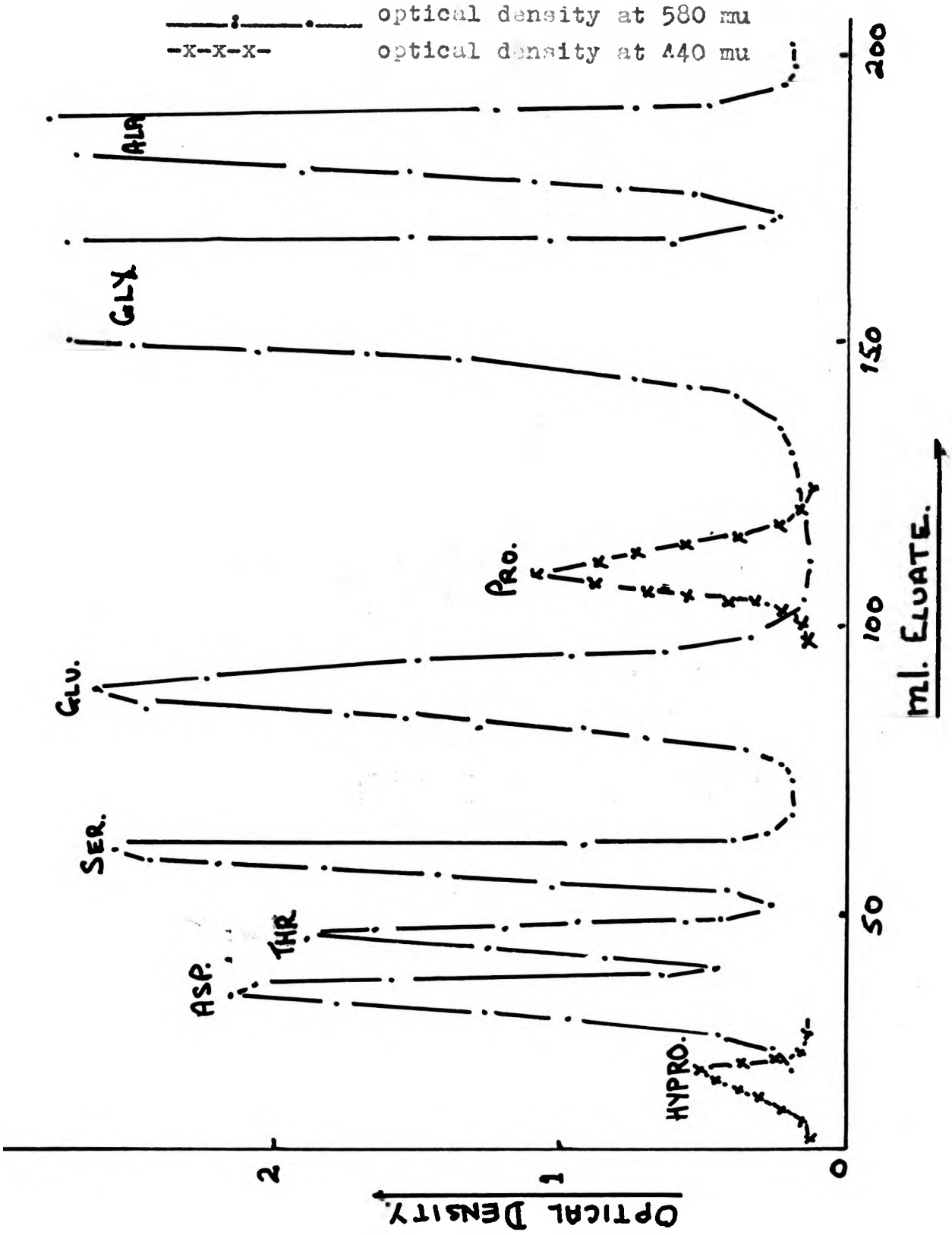


Table 3: Amino acid composition of Fish-scale proteins.

Amino acid	Tissue	
	Gelatin	Ichthylepidin
	Ave. of 3 determinations	Ave. of 4 determinations
Hydroxyproline	8.40 ± 0.80	9.8 ± 0.70
Aspartic acid	7.70 ± 0.30	6.5 ± 0.28
Threonine	1.62 ± 0.25	3.14 ± 0.29
Serine	1.44 ± 0.50	4.56 ± 0.23
Glutamic acid	10.5 ± 0.25	9.56 ± 0.50
Proline	12.9 ± 1.4	13.34 ± 0.18
Glycine	25.8 ± 0.98	24.0 ± 0.40
Alanine	12.6 ± 0.15	9.30 ± 0.93
Valine	2.20 ± 0.15	2.44 ± 0.13
Methionine	1.36 ± 0.10	2.06 ± 0.12
Isoleucine	1.27 ± 0.12	1.46 ± 0.06
Leucine	2.26 ± 0.21	2.84 ± 0.02
Tyrosine	0.50 * ± 0.21	1.80, 1.92 * ± 0.20
Phenylalanine	Not determined	2.96 ± 0.10
Histidine	1.70 ± 0.15	1.57 ± 0.10
Hydroxylysine	2.63 ± 0.80	1.63 ± 0.70
Lysine	3.15 ± 0.25	3.69 ± 0.13
Arginine	9.2 ± 2.0	7.33 ± 2.0
Tryptophan	0.00 * ± 0.10	0.50 * ± 0.1
Cystine	0.00 * ± 0.28	0.50 * ± 0.2
Cysteine	0.00 * ± 0.02	0.03 * ± 0.02
Amide nitrogen	0.68 ± 0.05	0.73 ± 0.05
Total nitrogen	16.0 ± 0.10	15.9 ± 0.10

Notes: 1. Over 98% of the total protein nitrogen was accounted for.

* 2. These values were obtained by the chemical and ultra-violet absorption methods.

The values obtained for the amino acid composition ~~xhxx~~ show that, as is well known (2c), there are marked differences

between/

Fig. 2 (ii): Elution curve contd.
Buffer changed from pH 3.27 to pH 4.25 at point X.
Temperature changed to 70°C at point Y.

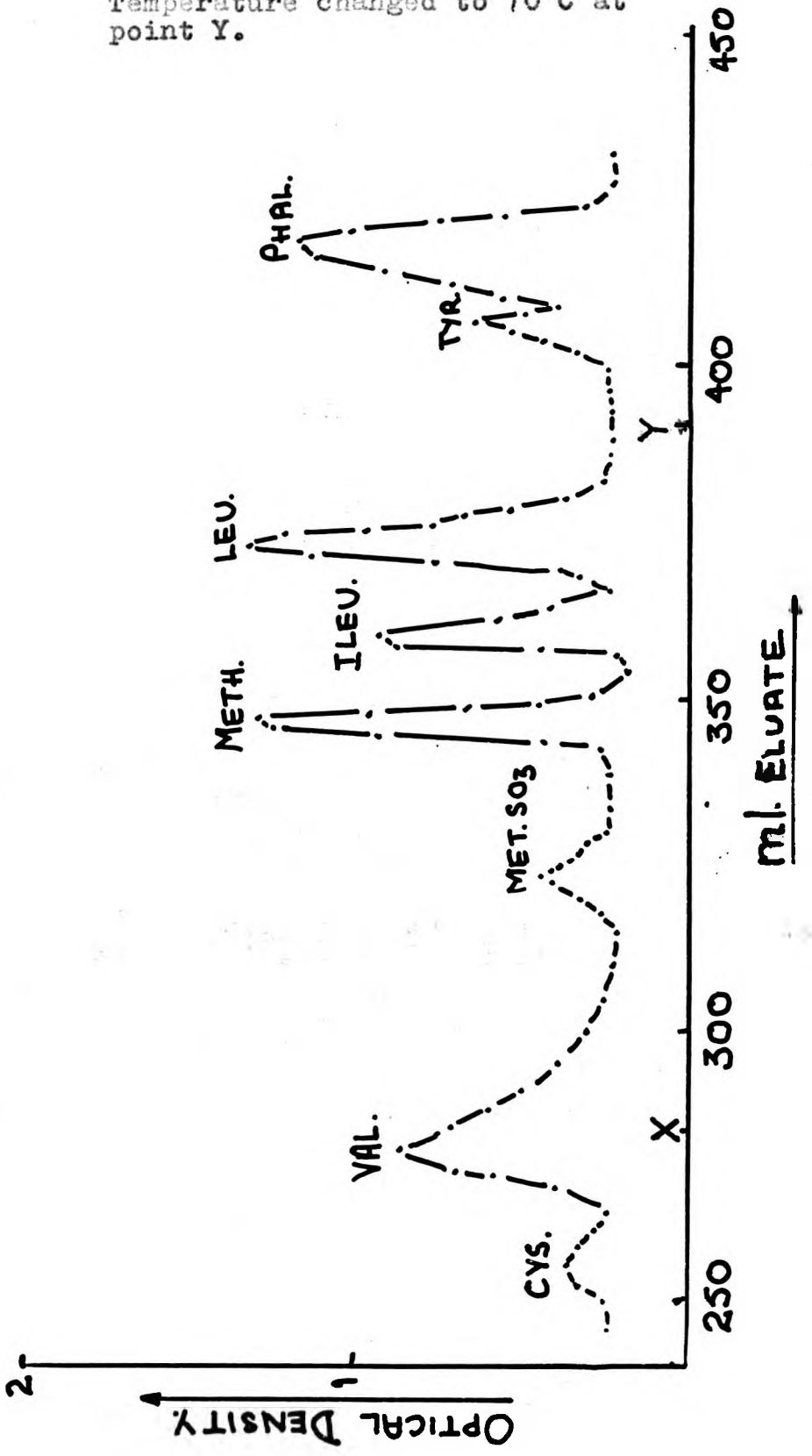
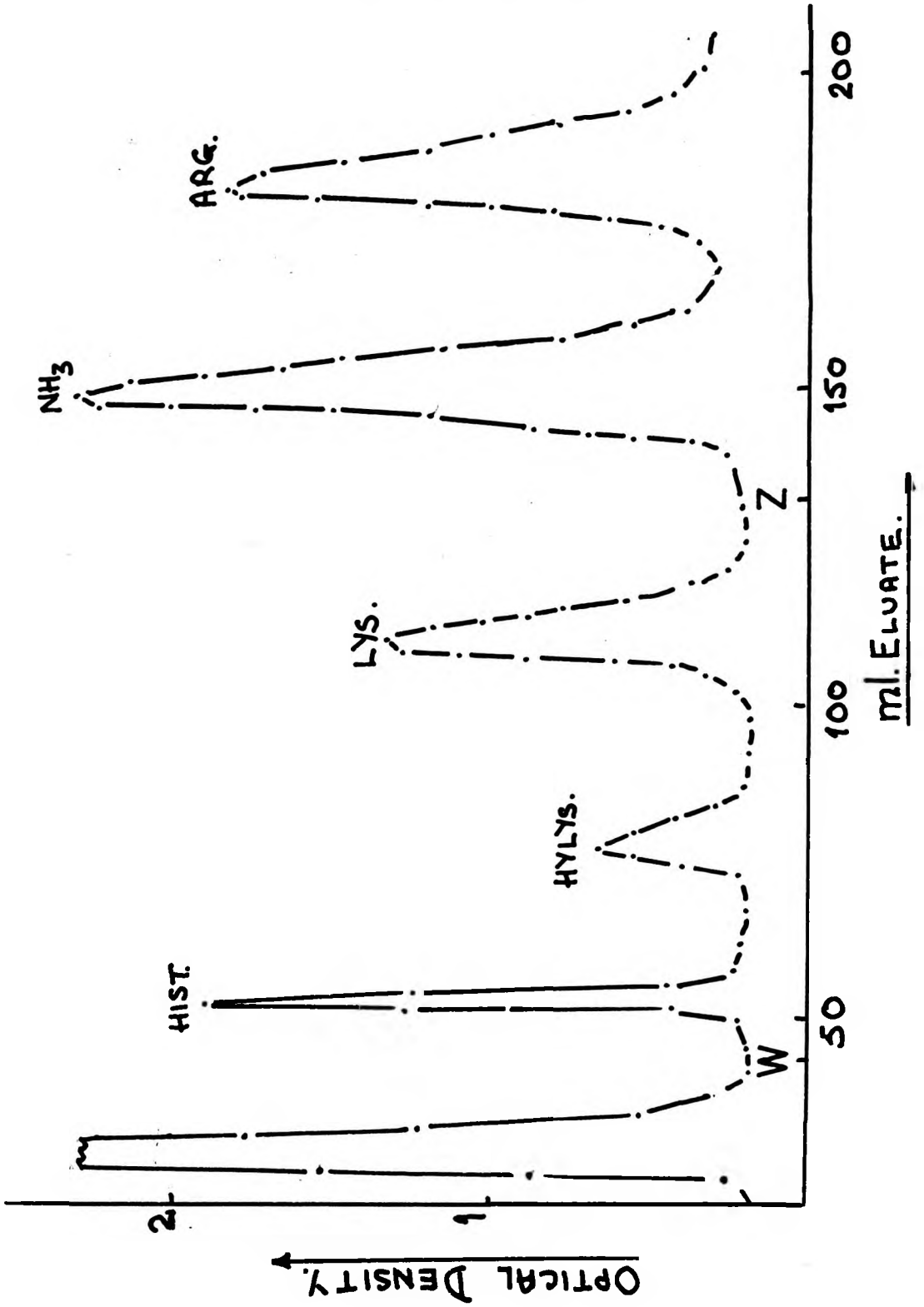


Fig. 2 (iii): Elution curve of hydrolysate of ichthylepidin chromatographed on 15 x 0.9 cm column of Dowex 50. Citrate Buffer pH 5.0 changed to phosphate buffer pH 6.8 at point W Phosphate buffer changed from pH 6.8 to pH 6.5 at point Z.



between mammalian collagen (bovine and human) and marine collagens. The main differences are the low content of hydroxyproline in the fish scale proteins and the higher amounts of the other hydroxy amino acids. Ichthylepidin was found to have a fairly typical fish-collagen amino acid composition, except that it contained appreciable amounts (0.5%) of cystine. The amino acid composition of elastoidin, an insoluble protein, found in shark fins (3), resembles that of ichthylepidin, except that elastoidin has three to four times as much tyrosine. The gelatin fraction, however, in addition to its low content of hydroxyproline had only very small quantities of the other hydroxy amino acids except for hydroxylysine

2. Demineralisation studies.

(i) Dissolution in acids and versene solutions:

The protein portion of the hard tissues was isolated by removing the mineral fraction. Table 4 and Figs. 3, 4, and 5 show the rate of demineralisation of the hard tissues in each of the decalcifying solutions at 4°C. The % dissolution was obtained by measuring the loss in weight. Ox-hide was similarly treated in order to determine the effect of the demineralisation reagents on soft tissue collagen

Table 4/

Fig. 3: Rate of dissolution of dentin treated at 4°C with:-

1N HCl --o-o-o

1N trichloracetic acid —·—·—

0.5M versene pH 7.3 --·---·---·--

The versene solution was renewed daily.

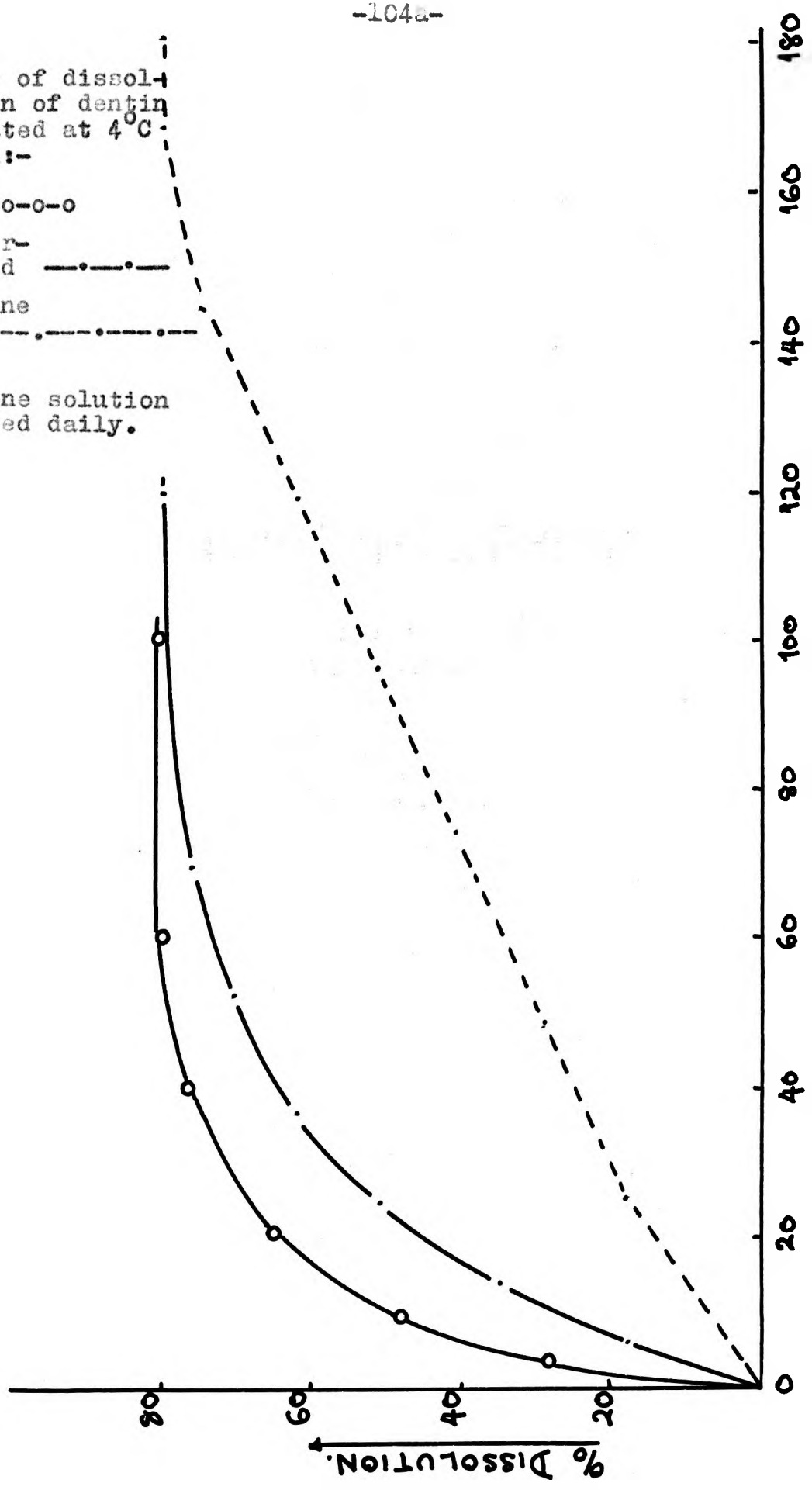


Table 4: Treatment of tissues at 4°C with demineralising reagents:

Tissue	Demineralising Agent																	
	1N Hydrochloric acid								1N Trichloracetic acid									
Human dentin:																		
Time(Hrs)	0	4	9	20	40	60	100		0	6	14	25	38	69	105			
% Dissolution:	0	28	48	64	76	80	80		0	18	35	51	62	76	80			
Ox-bone:																		
Time(Hrs)	0	1	2	4	6	8	10	12	0	1	2	4	6	8	10	12	14	18
% Dissolution:	0	20	34	51	62	70	76	77	0	10	20	35	46	55	62	68	73	77
Fish scales:																		
Time(Hrs)									0	.25	.5	1	1.5					
% Dissolution:									0	41	52	56	56					
Ox-hide:																		
Time(Hrs)	0	40	80	100	120				0	48	80	100	120					
% Dissolution:	0	1	1.5	2	2.2				0	1.1	1.3	2	21					

Tissue	Demineralising Agent								
	0.5M Versene pH=7.3.								
Human dentin:	Time(Hrs)	0	24	48	72	96	120	144	168	180
	% Dissolution:	0	18	29	41	51	62	75	80	80
Ox-bone:	Time(Hrs)	0	24	48	72	96	120	144		
	% Dissolution:	0	24	44	58	68	75	77		
Fish scales:	Time(Hrs)	0	10	20	30	40	50			
	% Dissolution:	0	37	45	62	56	56			
Ox-hide:	Time(Hrs)	0	40	80	100	120				
	% Dissolution:	0	0.5	0.6	1.0	1.1				

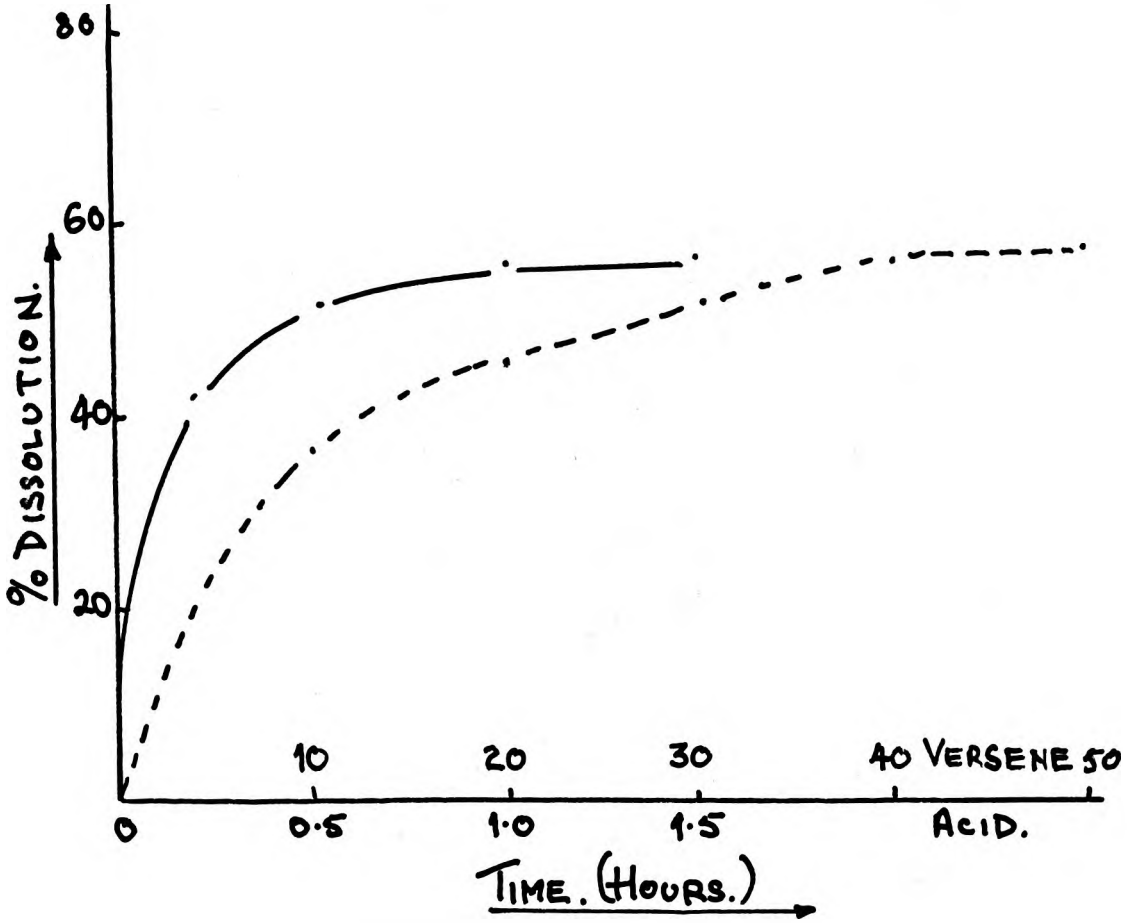


Fig. 5: Rate of dissolution of fish scales treated at 4°C with:- 1N trichloracetic acid ——•——•
0.5M Versene pH=7.3 ----------

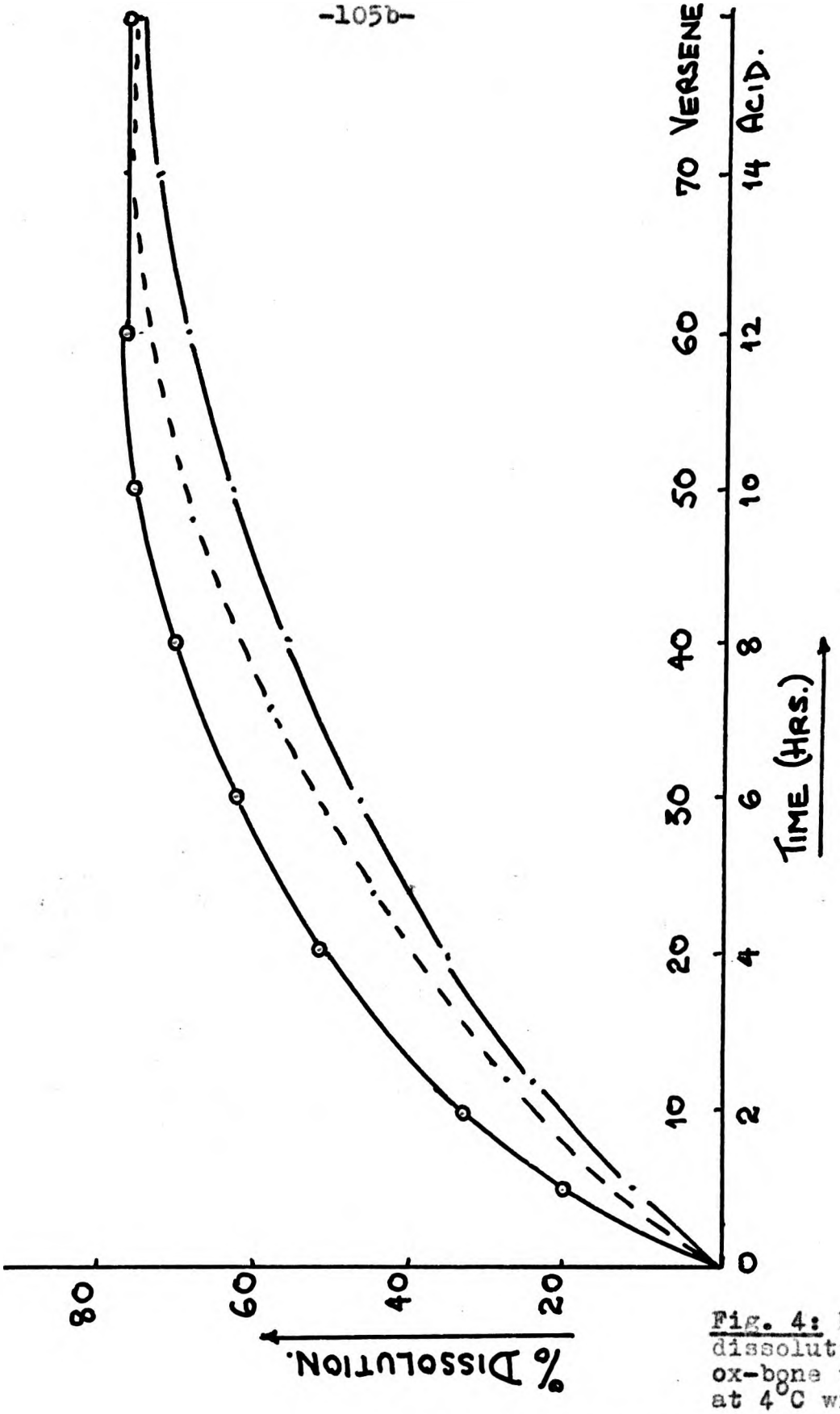


Fig. 4: Rate of dissolution of ox-bone treated at 4°C with:

1N Hydrochloric acid ——o——o——

1N trichloracetic acid —·—·—·—

0.5M versene ——·——·——·——

After being used for demineralisation, the acidic solutions were decanted from the tissue and were tested for the presence of free amino acids and carbohydrate material, by treating aliquots with ninhydrin and anthrone (Table 5). The dissolution of dentin in hydrochloric acid was followed by determining the amount of Ca^{++} and $PO_4^{\equiv}$ dissolved, and plotting this against the percentage weight loss (Fig. 6). It is seen that the ratio of amounts of calcium to the amounts of phosphorus dissolved by the acid, varied during demineralisation. The ratio was greater during the initial stages of decalcification implying that calcium ions were preferentially dissolved. This is in accordance with the view that hydrated hydrogen ions can easily replace calcium ions in the hydroxyapatite lattice (4).

Table 5/

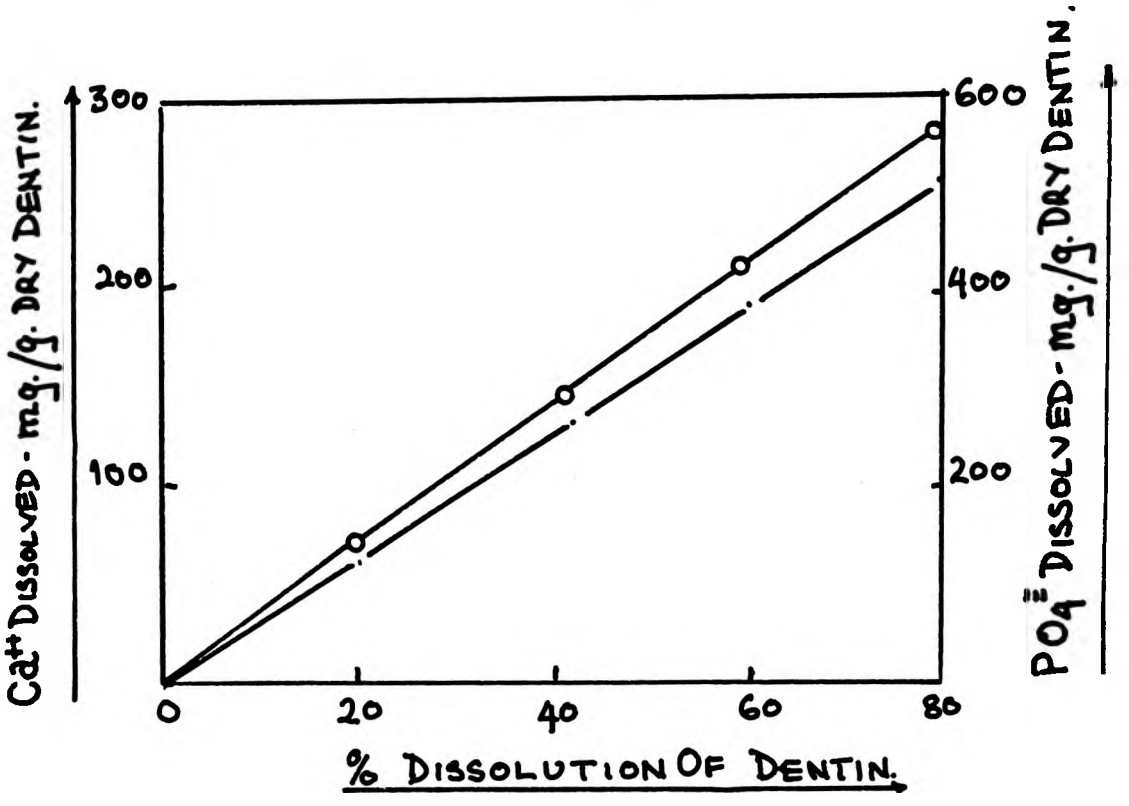


Fig. 6: Dissolution of Calcium —o—o—
and Phosphate —•—•—

from human dentin
treated with 1N hydrochloric
acid at 4°C.

Table 5: Analysis of Supernatant Solutions:

Tissue		Demineralising Agents.						
		1N Hydrochloric acid				1N Trichloracetic acid		
Ox-bone: (2g.sample)	% Dissolution	34	62	76		35	62	76
	Ninhydrin	1+	1^+	2^+		1^+	1^+	1^+
	Anthrone	0	0	0		0	0	0
Dentin: (.2g.sample)	% Dissolution	20	41	60	80	35	55	77
	Ca^{++} dissolved mg/g.dentin	80	145	215	290			
	$PO_4^{\equiv}$ dissolved mg/g.dentin	130	270	380	530			
	Ca/P ratio in supernatant	1.82	1.60	1.68	1.63			
	Ninhydrin	0	1^+	1^+	2^+	0	1^+	1^+
	Anthrone	0	0	1^+		0	1^+	0
Fish Scales: (.6g.sample)	% Dissolution					20	42	56
	Ninhydrin					0	0	0
	Anthrone					0	0	1^+
Ox-hide COLLAGEN (2g.sample)	Time of soaking	40	80	100	120	48	100	120
	Ninhydrin	0	1^+	1^+	2^+	0	1^+	1^+
	Anthrone	0	0	1^+		0	0	1^+

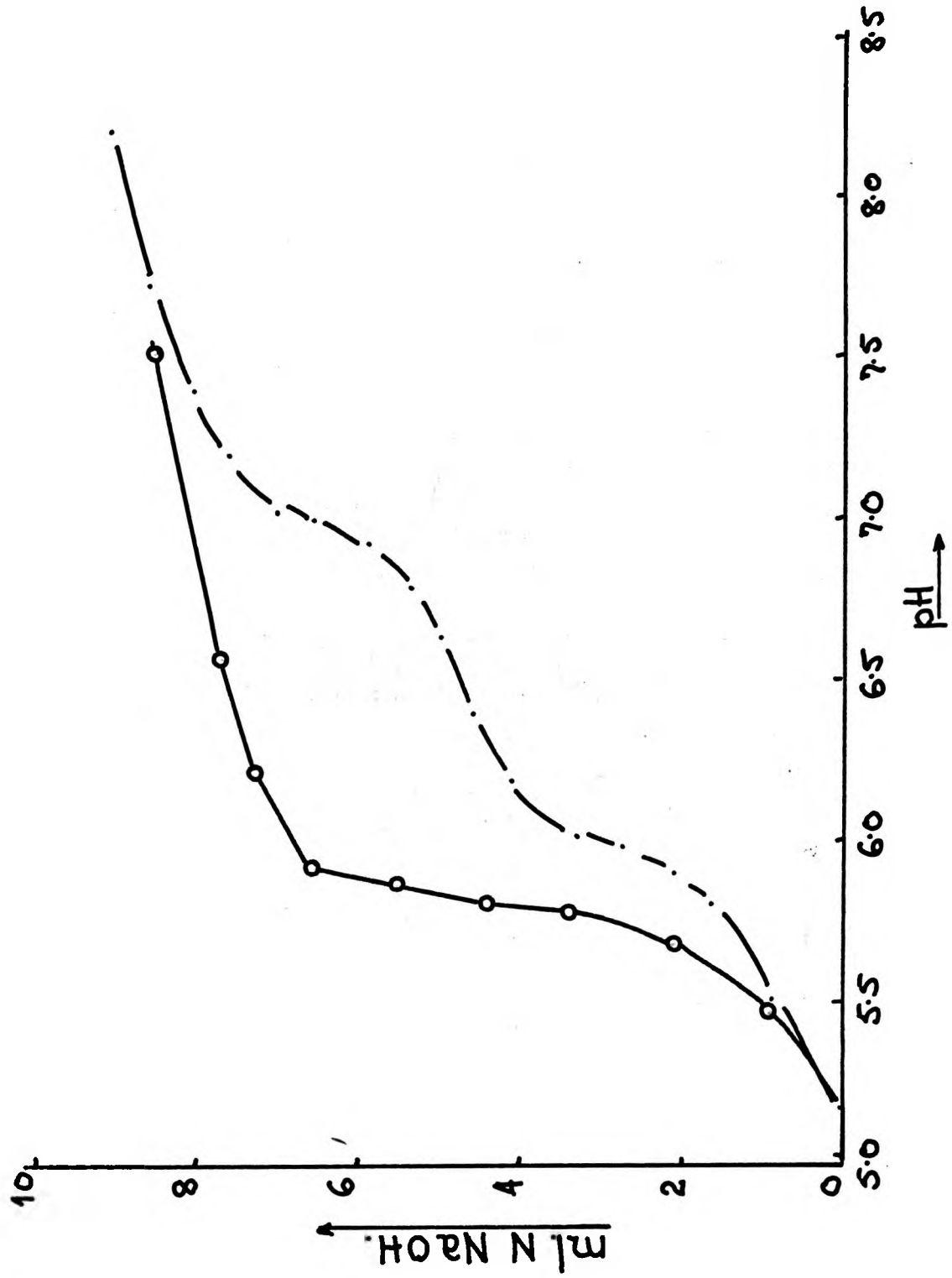
Notes: 1. 1^+ denotes 0.01 mg. nitrogen or 10 microgram Carbohydrate calculated as glucose.

2. The Ca^{++} and $PO_4^{\equiv}$ estimations were accurate to within 4%.

Paper chromatographic analysis showed that the versene supernatant solutions of contained only minute traces of ninhydrin and anthrone positive materials.

These/

Fig. 7: Titration curves of 1M phosphate buffer, -·-·-·-
and 1M acetate buffer —o—o—



These demineralisation studies show that hard tissues may be demineralised with acid at low temperatures, without large scale hydrolysis of protein peptide bonds, but decalcification with versene at neutral pH is preferable.

(ii) The dissolution of hard tissues in buffer solutions.

The dissolution of finely divided human dentin and ox-bone in 1M acetate and phosphate buffers at room temperature, was determined. Fig. 7 shows the titration curves of the buffers used. The percentage dissolution of the tissue after soaking in each buffer solution for 4 hours was recorded (Table 6) and plotted against the pH value of the buffer solution (Fig. 8). The pH of the buffer solution remained constant throughout the experiment. Bone and dentin gave almost identical results in both sets of buffers.

Table 6: Dissolution of human dentin and ox-bone in phosphate and acetate buffers:

pH value	% Dissolution in 4 hours.				
	Ox-bone	Dentin	Deorganicised bone	Deorganicised dentin	Synthetic hydroxyapatite
4.7	30	31	-	-	-
5.2	22	21	14	13	5
5.5	19	19	10	10	2
5.8	17.5	18	7.0	6.5	1
6.1	17.0	17.5	5.0	4.5	0.5
6.4	17.6	17.5	3.0	3.0	0.1
6.7	16.0	16.0	2.0	1.5	0.0
7.0	12.0	11.0	1.0	1.0	0.0
7.3	9.0	7.0	0.0	0.1	-
8.0	5.5	4.5	0.0	0.0	-

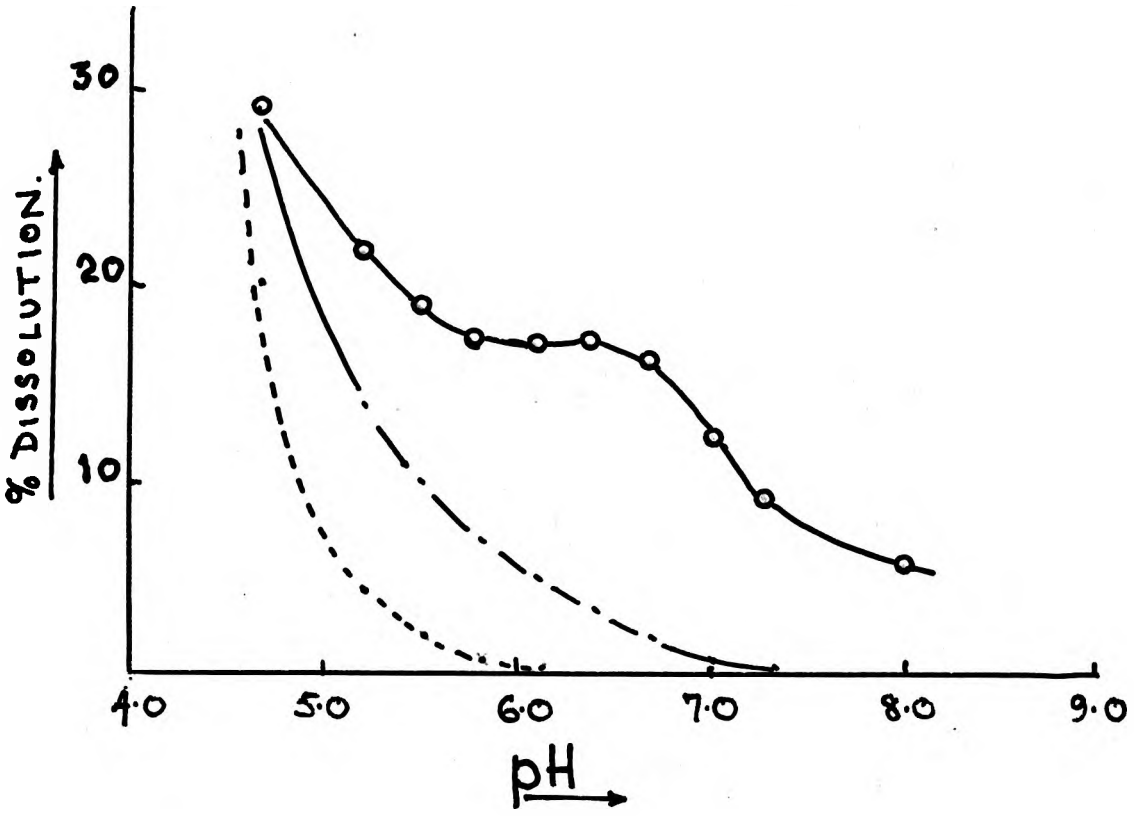


Fig. 8: The dissolution of human dentin in 1M phosphate and acetate buffers.

TABLE 6a: Dissolution of Ca^{++} and $PO_4^{=}$ in buffer solutions.

pH	Ca^{++} mg./g. dentin dissolved by phosphate buffer.	$PO_4^{=}$ mg./g. dentin dissolved by acetate buffer
6·1	68	110
6·4	65	105
6·7	64	100

Demineralisation of ox-bone and dentin with phosphate buffer solutions gave an unexpected plateau region in the pH range of 5.8 - 6.5 (Fig. 8). As this plateau was not obtained with 'synthetic' hydroxyapatite or 'deorganicised' bone and dentin, it is thought that it may be due to the presence of the organic fraction of the tissues. Small amounts of ninhydrin material were also dissolved by the buffer.

3. Moisture content.

The residual moisture contents of some of the tissues after drying in air at 40% humidity, were determined (Table 7)

Table 7: Residual moisture contents of bovine, human and marine proteins.

Tissue	% Moisture
Ox-hide	18
Human dentin	8
Demineralised dentin	16
Ox-bone	10
Demineralised Ox-bone	17
Fish scales	6.8
Demineralised Fish scales	9.0
Fish gelatin	8.6
Fish ichthylepidin	8.6

It/

It is seen that the moisture content of all the hard tissues is increased by the removal of mineral material. As the polar and ionic groups of collagen are capable of taking up water molecules, the increase in moisture content of hard tissues with the percentage demineralisation also suggests that polar groups may be involved in the combination of mineral material.

4. The concentrations of terminal and ϵ-amino groups of Connective Tissue Proteins:-

Soft and hard tissue collagen, both in the freshly prepared state, and after treatment with the demineralising solutions, were assayed for the presence of N-terminal and ϵ N amino groups by treatment with DNFB.

A. N-terminal studies.

(i) Ox-hide collagen:

1. No N-terminal amino acids were detected in ox-hide collagen in the native state. This result is in accordance with the results of other workers (5).

2. No liberation of N-terminal amino groups occurred when the ox-hide collagen was treated at $4^{o}C$ with versene at pH 7.3 or 8.5, under the same conditions of demineralisation that were used to decalcify the hard tissues. Soaking in 1N

hydrochloric/

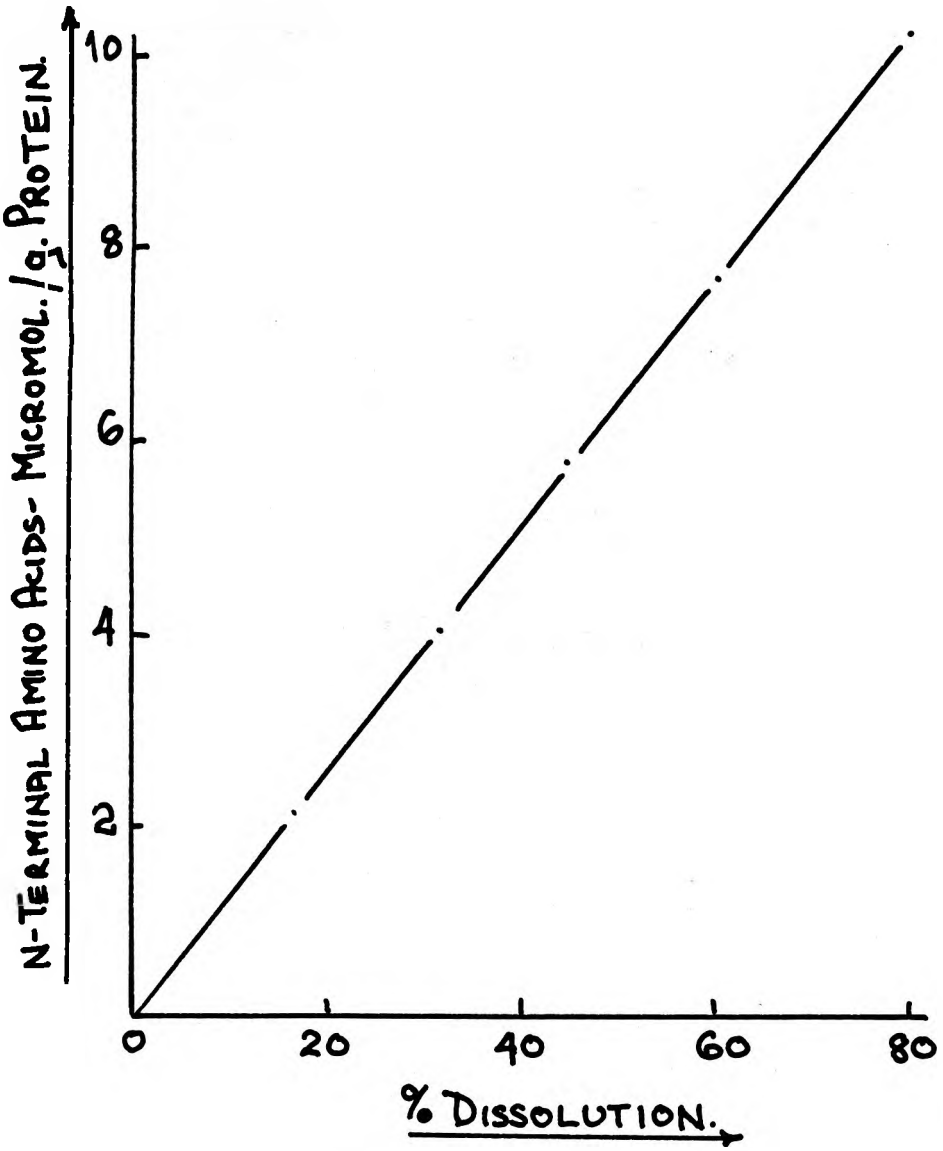


Fig. 9: Lib ration of N-terminal amino groups during demineralisation of dentin.

Table 8: N-terminal amino groups of human dentin:

% Dissolution	Micromol/g.
16	2.1
31	4.0
44	5.8
60	7.7
80	10.2

hydrochloric acid and 1N trichloracetic acid at 4°C for 100 hours produces a very small number of N-terminal groups, mainly those of aspartic and glutamic acids in a concentration of about 1 micromol/g. protein.

3. Modification of ox-hide collagen by heating in water or by treatment with urea, acetic acid, or hyaluronidase, did not increase the values for N-terminal amino acids, mainly aspartic and glutamic acids, to above 3 micromol/g. It is thought that these groups may be produced by a small amount of peptide bond hydrolysis.

4. No evidence for protein decomposition during dinitrophenylation was detected.

(ii) Human dentin:

1. Fully calcified dentin resembled ox-hide collagen in that no N-terminal groups were detected.

2. During the demineralisation of dentin small amounts of aspartic and glutamic acids were detected, and these increased in a linear manner to a maximum of 10 micromol/g. protein, when plotted against the percentage dissolution (Table 8)(Fig. 9). It is possible that these groups are the result of peptide bond hydrolysis, but this is unlikely when one considers the linearity of the graph, and the fact that no

increase/

increase in terminal groups took place in similarly treated ox-hide collagen treated with versene solutions. It is, therefore, possible that these amino groups are involved in the combination of mineral material with the organic matrix. The same results were obtained whether acid or neutralised versene (pH 7.3) were used as demineralising agents.

3. However, if fully demineralised dentin collagen was allowed to stand at room temperature for two weeks, or longer, the number of N-terminal amino groups available to DNFB decreased by 60%.

4. The same concentration of N-terminal amino acids was obtained whether demineralisation and dinitrophenylation were carried out separately, or simultaneously (see p. 93-94).

5. No evidence for protein decomposition during dinitrophenylation was detected.

(iii) Ox-bone:

In fully calcified ox-bone extremely small amounts of terminal aspartic acid, about 1-2 micromol/g. protein, were detected, but these remained constant during demineralisation. It is thought that these terminal groups may be due to the incomplete removal of some vascular components of bone tissue.

B. The/

B. The availability to DNFB of protein ε amino groups.

(i) Ox-hide collagen:

1. Only 55%, i.e. 182 micromol/g. protein, of the total number of lysyl and hydroxylysyl ε amino groups present in ox-hide collagen combined with DNFB. The remaining lysyl and hydroxylysyl residues reacted quantitatively with DNFB only after hydrolysis of the DNP protein. In this way it was possible to account for over 95% of the lysine and hydroxylysine originally present.

2. The percentage of ε amino groups available to DNFB was not changed by either increasing the pH to 10 during coupling with DNFB, or by performing the reaction in aqueous medium. Modification of the collagen by heat shrinkage, or treatment with urea and acetic acid and alkali had no effect, as reported by other investigators (5). Bowes & Moss suggest that all the lysyl residues combine with DNFB, but large losses of ε N DNP lysine occur during acid hydrolysis,(even after applying the predetermined correction factor). This is considered unlikely, as their own data show that prolonging the time of hydrolysis from 16 to 64 hours results in a slightly higher recovery of ε N DNP lysine. The existence of ε amino peptide links (13) is unlikely, as titration data show that these groups are readily discharged by hydroxyl ions. No evidence was found for the existence of α DNP lysine or α ε di DNP lysine, or resistant DNP lysine peptides.

3/

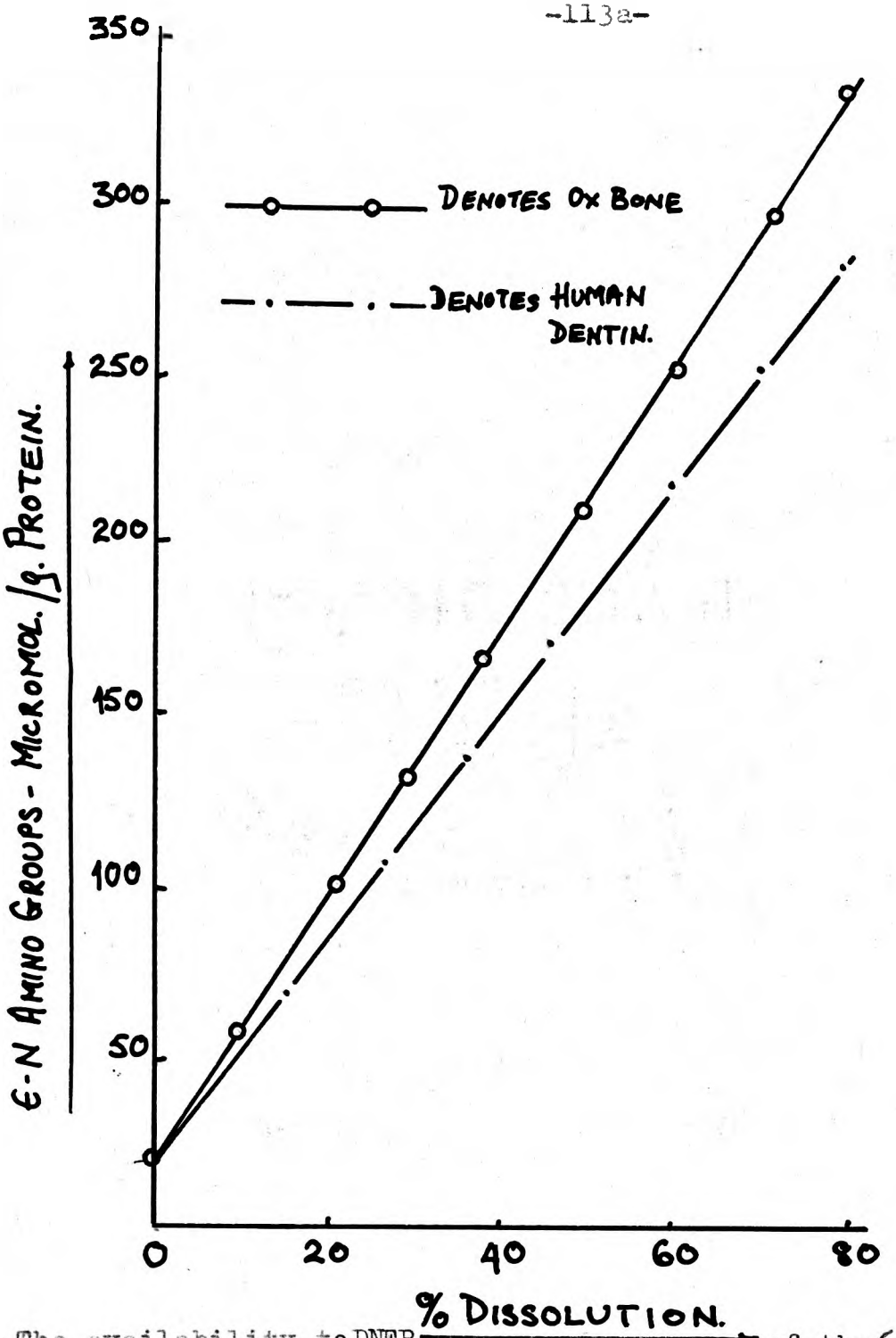


Fig.10: The availability to DNFB of the ε amino groups of lysine and hydroxylysine during demineralisation

Table 9: ε amino groups available to DNFB in ox-bone and dentin during demineralisation:

Ox-bone:	% Dissolution	0	10	21	30	38	50	60	72	80
	$[\varepsilon NH_2]$ in micromol/g. protein:	25	51	100	130	160	205	245	280	330
Dentin:	% Dissolution	15	26	36	455	599	72	80		
	$[\varepsilon NH_2]$ in micromol/g. protein	60	100	135	165	210	245	270		

3. Treatment of the protein with the demineralising solutions before coupling with DNFB also had no effect on the number of ε amino groups found.

4. The molar ratio of ε N DNP lysine to ε N DNP hydroxylysine was found to be 3.9 which is the same as the ratio of lysine to hydroxylysine calculated from the amino acid composition of the protein (3).

(ii) Dentin:

1. Fully calcified dentin possessed small amounts of ε amino groups, about 25 to 30 micromol/g. protein, and in this connection it is noted that the presence of some uncalcified collagen fibres have been previously reported in mature dentin (6).

2. As demineralisation progressed, the number of ε amino groups available to DNFB increased in a linear manner from 26 to 270 micromol/g. protein, when plotted against the percentage dissolution (Table 9, Fig. 10). Thus in the fully demineralised state over 93% of the ε amino groups reacted with DNFB. However, on allowing the demineralised protein to stand at room temperature, the availability of the ε amino groups to DNFB slowly decreased to 160 micromol/g. after two weeks, and gradually to 110 micromol/g. after 1 year.

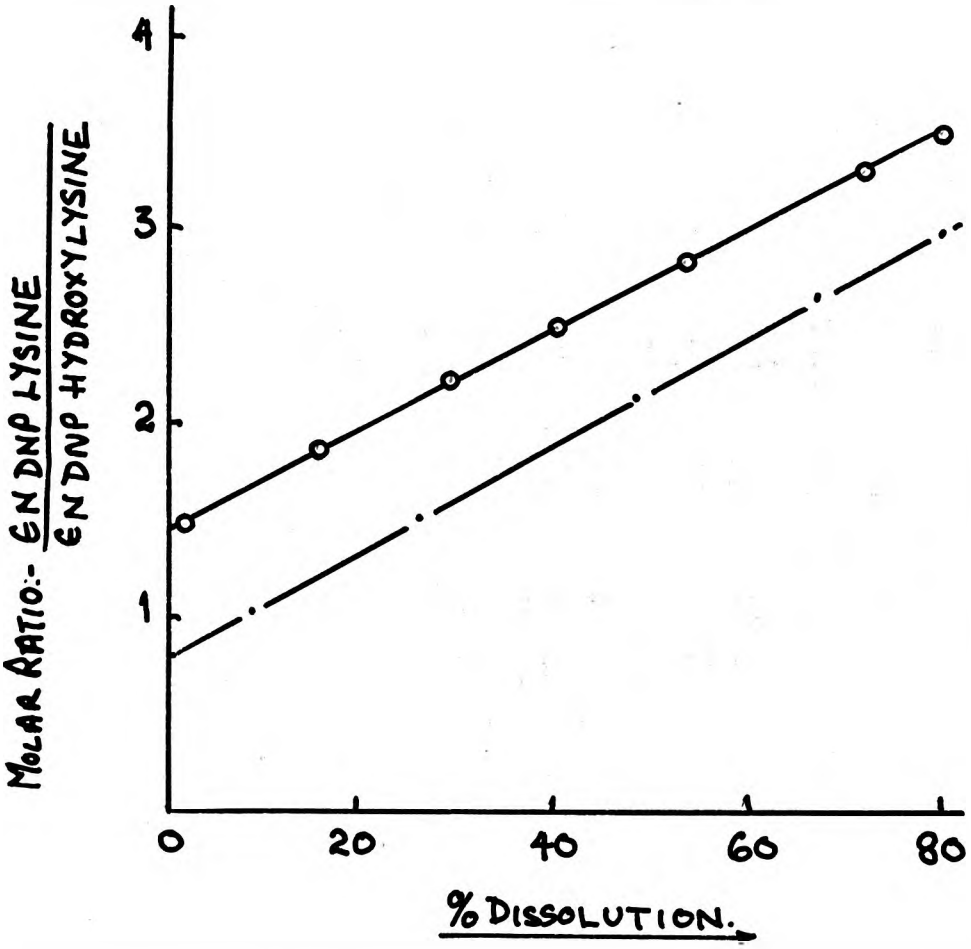


Fig. 11: Relative availability to DNFB of lysyl and hydroxylysyl ε amino groups of dentin collagen during demineralisation:

—·—·— Denotes dentin treated first with demineralising reagents and then with DNFB

—o—·—o Denotes dentin treated simultaneously with versene (pH 8.8) and DNFB.

Table 10; Molar ratios of εN DNP lysine and εN DNP hydroxylysine obtained from the dinitrophenylation of human dentin

Simultaneous demineralisation and dinitrophenylation	% Dissolution	0	16	30	41	54	62	80
	Ratio	1.5	.9	2.2	2.5	2.8	3.3	3.5
Dinitrophenylation and demineralisation carried out separately	% Dissolution	0	10	26	49	67	80	
	Ratio	0.81	1.0	1.5	2.1	2.6	2.9	

3. The molar ratio of ε N DNP lysine to ε N DNP hydroxylysine obtained from the dinitrophenylation of dentin collagen increased in a linear manner from 0.8 to 2.9 (theoretical maximum 3.7 (2b)), when plotted against the percentage dissolution of mineral material in each of the demineralising solutions (Fig. 11). If demineralisation and dinitrophenylation were carried out simultaneously the ratio increased linearly from 1.5 to 3.5 (Fig. 11). This implies that, especially in the early stages of decalcification, the availability to DNFB of hydroxylysyl ε amino groups exceeds that of the lysyl ε-amino groups in spite of the much higher concentration of lysine residues. Klotz (11) has shown that the polar side chains of amino acid residues of many different proteins may act as binding sites for ions. As the ~~xxx~~ anionic binding power of hydroxylysine is weaker than that of lysine (14), it is possible that if both amino groups bind lime salts, the anions bound to hydroxylysine would be liberated preferentially from hydroxylysine than from lysyl amino groups during demineralisation.

(iii) Ox-bone:

In the fully calcified tissue, only 20 to 25 micromols/g. of protein of ε amino groups was detected. This value increased with demineralisation, but here it was found that the number of ε amino groups available to DNFB was profoundly influenced by the demineralising agent used, and whether dinitrophenylation was performed some time after, or simultaneously with the

demineralisation/

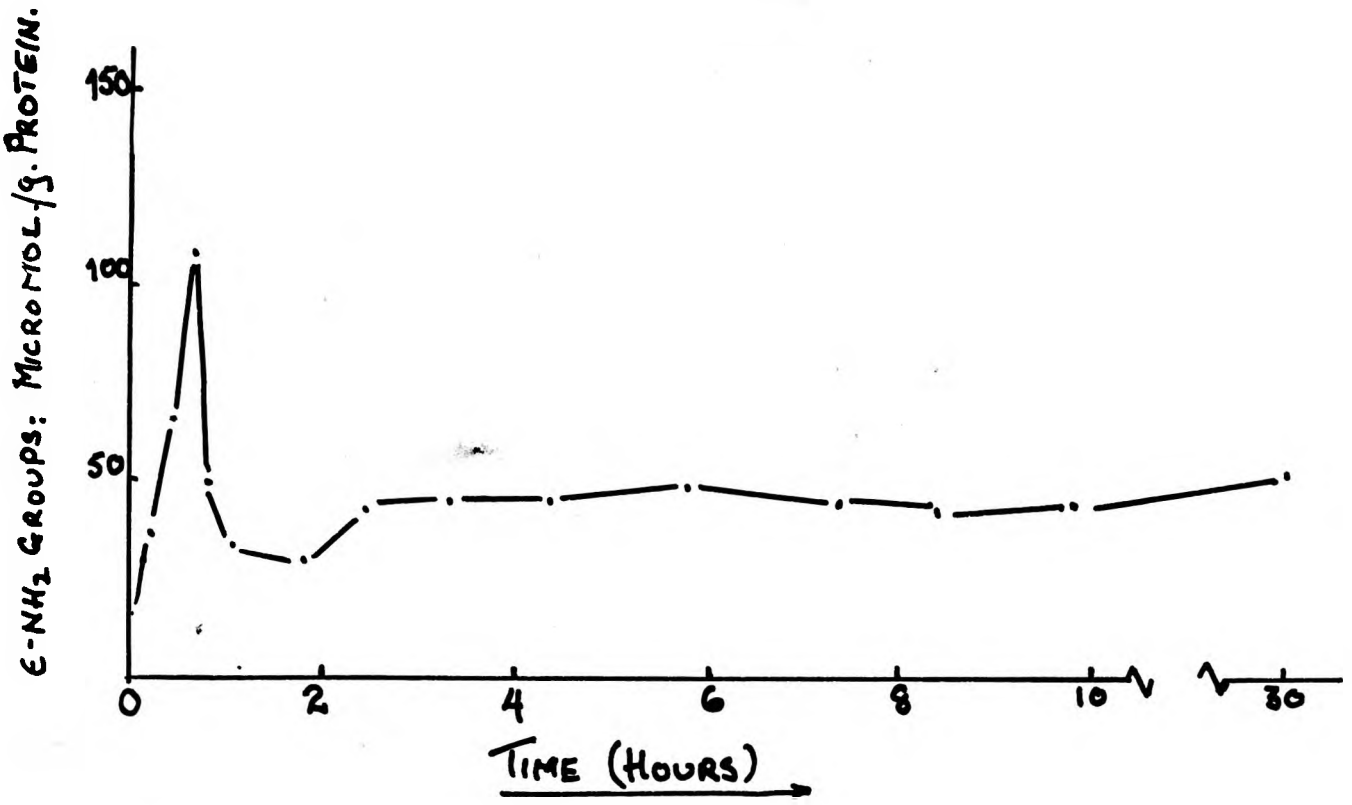


Fig. 12: ε amino groups of acid treated ox-bone collagen.

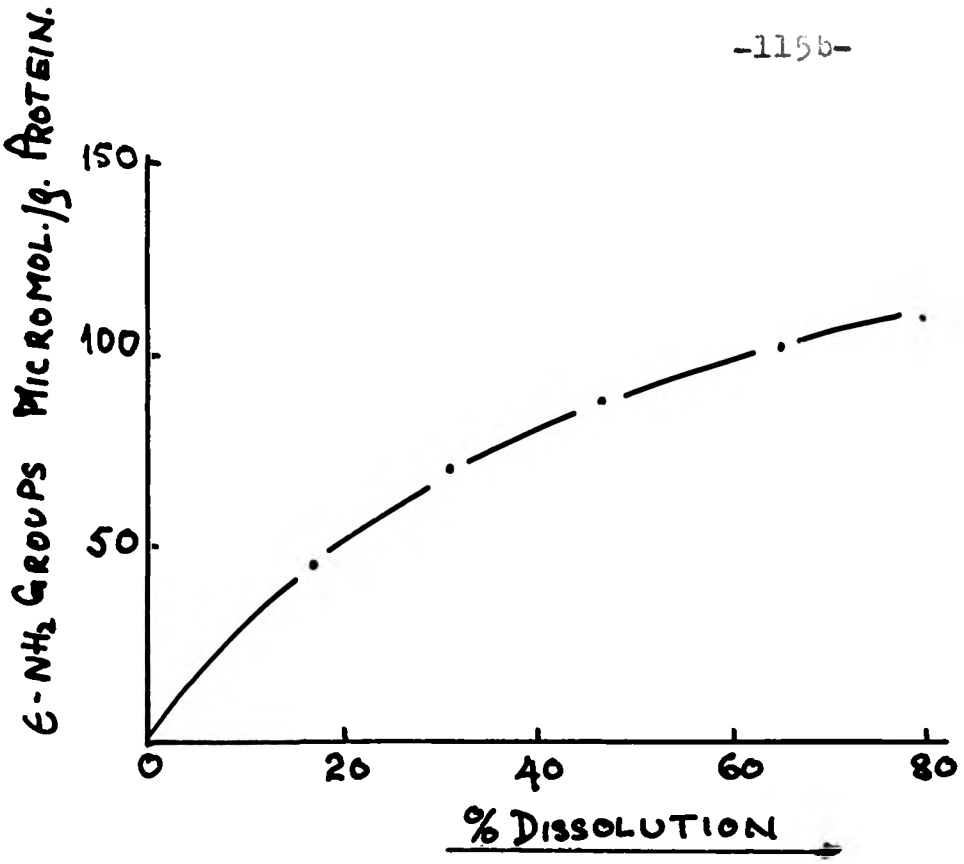


Fig. 13: ε amino groups of versene decalcified ox-bone collagen

Table 12: Availability of ε amino groups of ox-bone collagen during demineralisation with versene:

% Dissolution	ε amino groups micromol/g.protein
17	45
30	70
46	85
65	105
80	110

demineralisation process:-

a) If demineralisation was first effected by means of hydrochloric acid, and the tissue then treated with DNFB, the availability of the ε amino groups first increased, but then rapidly decreased to values between 40 and 60 micromol/g. protein (Fig. 12). Occasionally, values as low as 15 and as high as 120 micromol/g. protein were obtained. These results were apparently not related to the degree of demineralisation or the time taken for demineralisation. Similar results were obtained using trichloracetic acid.

b) If demineralisation was effected with versene, and the tissue then treated with DNFB, the availability of the ε amino groups increased with the percentage dissolution but not in a linear manner, and the value at complete demineralisation was only about 35-40% of the total number present. (Table 12)(Fig. 13).

c) If, however, demineralisation with versene at pH 8.5 and dinitrophenylation were carried out simultaneously, the availability of the ε amino groups increased in a linear manner from 25 to 330 micromol/g. protein when plotted against the percentage dissolution (Fig. 10). Thus in the fully demineralised state over 93% of the ε amino groups reacted with DNFB. This result is in agreement with x that obtained on dentin.

These/

These experiments show that there are distinct differences in chemical reactivity between collagens which have the similar amino acid compositions but differ in their being obtained from hard and soft tissues.

Articular cartilage:

Ox-articular cartilage was treated with DNFB before and after extraction of the mucopolysaccharide fraction. Results were obtained which indicated that only about half the ϵ amino groups were available to DNFB, assuming the percentages of lysine and hydroxylysine to be the same as in ox-hide collagen. No N-terminal amino groups were detected. These figures were not changed by the extraction of the mucopolysaccharide fraction. Thus the collagen of cartilage resembles that of skin but these results can only be regarded as approximate, since it was not established whether the DNFB reagent had reacted with amino sugars known to be present in cartilage.

Fish scale proteins:

The reaction of DNFB with ichthylepidin, a protein present in pilchard scales, was studied. Ichthylepidin is a stable protein notable for its insolubility in boiling water, and the usual solvents for proteins (7). On attempting to dinitrophenylate ichthylepidin, it was observed that the action

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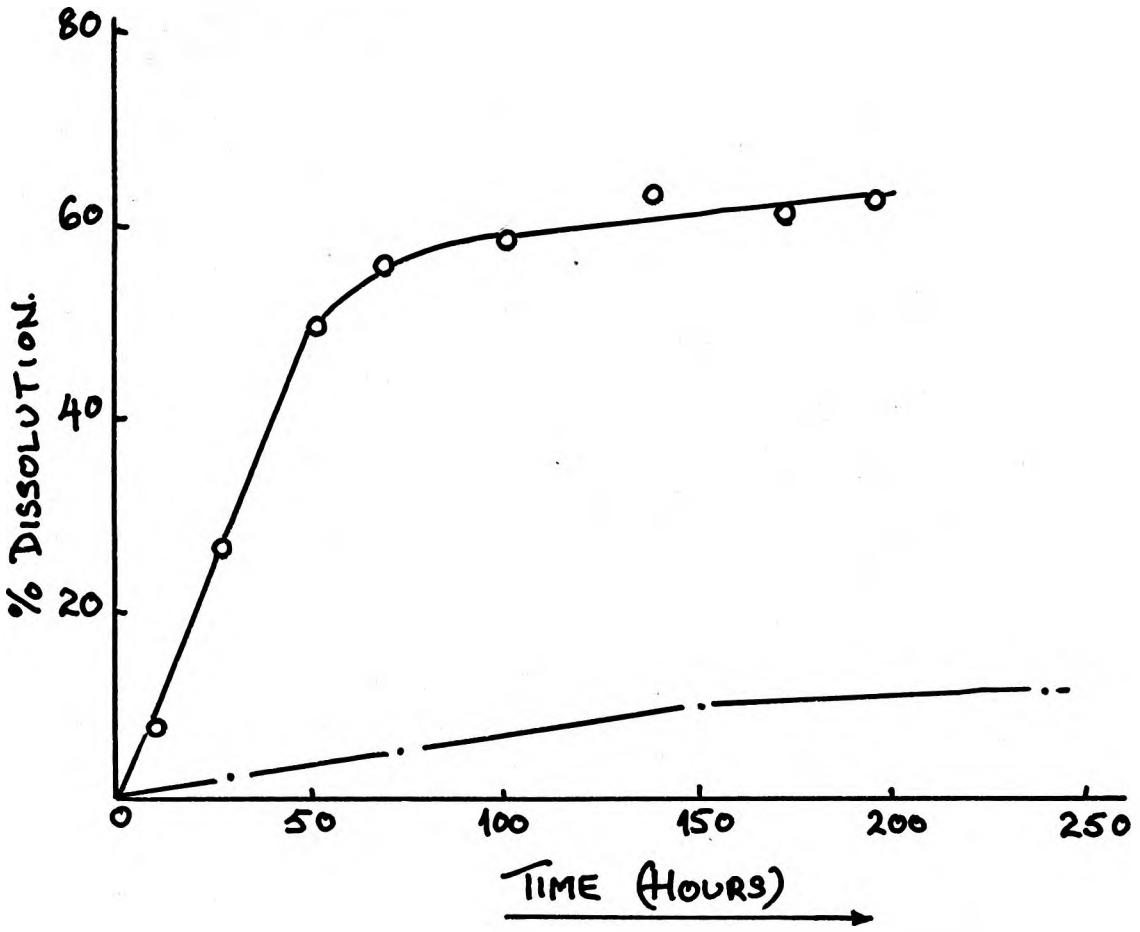

Figure 14: Dissolution of ichthylepidin in the presence of DNFB ——o——o——o——

in 66% ethanol saturated with $NaHCO_3$ ——•——•——•——

of DNFB in an aqueous alcoholic solution of sodium bicarbonate brought about the dissolution of more than half the protein, whereas very little is dissolved in the same mixture without DNFB. (Table 13 i) & ii))(Fig. 14)

Table 13 i): The action of DNFB on Ichthylepidin:

Time (Hours)	% Dissolution
10	6
35	25
50	48
65	56
100	60
135	64
170	60
190	63

Table 13 ii): The dissolution of ichthylepidin in 66% ethanol saturated with $NaHCO_3$

Time (Hours)	% Dissolution
25	2
70	4
150	10
240	11

If alcohol was omitted from the solution the rate of dissolution was slower, but about the same amount dissolved. It is known that in bicarbonate solution, DNFB can split certain labile bonds (8) but analysis of the dissolved DNP protein showed that degradation was extensive. The dissolved protein was deep

yellow/

yellow, it could not be precipitated by trichloracetic acid or ammonium sulphate, and it was separated into a number of fractions on a talc column (9). The peptide fraction present in the largest amount was eluted from the column by alcohol-water mixtures, and evaporated to dryness. Its C-terminal residue, determined by the thiohydantoin method, was found to be that of serine. Many of the fractions were of low molecular weight and easily passed through a dialysis membrane. These results indicate that DNFB was responsible for the dissolution and probably the degradation of an insoluble and relatively stable protein. As fish scale proteins rely largely on interchain salt links for their stability (10), it is possible that the rupture of these links involving free amino groups in the reaction with DNFB, weakened the structure sufficiently for dissolution to take place.

5. Dye binding experiments.

The dye, Orange G, has the property of combining ionically with the basic groups of protein molecules (15). These groups include the side chains of lysine, hydroxylysine, arginine and histidine, as well as N-terminal groups. As DNFB does not react with the guanidino group of arginine, and the DNP derivative of histidine is colorless (9), the dye binding method was applied to the study of hard tissues partly in an attempt to determine the availability of these groups to the dye molecule, and partly to confirm the DNFB results by

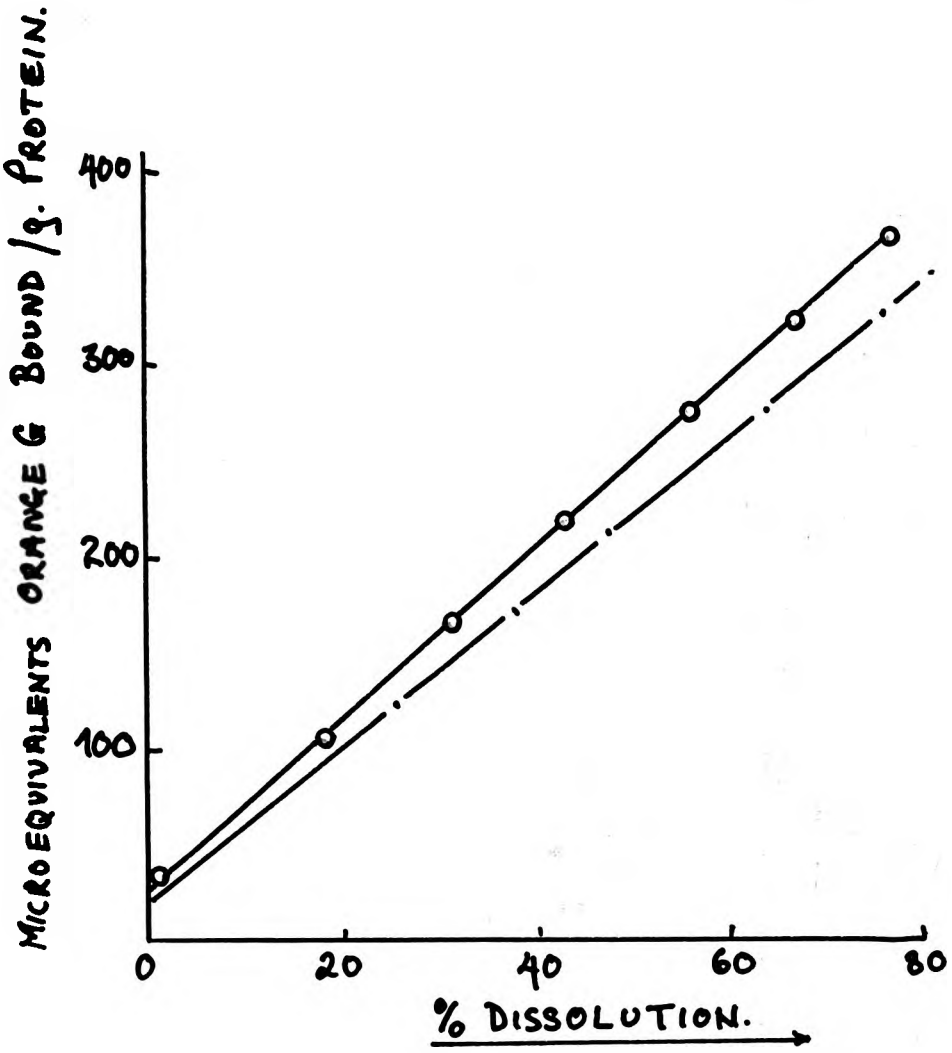

Fig. 15: The binding of Orange G by
Ox-bone collagen ——o——o——
and human dentin collagen ——•——•——
during the demineralisation of these tissues by citric acid.

an independant method.

In this work the calcified tissues were treated with dye and demineralising reagent simultaneously. Combination of the anionic protein groups with Safranine O was also studied.

In bone and dentin it was found that as demineralisation in citric acid (pH 1.5) progressed, the number of microequivalents of Orange G bound per g. of protein increased in a linear manner from 20 to a maximum of 336, when plotted against the percentage dissolution of mineral material (Table 14)(Fig. 15).

Table 14: Binding of Orange G by human dentin collagen and ox-bone collagen during demineralisation with citric acid:

Tissue	% Dissolution	Microequiv.dye bound per g. protein
Ox-bone	0	20
	18	105
	31	170
	44	220
	56	280
	67	325
	77	360
Dentin	0	20
	26	120
	38	170
	48	210
	64	370
	77	325
	80	340

Thus these results numerically confirm those obtained by the DNFB method for free ε amino groups (see Fig. ~~10~~ 10)

Ox-hide collagen, in the native state, bound Orange G to the extent of 264 microequivalents/g. protein. This figure corresponds to about 70% of the total number of lysyl and hydroxylysyl amino groups although there is no proof that the dye reacted with these residues only.

In interpreting these results it must be remembered that low values may be obtained as the result of competition between buffer ions and the dye for the binding sites on the protein molecule (11).

The figures obtained for the basic side groups in ox-hide collagen, decalcified ox-bone, and human dentin, represent only a fraction of the total number of basic groups which is 858 microequivalents/g. in ox-hide collagen, and 766 micro-equivalents/g. protein in dentin. On denaturation of the protein, the uptake of Orange G was increased (Table 15):

Table 15: The acid and base binding capacities of collagen in the native state and after treatment with various reagents:

Tissue	Treatment	Binding of Orange G	Binding of Safranine O
Ox-hide collagen:	Heat shrunk	510	427
	1N Acetic acid	621	512
	8M urea	800	618
Ox-bone Collagen:	Heat shrunk	560	436
	1N Acetic acid	744	523
	8M Urea	820	590
Human Dentin Collagen:	Heat shrunk	570	520
	1N Acetic acid	743	690
	8M Urea	788	684

Note: The degree of accuracy and reproducibility of these values is 10 to 15%

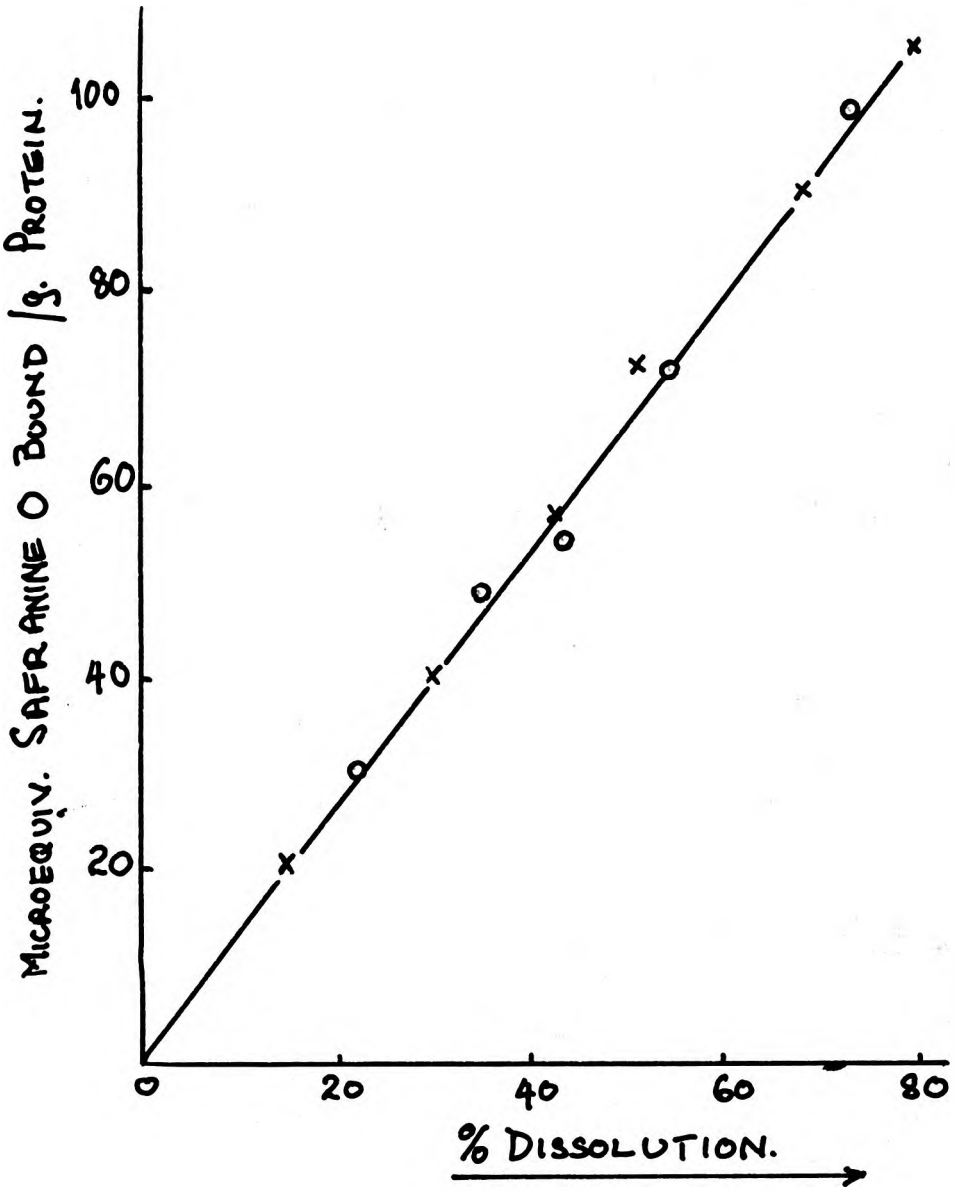

Fig. 16: Binding of Safranine O by human dentin collagen ——x——x——x and ox-bone collagen ——o——o——o during demineralisation with versene pH 11.0.

It was observed that the number of microequivalents of Safranine O bound per g. of protein also increased in a linear manner from 0 to 100 when plotted against the percentage dissolution of ox-bone and dentin in versene solutions (pH 11.0)(Table 16) (Fig. 16)

Table 16: Binding of Safranine O by human dentin collagen and ox-bone collagen:

Tissue	% Dissolution	Microequivs.dye bound/g.protein
Human dentin:	0	0
	15	20
	32	40
	43	56
	51	72
	68	90
	80	105
Ox-bone:	0	0
	22	30
	36	48
	43	54
	56	72
	77	99

The maximum amount of Safranine O bound under these conditions represents about 12% of the total number of acidic side chains (i.e. aspartic + glutamic acids - amide nitrogen). In native ox-hide collagen, no combination of Safranine O with the protein took place. These figures were increased after denaturation of the protein (Table 15).

Veis and Cohen (12) have found about 50% of the anionic groups to be reactive in preswelled ox-hide collagen,

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and this figure was increased to 100% by heating the collagen in water. The high degree of binding of both acid and basic dyes found by these authors may be the result of their drastic pretreatment of the collagen.

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Chapter VII

Discussion of the Results in relation to 'in vivo' Calcification.

In recent years, much importance has been attached to the function of acid mucopolysaccharides such as chondroitin sulphate as one of the 'local factors' necessary for 'in vivo' calcification and ossification (1).

It has been shown, in rachitic cartilage ('in vitro')(2) that chondroitin sulphate can take up considerable amounts of calcium and phosphate ions. In areas of cartilage about to become bone, there is an increase of metachromasia. This suggests the formation or modification of the mucopolysaccharide fraction, thereby conferring calcifiability on the matrix. However, the amount of carbohydrate in bone is extremely small, and it should be remembered that the chemistry of metachromasia is not fully understood. Minor variations in technique will change the sites and the intensity of metachromasia (3). Autoradiographic studies of the formation of bones and teeth have been carried out using the radioactive C^{14} bicarbonate and S^{35} sulphate (4). These studies show that in cartilage and bone C^{14} and S^{35} are first present in the chondrocytes and osteoblasts, and are later deposited in the matrix by the activity of the cells. In dentin and possibly in bone, the matrix is formed in two steps: First C^{14} is incorporated into the predentin and preossein and then the addition of S^{35}, which becomes associated with the metachromatic regions, confers

calcifiability/

calcifiability on the matrix. Sections treated with hyaluronidase before autoradiography showed no reaction. S^{35} 'in vitro' is also incorporated into the mineral fraction, presumably by a process of physical adsorption (5).

However, chemical and autoradiographic studies show that the calcification of cartilage is preceded by a loss of organic sulphate (6). This evidence is in favour of the suggestion that chondroitin sulphate may be utilised in the process of calcification of cartilage, or else that its removal from cartilage is indispensable for the process of fixation of calcium salts on the cartilage matrix.(7). Healing skin and tendon elaborate considerable amounts of mucopolysaccharides, but this process is not normally followed by calcification. Thus, more work must be done to elucidate the significance of mucopolysaccharides in calcification. Many of the ideas put forward have been based on studies of cartilage or of osteoid and cartilage together. What happens in the one during calcification, does not necessarily happen in the other, as is evident from electromicroscopic studies (8). The deposition of organic salts in the two areas differs in several ways:-

Calcification of osteoid occurs immediately after the deposition of the collagenous matrix by the osteoblasts, whereas in cartilage calcification appears after degeneration of the cells. In cartilage the crystals are laid down at a distance from the edge of the cell capsule but in osteoid, within a fraction of a micron of the osteoblasts. The initial crystal

arrangement/

arrangement in bone at the epiphyseal line is at first irregular, as in cartilage, but the inorganic fraction soon becomes coaligned with the banded regions of the collagen fibres, so that the periodicity of the underlying fibres is emphasized. In calcified cartilage, however, the mineral material remains haphazardly deposited in the ground substance and has no obvious relationship to the collagen fibres. The collagen fibres in cartilage, although described as collagenous do not exhibit the typical collagen banding, are only $\frac{1}{2}$ to $\frac{1}{5}$ of the diameter of those in bone, and are widely separated both before and after calcification. Thus it is evident that the mechanism of calcification is different in the two tissues. Furthermore, calcifying osteoid and calcified cartilage are distinctly separated from each other by a 'double membrane'.

The first appearance of inorganic material in bone is observed in conjunction with the 100Å bands of collagen in newly formed human bone and the doublet bands of the longer established bone. Later, crystals gradually fill in the cement substance between fibres. Thus calcification may depend on a chemical feature of the band regions, namely, a high concentration of acidic and basic polar groups. The relatively large amounts of mineral material oriented and accumulated in the banded regions of the fibre suggests that the collagen fraction of these tissues play an active x role in the acquisition of mineral material.

From X-ray diffraction, the reaction with tanning

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and staining agents (9), and the osmotic swelling of collagen, it has been postulated that the banded regions contain a large proportion of the basic and acidic amino acids which possess bulky side chains viz: lysine, arginine, hydroxylysine, histidine, aspartic and glutamic acids. By treating tendon collagen with the difunctional agent, difluorodinitrodiphenyl sulphone, (Fig. 1), the Ts was elevated 10° and the high yields of sulphone bislysine, sulphone bishydroxylysine and sulphone lysine-hydroxylysine obtained, were interpreted as evidence for the occurence of these two basic amino acids in close proximity on adjacent polypeptide chains. These observations support Bear's concept (9) that the bulky polar side chains are confined to the open structural band section of the collagen fibrils.

The results obtained by the present writer in treating the collagen fraction of bone and dentin during demineralisation with DNFB and dyes, show that nearly all the lysyl and hydroxylysyl groups as will well as some of the carboxyl groups of collagen may be involved in the combination of mineral to the organic matrix. In ox-skin, which contains large amounts of typical collagen, but does not normally calcify, it was found that only about ½ of the total number of ε amino lysyl and hydroxylysyl groups were able to react with DNFB. The reason for this was not definitely established, but steric hindrance or large decomposition losses during hydrolysis of the DNP collagen were discounted. This finding indicated that there is a marked difference in chemical reactivity of some of the side chains

between/

between collagens which have the same amino acid composition and electronoptic appearance, but differ in their being obtained from calcified and non-calcified connective tissues. In ox-hide collagen treated with DNFB, the ratio of ε N DNP lysine to ε N DNP hydroxylysine was almost the same as that obtained by calculation from the amino acid composition, viz: 3.9, but in dentin and bone, this ratio increased linearly with the percentage dissolution in demineralising solutions from about 1 to 3.2. This indicates that, during decalcification, hydroxylysyl amino groups are preferentially made more available to DNFB than the lysyl ε amino groups, in spite of the much large concentration of the latter in collagen. Thus, if lime salts are in some way bound to ε amino groups of both the amino acids, those bound to the ε amino groups of hydroxylysine are the more easily removed by decalcification procedures. This is in accordance with the fact that the pK of the hydroxylysyl ε amino group is appreciably lower than that of the lysyl ε amino group (10). In ox-bone, but not in human dentin, the number of ε amino groups available to DNFB was profoundly influenced by the demineralising agent used, and whether dinitrophenylation was performed some time after or simultaneously with, the demineralisation process. Speculating on the reasons for the low values obtained for free ε amino groups when bone was first demineralised in acid and then dinitrophenylated, it is possible that the removal of mineral material causes a 'reorientation'

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of the protein which renders the ε amino group unavailable to DNFB. This unusual effect was seen to a lesser extent when demineralisation was effected by versene and not at all if demineralisation and dinitrophenylation were carried out simultaneously. In ox-hide collagen, however, no change in the availability of the ε amino groups was detected after treatment with acid or versene but in dentin, a slow decrease was noted after demineralisation was complete. These observations suggest that the removal of mineral material by acid causes different degrees of structural changes in bone and dentin collagens. The changes are apparently not reversed by the alkaline conditions of dinitrophenylation.

The solubility curve of dentin and bone in buffer solution shows a plateau region in the range of 5.8 to 6.5 which is not exhibited by hard tissues 'deorganicised' with ethylene diamine. The plateau region may be caused by the presence of organic material, but it is possible that its disappearance in the deorganicised tissues is due to a change in the properties of the mineral component during boiling in ethylene diamine. It is known, for example, that this treatment increases the size of the crystallites. Only a portion of the elements in the atomic crystal lattice can be expected to be protein bound. If they were all bound to organic components, it would not seem possible that the large apatite crystals found in senile bone could be formed. A theory of 'seeding' has been proposed (11) in which the organic phase of osteoid or dentin may bind either

calcium/

calcium or phosphate ions in the space relationships of the apatite crystal lattice and thus initiate the formation of crystals which would, therefore, be oriented with respect to the organic structure. It is possible that hard tissue collagen, in which almost all the lysyl ε amino groups can form covalent links with DNFB after decalcification, and which has some terminal amino groups, is a more suitable 'template' for the crystallisation of hydroxyapatite than similarly treated skin collagen, in which only about half of the lysyl and hydroxylysyl ε amino groups react with DNFB, and no terminal groups were detected. Negatively charged groups such as phosphate or hydroxyl ions would be attracted to the ε amino groups of collagen, whilst side chain carboxyls can bind calcium ions. It is difficult to test this theory at the molecular level, as no final structure for the collagen molecule has been proposed, nor is the amino acid sequence of the protein chain known.

The view that basic and acidic amino acids are involved in calcification is supported by the values obtained for the amino acid composition of normal and calcified aortas (12). It is seen in Table 1. that the percentage of polar amino acids is much greater in the older, calcified aorta, than in the normal one. It is possible that this change facilitates pathological calcification.

Table 1/

Table 1: Amino acid composition of young and old aortas:

Tissue	g.Ca/100g. Nitrogen	Asp.	Glu.	lys.	Arg.	Hist	Ser.	Thr.
Young aortas 15-20 years:	0.35	0.31	1.51	0.49	1.78	1.15	0.29	0.65
Old aortas 55-75 years:	5.93	1.07	2.48	1.17	4.35	0.75	0.70	1.13

Note: Gly., val., pro., leu., and ileu. remained relatively constant in both young and old aortas (12).

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Chapter VIII

Summary and Conclusions.

In this work, some of the properties of the organic and the inorganic intercellular substances of ox-skin, ox-cartilage, ox-bone and human dentin, are described, and information concerning the combination of mineral material with the organic matrix of the hard tissues is discussed.

Modern methods of chemical analysis have been applied to the study of intercellular substances in both hard and soft connective tissues of bovine, human and marine origin. Care was taken to isolate the tissues with the minimum of chemical treatment. The experimental work was mainly directed at obtaining more information on the composition and structure of collagen. A fairly complete amino acid analysis of two fish scale proteins was carried out using the accurate ion exchange chromatographic method of Moore & Stein. Systems of paper and column chromatography of free amino acids and their yellow dinitrophenyl derivatives were used for the determination of some of the amino acids in bovine and human collagens. Colorimetric and spectrophotometric methods were also used. The amino acid compositions of ox-hide collagen and human dentin collagen were found to be very similar, but they both differed from that of the fish scale collagens. The results obtained agreed well with those reported in the

literature/

literature.

The structure and chemical reactivity of collagens obtained from hard and soft tissues were also studied. This was done by making use of Sanger's dinitrofluorobenzene (DNFB)method for the identification and quantitative determination of the N-terminal amino acid residues, in the intact protein. The DNFB reagent also reacts with non-terminal free amino groups such as the ϵN amino groups of lysine and hydroxylysine, indicating the availability of these groups in the protein molecule. The reactivity of polar protein side chains was also investigated using acidic and basic dyes. The collagens obtained from hard and soft tissues were found to differ markedly with regard to the availability of some of their ϵ-amino groups to DNFB and the concentration of terminal amino acid residues, before, during and after treatment with decalcifying reagents. It was found that the method of decalcification could influence the reactivity of the ϵ-amino group of hard tissue collagen, particularly that of bone, and a method was devised whereby decalcification and coupling with DNFB could be performed simultaneously. Similar methods were used for the combination of proteins with acidic and basic dyes during demineralisation. The concentrations of N-terminal and ϵ-amino groups available to DNFB were higher in collagen obtained from bone and dentin, than those of the similarly treated ox-hide collagen.

On the basis of the experimental work, it was concluded that in hard and soft tissues, the presence of, or lack or calcification may be due to differences in the properties of the collagen fraction of these tissues. Some suggestions concerning the modus operandi of this idea in bone and dentin were made.