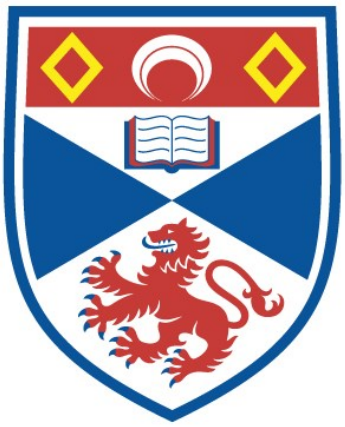
THE ACTION OF SOME ENZYMES ON BOVINE COLLAGEN

Ian Richardson Tyson

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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THE ACTION OF SOME ENZYMES ON BOVINE COLLAGEN

by

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A Thesis

presented to the University of St. Andrews for the

Degree of Doctor of Philosophy.

DECLARATION

I hereby declare that the following Thesis is based on work carried out by me, that the Thesis is my own composition, and that no part of it has been presented previously for a higher degree.

The research was carried out in the Department of Physiology and Biochemistry in the United College of St. Salvator and St. Leonard, St. Andrews, under the direction of Dr. G. R. Tristram.

CERTIFICATE

I hereby certify that IAN RICHARDSON TYSON has spent nine terms engaged on research work under my direction, that he has fulfilled the conditions of Ordinance No.16 (St. Andrews), and that he is qualified to submit the accompanying Thesis for the degree of Doctor of Philosophy.

ACADEMIC RECORD

I matriculated at the University of St. Andrews in October 1956, passed the examination for the degree of Bachelor of Science in Chemistry and Biochemistry, and graduated in June 1959.

I obtained a Second Class Honours Degree in Biochemistry in June 1960, and was admitted as a research student to the Department of Physiology and Biochemistry in the United College. Since that time I have been engaged in the research, the results of which are now submitted for the Degree of Doctor of Philosophy.

I have been supported throughout this work by a scholarship from Unilever Ltd.

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SECTION I

INTRODUCTION.

Throughout the animal kingdom the fibrous proteins comprise a large part of the structural elements of the body. The fibrous proteins were divided into two main groups by Astbury on the basis of their wide-angle X-ray diffraction patterns. The first of these groups was termed the keratin-myosin-epidermin-fibrinogen (k-m-e-f) group, comprising fibrous proteins from hair, muscle, epidermis and blood. The second group consists of collagens, the fibrous components of connective tissue.

Collagens have been found in almost all classes of animals, but mammalian collagen, and ichthyocol, the collagen of the Teleostomi, have been studied most extensively. Collagen is widely distributed throughout the body and in mammals is found in tendon, cartilage, bone, skin, and other tissues having a structural function, e.g. blood vessel walls and the sclera of the eye. In all tissues the collagen is associated with other components, the nature and quantity of these components varying with the particular function of the tissue. In bone the collagen is in specific association with calcium salts, and in the soft connective tissues mucopolysaccharides are found in association with the collagen.

As a result of its several distinctive properties collagen is of interest and importance from a variety of aspects. Its unique structure presents a challenge to the chemist, and its inertness is of interest to the enzymologist. From the medical aspect the role of collagen in disorders of connective tissue is of great importance.

Composition and Structure of Collagen.

Initially amino acid analyses were performed on gelatin rather than collagen, e.g. by Dakin (1918). More recently analyses have been carried out on collagens from a wide variety of sources. Although there are some differences in amino acid composition of collagens from different species, especially in hydroxyproline content, and even slight variations in composition of collagens from different organs in the same animal, all collagens have a very similar composition.

There is a predominance of non-polar amino acids (glycine alone accounts for almost one-third of the total residues) and an unusually low content of aromatic amino acids. Of the sulphur-containing amino acids, only methionine is present, and this in small amounts. The imino acids, proline and hydroxyproline, account for almost one-quarter of the residues, and the unusually high content

of hydroxyproline is characteristic, although recently a non-collagenous protein from plant cell walls has been shown to have a high hydroxyproline content (Lamport and Northcote 1960). Hydroxylysine is also found almost solely in collagen, although it has been reported in small amounts in wool (Kerston and Zürn, 1959). The amino acid compositions of some collagens are tabulated below.

Table 1.

Amino acid composition of some collagens. Compositions expressed as moles of amino acid residue per 1000moles.

Amino Acid Residue	A Calf Skin collagen	B Calf Skin collagen	C Calf Skin tropo-collagen	D Calf Skin soluble collagen
Alanine	112.0	95.4	112.0	105.6
Glycine	320.0	350.0	330.0	331.0
Valine	20.0	21.7	19.0	20.8
Leucine	25.0	23.1	22.0	26.6
Isoleucine	11.0	14.2	11.0	14.1
Proline	138.0	119.0	141.0	131.8
Phenylalanine	13.0	11.3	15.0	12.6
Tyrosine	2.6	0.8	3.0	3.9
Serine	36.0	27.0	32.0	31.8
Threonine	18.0	18.1	16.0	17.9
Methionine	4.3	5.6	2.0	3.1
Arginine	50.0	35.9	47.0	48.2
Histidine	5.0	4.0	7.0	5.8
Lysine	27.0	34.7	29.0	28.4
Aspartic acid	45.0	50.6	43.0	46.5
Glutamic acid	72.0	80.5	70.0	76.8
Hydroxyproline	94.0	107.5	93.0	89.6
Hydroxylysine	7.4	-	10.0	6.0

A. Piez, K. A., Gross, J. (1960)

B. Neumann, R.E. (1949)

C. Rubin, A. L. et al. (1963)

D. Kühn, K., Kühn, J., Hannig, K. (1961)

Collagen is essentially an insoluble protein, and physical studies in the solid state produced the first evidence for the highly ordered structure of the protein.

Wide-angle X-ray diffraction techniques show a well-defined pattern, similar for all collagens, indicating an ordered arrangement of amino acids within the structure. The most significant data derived from such techniques was the meridional spacing of 2.86Å, this distance, the mean length of one residue in the peptide chain, being unusually small (Gustavson 1956).

Various arrangements of amino acids were proposed, in an attempt to explain this meridional spacing, and also to accommodate the large number of imino acids, e.g. Astbury proposed a repeating sequence P-G-R, (where P = proline or hydroxyproline, G = glycine, and R = any other amino acid residue) with alternate peptide bonds having a cis-trans arrangement. Pauling and Corey (1951) proposed a structure based on a similar repeating sequence, and also containing cis-peptide bonds, but consisting of three peptide chains, each in a helical configuration, with the three helices having a common axis. Although such structures can provide an explanation for the X-ray data they are inconsistent with other data. The cis-configuration, suggested for some of the peptide bonds, is unusual in proteins, and it was shown that collagen contains no such

bonds (Badger and Pullin, 1959). In addition the P-G-R repeating sequence requires that one-third of the total residues be imino acid residues but in fact only one-quarter of the residues are of this type and thus the above sequence cannot hold throughout the length of the peptide chain. This has been confirmed, e.g. by Kroner et al. (1953) who isolated peptides inconsistent with the above sequence. In 1955 Ramachandran and Kartha put forward a modification of an earlier structure (same authors 1954) which was consistent with almost all the experimental data. Rich and Crick (1955) and Cowan, McGavin and North (1955) working from model structures of poly-glycine and poly-proline respectively, proposed structures essentially similar to that of Ramachandran.

The proposed structure was still based on a repeating sequence of amino acids, the allowed sequence being G-R-R, where G = glycine and R = any other residue, including imino acids. The structure comprises three polypeptide chains, each having the above repeating sequence, each chain being in the form of a left-handed helix of 3.3 residues per turn. These three helices are linked together by hydrogen bonds, and twisted in a major right-handed helix of 30 residues per turn.

There are two possible variations of the triple helix

structure, (designated collagen I and collagen II) differing in the stereochemical phasing of the 3 chains with respect to one another, and in the orientation of the inter-linking hydrogen bonds, especially the hydrogen-bond which can be made from the hydroxyl group of a hydroxyproline residue in position (3) of the sequence G - R - R. In structure I this hydroxyl group can form a hydrogen bond within the group of three chains, with slight deformation of the structure, whereas in structure II such an intrahelical hydrogen bond is not possible. Rich and Crick (1958) favour structure II. Although the wide-angle X-ray technique provided evidence for the ordered configuration, further physical studies were necessary for examination of the collagen molecule and its arrangement in collagen fibres.

(1) (2) (3)

Low-angle X-ray diffraction techniques, and electron microscopy (Hall, 1942) reveal a characteristic periodicity in the collagen fibre, a repeating period of approximately 640Å being observed. In addition to this 640Å period, collagen fibres stained with phosphotungstic acid reveal a detailed intraperiod banded arrangement of light and dark bands (Schmitt et al. 1948). Bear (1952) suggested that the dark bands correspond to regions containing amino acids with large side chains, and the non-stained interbands to regions of small amino acids. Hodge and Schmitt (1960)

have examined fibres stained with phosphotungstic acid, which binds with basic groups, and chromium or uranyl salts, which bind with acidic groups, and have shown that in each case the bands occur in the same relative positions, confirming the hypothesis of Bear that the polar residues occur at the same select locations along the fibre, and therefore that the collagen molecules must be aligned in ordered array within the fibre.

Analyses of peptides produced by enzymic digestions of collagen provided further evidence for a molecule composed of alternating regions of polar and non-polar amino acids. Schrohenloher et al. (1959) isolated two peptides, Gly-Pro-Hypro and Gly-Pro-Ala and Grassmann et al. (1961) isolated the sequences Gly-Pro-Gly and Gly-Pro-Ala and showed that these two peptides, together with the sequence Gly-Pro-Hypro contained 95% of the total proline. Grassmann et al. (1960) also isolated two peptides, of 21 and 22 residues, containing no imino acids, and Manahan and Mandl (1961) found a sequence of 24 residues containing no hydroxyproline and very little proline. In all peptides examined glycine comprises roughly one-third of the total residues, confirming the picture of peptide chains with glycine occurring every third residue.

Although collagen fibres are in the main insoluble, a small proportion can be solubilised. A brief account of

soluble collagens will be given later. Nageotte (1927) first obtained solutions of collagen in weak acids, and showed that collagen could be reconstituted from these solutions, in the form of fibres, by addition of salt. Examination of such fibres by electron microscopy (Schmitt et al. 1942) revealed the characteristic periodicity and fine structure typical of native collagen. However, by careful selection of appropriate conditions, precipitates can be formed in which the 640Å spacing is absent, but other morphological characteristics apparent. Highberger et al. (1950) described a fibrous precipitate with an axial period of 2800Å. This material, precipitated in the presence of glycoprotein, was termed "Fibrous Long Spacing" or FLS. Another type of precipitate, produced by the addition of ATP, was described by Schmitt et al. (1953). This precipitate consisted of short segments of varying width, and length about 2800Å and was designated "Segment Long Spacing" or SLS.

To explain these results Gross et al.(1954) postulated a hypothetical collagen unit of length 2800Å and width 15-20Å which was called tropocollagen (TC). According to these workers the particular periodicity observed is dependent on different alignments of this tropocollagen unit, viz:- a) TC units in parallel, with the ends in

register, but no end-to-end association, giving SLS.

b) TC units antiparallel, with ends in register, and end-to-end aggregation, giving FLS.

c) In parallel array, with parallel adjacent tropocollagen units staggered by one-fourth of their length, giving rise to the native-type 640Å spacing.

Examination of acid solutions of ichthyocol by physical methods reveal that the particle in acid solution has a length of about 3000Å and a diameter of 13.6Å (Boedkter and Doty, 1955), dimensions which agree with those given to the postulated tropocollagen unit of Gross et al., providing experimental evidence for the existence of such a unit. Boedkter and Doty also suggested that short peptide chain appendages extended beyond the triple helix body of the molecule.

Evidence for the existence of such "end-chains" was provided by Hodge and Schmitt (1958), who found that solutions of collagen subjected to ultrasonics would no longer form end-to-end aggregates, but would still form lateral aggregates of the SLS type. They suggested that the ends of the molecule are susceptible to sonic irradiation, and postulated that end-to-end aggregation involved intercoiling of the terminal "end-chains", which were later suggested to have the following characteristics,

viz:- the "end-chain" at one end of the molecule is about 100Å long when coiled, and contains few, if any, basic residues, while the end-chain at the other end of the molecule is about 200Å long, and contains basic residues.

In an attempt to isolate these "end-chains" solutions of collagen were treated with trypsin (Hodge et al., 1960). These workers reported that the enzyme destroyed the end-to-end aggregation properties of the collagen and released small peptides. Examination of the peptide fraction revealed a large proportion of acidic amino acids, few basic amino acids, and a considerable amount of tyrosine. The presence of tyrosine in the "end-chains" was thought to be significant since Bensusan and Scanu (1960) had shown that tyrosine residues play an important role in fibre formation.

Denaturation: The sub-units of collagen.

Degradation of collagen by physical or chemical means produces products collectively termed gelatins. The discussion here will be limited to simple depolymerisation of the collagen structure. The more extensive degradation employed commercially will be discussed elsewhere.

Thermal denaturation of collagen, brought about by mild heating of solutions of collagen at neutral pH results in the formation of parent gelatin. Under these conditions randomisation of the helical collagen configuration is

achieved by cleavage of the inter-helical hydrogen bonds, which can also be brought about by cleavage of the hydrogen bonds with specific reagents, e.g. urea.

Under certain conditions gelatin will revert, at least in part, from a random configuration to a more organised structure, this reversion to the collagen fold being manifested by a large increase in laevorotation on cooling a solution of gelatin (Robinson 1953). Harrington and von Hippel (1959) have studied the collagen-gelatin transition and shown that the reversion to the ordered collagen fold is a three stage process, each stage being manifested by a characteristic change in property.

i) The first stage consists of a local locking of the non-polar regions of the peptide chains into a poly-proline II configuration, this change being manifested by a change in the kinetics of proteolysis.

ii) As a result of (i) the polar regions of the peptide chain are induced to take up a loose poly-proline II-type helical configuration. This change is characterised by an increase in specific rotation.

(iii) Finally there occurs specific association between individual poly-peptide helices, resulting in an increase in viscosity.

Orekhovitch and Shpikiter (1955) first showed that denaturation of rat skin procollagen resulted in two species

of particles which differed widely in their molecular weights, as shown in the ultracentrifuge. These two components were termed α and β, and it was shown that the β-component had a molecular weight approximately twice that of the α-component. A similar two-component composition has since been reported for denatured collagens from a variety of other sources (e.g. Doty and Nishihara, 1958). A more complex sub-unit composition was reported by Piez, Eigner and Lewis (1963). The denatured rat-skin collagen used by these workers showed the usual two species of sub-units on ultracentrifugation, but chromatography of the gelatin resulted in a separation into four components. Of these four components, two showed the same sedimentation rate as the α-component, and were designated α_1 and α_2, and the other two components, having a sedimentation rate the same as the β-component, were termed β_1 and β_2. Amino acid analysis of the four components revealed significant variations in composition, these variations being consistent with the β_1 component comprising one α_1 unit and one α_2 unit, and the β_2 component consisting of two α_1 units.

Although many workers report an α:β ratio of 1:1 by weight in gelatins, Piez *et al.* (1963) consider such a ratio to be due to incomplete separation of the α- and β-

units, and have proposed a structure for the collagen molecule based on a stoichiometric ratio of 1:1 for α:β. This concept envisages the molecule as consisting of three α-units (two α_1 and one α_2), two of the α-units being covalently linked together as a β-unit, the remaining α-unit being linked to the β-unit by hydrogen bonds, and therefore separable on denaturation. Piez also suggests that the three α-units represent the three polypeptide chains comprising the collagen triple-helix.

Grassmann, Hannig and Engel (1961) have reported another species of particle present in denatured collagens. This particle has a sedimentation coefficient higher than both α- and β-units, and a molecular weight equivalent to that of the tropocollagen molecule, and is termed the γ-component. Grassmann et al. consider that this component consists of three α-units covalently linked together, and Altgelt et al. (1961) consider that the increased cross-linking present in this component is a manifestation of a more mature form of collagen. The concept of increasing intramolecular cross-linking with age is further demonstrated by the sub-unit composition of the most immature form of collagen (neutral salt soluble collagen). On denaturation of this collagen predominantly α-units are produced, with very little β, (Orekhovitch 1960) in

contrast to the 1:1 α:β ratio found for the more mature acid soluble collagen.

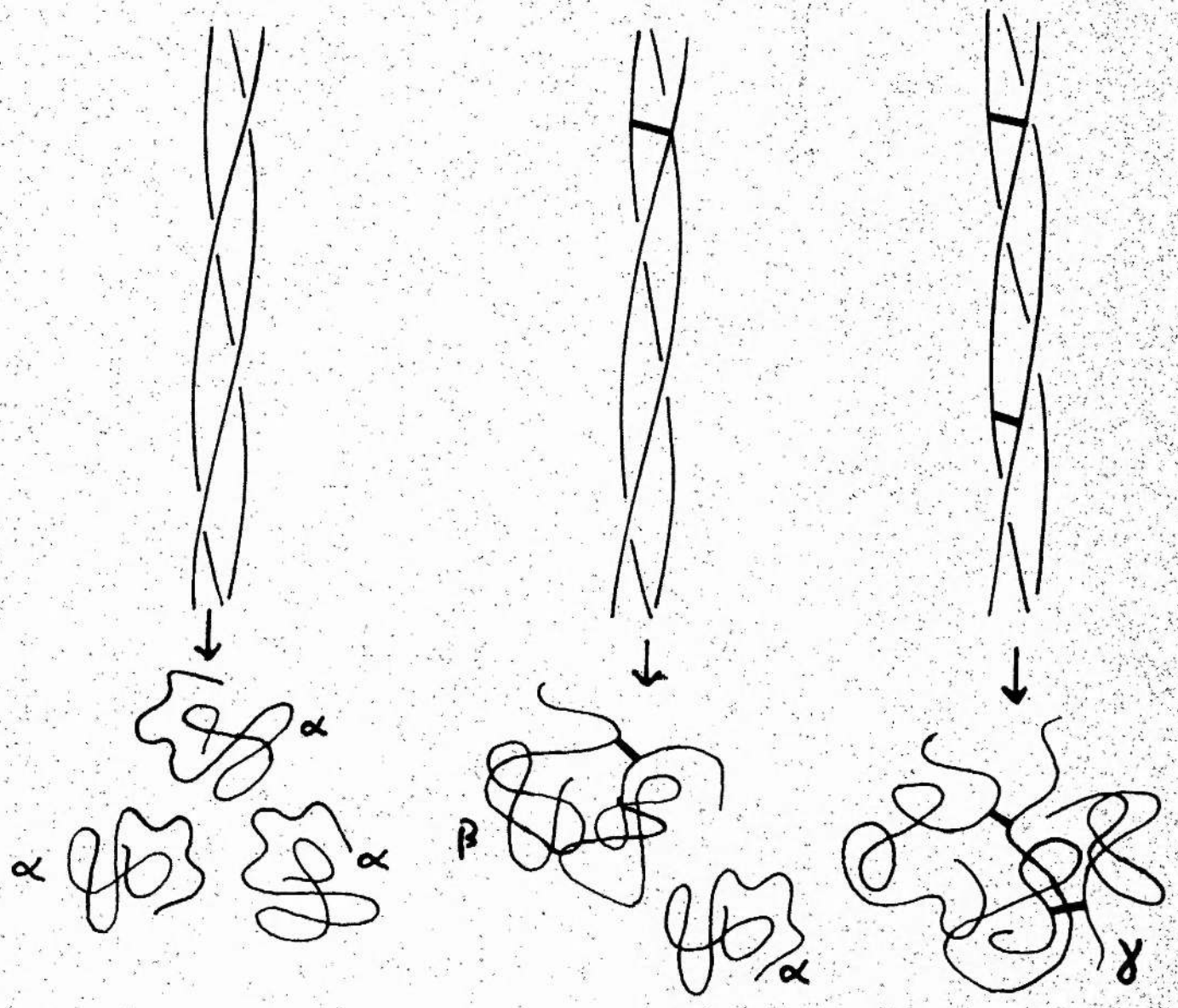
The inter-relationship of the various collagen sub-units has been summarised diagrammatically by Grassmann (1961).

Cross-links of the collagen molecule: the carbohydrate component.

The nature of the covalent cross-links of collagen is of great interest, since collagen contains no cysteine or cystine, thus eliminating the possibility of disulphide bridges, which are commonly found as cross-links in other proteins. Various possibilities exist for the nature of the cross-link and a brief account of these is given.

The presence of peptide bonds involving the ϵ-NH_2 group of lysine was suggested by Bowes and Kenten (1948) from quantitative data on the number of such groups available for titration. These workers also reported that only about one-half of the total ϵ-NH_2 groups were available for reaction with FDNB, an observation borne out by work of Solomons and Irving (1956), suggesting that the unreactive groups were involved in some type of linkage. The unavailability of some of the ϵ-NH_2 groups was not substantiated by the work of Bethell and Gallop (1960) who reported almost complete reactivity of these groups.

INTER-RELATIONSHIP OF COLLAGEN SUBUNITS



Grassmann, Hannig, Engel (1961)

However the presence of ϵ-NH_2 links has been confirmed by Mechanic and Levy (1959) who isolated a tripeptide containing lysine, in which the ϵ-NH_2 group of the lysine residue was involved in a peptide-linkage.

The involvement of side chain carboxyl groups in peptide linkages has also been suggested, and from data on the reactivity of side-chain carboxyl groups, Franzblau, Gallop and Seifter (1960) have reported the presence of 20-30 γ-glutamyl peptide links per 100,000 molecular weight.

Grassmann et al. (1957) reacted calf-skin procollagen with lithium borohydride and obtained substantial amounts of amino alcohols. This reagent produces amino alcohols only from esterified carboxyl groups, and does not react with free carboxyl groups. Gallop, Seifter and Meilman (1959) reacted collagen with hydroxylamine in the presence of hydrogen-bond breaking reagents and found that a small number of covalent bonds were split, with concurrent production of hydroxamic acids, and they suggested that the bonds cleaved were ester bonds.

Grassmann et al. (1957) reported that sodium periodate treatment, which degrades hexoses, would solubilise native collagens, and suggested that the hexose component of collagen may be involved in ester linkages, although the

number of periodate-sensitive links was much in excess of the total carbohydrate content of the collagen. Although there is no direct evidence for the involvement of the carbohydrate moiety in the ester linkages (indeed Blumenfeld, O'Paz, Gallop and Seifter (1963) have suggested that in ichthyocol the hexoses are linked only by glycosidic bonds), Hörmann (1961) considers that the carbohydrate is involved, and shows that in tropocollagen (i.e. procollagen) the number of hexose units is almost the same as the number of hydroxylamine-sensitive links. In mature collagen the ratio of hydroxylamine-sensitive links to hexose units is 2:1 or 3:1. On this basis Hörmann considers that in tropocollagen the hexose unit is linked only by one ester bond, whereas in native collagen the hexose is involved in more than one ester link.

At this juncture it is convenient to consider more fully the carbohydrate component of collagen, already mentioned as having a possible role in the formation of ester bonds.

It has been known for a long time that all collagenous materials contain small amounts of a carbohydrate constituent. Grassmann and Schleich (1935) found 0.65% carbohydrate in hide collagen, and detected the presence of glucose and galactose. Gross, Highberger and Schmitt (1952) reported

the presence of mannose, and Clegg, Eidinger and Leblond (1953) found the above three sugars, together with fucose, in hydrolysates of reticular tissue. In addition to these four hexose units, a hexosamine component is also found in collagens, and this was identified by Schneider (1940) as glucosamine.

Purification of procollagen by repeated reconstitution from solution results in a reduction of the hexose content of the material (Grassmann; Hofmann; Kühn; Hörmann; Endres; and Wolf 1957) and almost completely eliminates the hexosamine. Hexosamine can also be eliminated by liming, or by treatment with dilute sodium hydroxide (Hörmann, 1960) and largely removed by recrystallisation via the SLS type of precipitate, or by treatment with trypsin (Kühn, Hannig and Hörmann 1961). With respect to trypsin treatment, a more comprehensive account of the action of the enzyme will be given later.

Purified collagens, although they may be devoid of hexosamine, still contain hexose to about 0.7% of the total weight (Grassmann et al. 1957). Although this hexose was considered by Moss (1955) to be present as an impurity, most other workers suggest that the hexose has a more positive functional role in the structure.

Grassmann et al. (1957) suggested three possible

linkages between hexose and protein, viz:-

1. N-glycoside links between sugar and amino groups of protein,
2. O-glycoside links between sugar and hydroxyl groups of protein,
3. Ester links,

and give evidence which indicates that N-glycoside links are unlikely.

Hörmann (1960) suggests that hexoses form interchain links in the collagen molecule, by forming a glycosidic link with one peptide chain, and an ester-type link with a second or even third peptide chain, but the same author (1961) concludes that a hexose cross-link between two peptide chains involves the formation of an ester link with each chain, although the involvement of a glycosidic link is not ignored.

Oneson and Zacharias (1960) have carried out viscosity studies on dispersion of tendon collagens, and have shown that the viscosities of such dispersions vary inversely with the carbohydrate content of the collagen. These authors interpret their results as supporting the hypothesis of a cross-linking role for the hexose moiety.

The action of enzymes.

General.

Enzymic degradation of proteins is a result of the cleavage of peptide bonds by specific proteolytic enzymes. These enzymes have been classified into two broad groups, based on the location of the hydrolysed peptide bond within the polypeptide chain of the substrate (Bergmann 1942). Thus exopeptidases will hydrolyse peptide bonds adjacent to a terminal α-amino or α-carboxyl group, whereas endopeptidases (proteinases) will hydrolyse peptide bonds located centrally within the polypeptide chain of the substrate. The specificity of proteolytic enzyme is dependent to a large extent upon the nature of the side chain groupings adjacent to the peptide bond, e.g. pepsin hydrolyses peptide bonds formed from the α-amino group of aromatic amino acids, and trypsin is specific for peptide bonds involving the α-carboxyl group of amino acids having a basic side chain. Another important factor affecting the enzymic hydrolysis of proteins is the steric configuration of the polypeptide chains of the substrate. It has been known for a long time that native proteins are often attacked extremely slowly, and in some cases not at all, by enzymes which will readily digest the same protein after it has been denatured. That this is the case with collagen was demonstrated by Grassmann (1936)

who reported that trypsin did not attack native collagen fibres, but would readily digest such fibres after they had been denatured by thermal shrinkage.

Native collagen fibres are extremely resistant towards almost all proteolytic enzymes, indeed Mandl (1961) considers that there is only one enzyme which has any collagenolytic activity. It is convenient therefore to consider this enzyme first.

The Collagenase of Clostridium histolyticum.

The collagenolytic activity of Clostridium histolyticum was demonstrated in 1931 by Weinberg and Randin, who found that native Achilles tendon was completely digested by several strains of this bacteria. These workers (1932) established that the Clostridium histolyticum enzyme was secreted by the bacteria, and they made attempts to isolate and purify the active component from culture filtrates. A similar collagenolytic activity was reported in strains of Clostridium perfringens (Macfarlane and MacLennan, 1945). The collagenase of Clostridium histolyticum has now been successfully isolated and purified (Mandl; Zipper and Ferguson 1958; Seifter, Gallop, Klein and Meilmann 1959) and its action on collagen extensively studied.

[N.B. Hereafter the collagenolytic enzyme of Cl. histolyticum will be referred to as collagenase].

Collagenase completely dissolves native insoluble

collagen fibres, and its action on solutions of soluble collagen is manifested by a rapid decrease in the viscosity of the solution, and a lowering of the specific rotation. These changes indicate a decrease in the axial ratio of the collagen unit in solution (i.e. decrease in length) and a loss of ordered configuration within the molecule (Seifter, Gallop and Meilmann, 1958).

Harrington and Von Hippel (1959) have investigated the kinetics of the proteolysis of solutions of ichthyocol, above and below the critical temperature Tc at which the collagen-gelatin configuration transition occurs. They have shown that above the Tc, where the structure is random, the proteolysis follows simple first order reaction kinetics, whereas below Tc the kinetics of the reaction are more complex, although they can be reduced to the sum of two first order reactions. They have suggested that the collagenase-sensitive bonds of collagen can be divided into two groups, on the basis of susceptibility to proteolysis, this difference in availability being dependent upon the configuration of the substrate in the region of the hydrolysed bonds. In addition to this dependence upon configuration, collagenase exhibits specificity with respect to the peptide bonds which are hydrolysed. This specificity is dependent upon the nature of the amino acids

in the proximity of the susceptible bond. Examination of the products of enzymic digestions has led to the elucidation of the specificity requirements of the enzyme, in addition to giving information on the nature of collagenolysis, and the structure of collagen.

The products of collagenase digestions of collagen are predominantly small peptides of molecular weight approximately 500 (Michaels, Gallop, Seifter and Meilmann 1958), although a small proportion of larger peptides are produced (Franzblau, Seifter and Gallop 1961). It has been reported that at least 30 peptides are produced (Michaels et al. 1958) and that glycine is present as the N-terminal amino acid of almost all peptides (Michaels et al. 1958; Grassmann, Nordwig and Hörmann, 1961). At the C-terminal end hydroxyproline and alanine are found, with hydroxyproline predominating (Heyns and Legler 1959; Nagai 1961; Manahan and Mandl 1961). The tripeptides Gly-Pro-Hypro and Gly-Pro-Ala have been isolated in large amounts (Grassmann, Hörmann, Nordwig and Wünch (1961); Schrohenloher, Ogle and Logan (1959)) accounting for a large percentage of the total imino acid, glycine and alanine content of the collagen. Analyses of this type, together with investigations on the effect of collagenase on various synthetic substrates reveal that the required sequence for collagenolytic cleavage is Pro-R-Gly-Pro,

with the hydrolysis occurring between R and Gly. (Heyns and Legler 1959; Grassmann et al. 1959). Although the proline in the above sequence can be replaced by hydroxyproline, this is accompanied by a decrease in activity, and a similar decrease in activity occurs if glycine is replaced by alanine, but the more general sequence X-P-R_1-R_2-P has been suggested for collagenase specificity (Manahan and Mandl, 1961).

The isolation of large amounts of the peptides described above indicates that collagenase hydrolyses peptide bonds in the non-polar regions of the peptide chains of collagen, and the isolation of large peptides containing negligible amounts of imino acids (e.g. Manahan and Mandl 1961) suggests that the polar regions of the peptide chains are relatively undegraded. Electron microscope studies on tanned collagen fibres during collagenolysis (Nordwig, Hörmann, Kühn and Grassmann 1961) have substantiated these conclusions. In the initial stages of collagenolysis the contrast between the light and dark cross-striations of the collagen fibre is increased; this being due to the digestion of the untanned apolar regions.

Collagenolytic action of other enzymes.

In addition to the collagenase of Clostridium histolyticum there have been numerous reports of other

enzymes possessing collagenolytic activity. In many instances such reports have been based on experiments in which the substrate used has been acid solutions of soluble collagen, or insoluble collagen in suspension at an acid pH. Mandl (1961) considers that activity under such conditions is not indicative of true collagenolytic activity, and required that the substrate used should be native insoluble collagen under physiological pH conditions.

Although collagen in solution in acid buffers, or insoluble collagen suspended in solutions of an acid pH, is not denatured (acid solutions of collagen exhibit the high laevorotation of the native protein) the swelling effect produced by acid pH conditions (or solutions of lyotropic salts) renders the protein more susceptible to proteolysis. According to Gustavson (1956) the swelling of the protein under such conditions is due to a weakening of the secondary valency forces. An example of a report of collagenolysis under such conditions is provided by the work of Sherry, Troll and Rosenblum (1954), who examined the activity of the plant cathepsins papain and ficin with respect to collagen. Although these enzymes have a pH optimum near to neutrality, it was found that they would not digest collagen at neutral pHs. However they will readily solubilise collagen at pHs below 4.5. Sherry et al.(1954)postulated that the digestion under these

conditions was not a true enzymic digestion, but rather the result of a two-stage process, the first stage being a physical alteration of the collagen, under the conditions employed, thus rendering it more susceptible to enzymic attack. That the swelling produced by the acid conditions was indeed a necessary factor was confirmed when it was shown that the digestion was inhibited when swelling of the collagen was prevented by the addition of salt. Hinrichs and Whitaker (1962) have confirmed the work reported by Sherry et al. and have also shown that bromelain, another plant enzyme, has a similar action to papain and ficin.

A brief account will now be given of some of the other enzymes which have been reported to have collagenolytic activity.

The action of the digestive enzymes trypsin and pepsin, upon collagen, has been extensively investigated. Sizer (1949) reported that native tendon was refractory to the action of trypsin, but that some digestion occurred when the collagen fibrils were cut into small segments thus freeing the ends. Extensive digestion occurred when the substrate used was a hide-powder, but Gustavson (1956) showed that trypsin had no action when the hide-powder used as substrate was prepared under conditions which

eliminated any denaturation of the collagen. Thaureaux (1945) found that native tendon, after treatment with trypsin, showed enhanced water solubility at the shrinkage temperature, and Gustavson (1949) reported similar results for hide collagen. Grant and Alburn (1960) reported that trypsin would solubilise collagen fibres from rat tail tendon at neutral pHs in the presence of calcium chloride and centain other reagents, e.g. arginine, salicylates, guanidine. However, calcium chloride itself had some solubilising effect, and the solubilisation was probably the result of a combined effect, as in the case of the action of the plant cathepsins at acid pH values.

The action of trypsin on solutions of collagen has also been investigated. Gross (1957) examined its effect upon solutions of neutral salt soluble collagen, and reported a slight activity, manifested by a small reduction in viscosity, and an inhibition of gelation on warming to 37°C. Hodge, Highberger, Deffner and Schmitt (1960) treated solutions of collagen with trypsin in the presence of 0.5M calcium chloride. The enzyme produced a small decrease in the viscosity of the solution, and the ability of the collagen to be reconstituted as native type (640Å spacing) fibres was inhibited. They have isolated an acidic peptide fraction from such digests and have shown that this fraction contains tyrosine. These results

constituted an important piece of evidence for the existence of the terminal "end-chains" described earlier. Kühn, Kühn and Hannig (1961) also digested tropocollagen solutions with trypsin in the presence of calcium chloride, and obtained results which conflict with those of Hodge et al. A decrease in the tyrosine content of the collagen was reported, and, in addition, a decrease in the content of hexose and hexosamine, but similar decreases in the content of these components could also be obtained by recrystallising the collagen via the SLS form of precipitate. The trypsin-treated collagen was found to be capable of normal fibre formation, and it was inferred that trypsin had no action upon the tropocollagen molecule in solution. Nishihara (1960) treated cowhide, after removal of soluble protein and hair, with trypsin at pH 8.0 and 25°C. No solubilisation of protein occurred. However, after removal of trypsin by washing, the pH was lowered to approximately pH 3.0, and it was found that the hide was completely solubilised. The material in solution was reported to have physical dimensions and properties almost identical to those of normal tropocollagen molecules, and could be precipitated from solution in the form of normal fibres having a repeating period of 700Å.

Investigations into the collagenolytic activity of

pepsin correspond closely to those performed with trypsin. Sizer (1949) showed that pepsin would readily digest native tendon at 37°C and pH 2.0, and Nishihara (1960) reported that pepsin solubilised cowhide at pH 2.0, producing a solution of collagen having identical properties to that produced by the action of trypsin. Kühn, Hannig and Hörmann (1961) treated solutions of tropocollagen with pepsin and reported similar changes to those produced by the action of trypsin, i.e. a decrease in tyrosine content, but no inhibition of fibre formation. Rubin, Pfahl, Speakman, Davison and Schmitt (1963) treated solutions of procollagen with pepsin, and found that pepsin had similar effects to those reported for trypsin by Hodge et al. (1960). They showed that the dialysable peptide fraction produced by peptic digestion contained large amounts of tyrosine, glutamic acid and aspartic acid, but no hydroxyproline. The pepsin treated collagen on denaturation yielded α:β subunit ratio of 85:15.

In addition to pepsin and trypsin, collagenolytic activity has also been found in other mammalian tissues. Frankland and Wynn (1961) found that the lysosomal fraction of rat liver homogenates would reduce the viscosity of acid-soluble collagen at pH 4.0, and Houck and Patel (1962)

reported a collagenolytic activity in pancreas. This enzyme reduced the viscosity and optical rotation of collagen solutions, and produced a hydroxyproline-rich dialysable fraction.

Enzymes from a wide variety of other sources have also been found to have some collagenolytic activity, e.g. Gross and Lapiere (1962) reported that certain tissues of tadpoles possessed a collagenolytic activity, and would degrade undenatured calf-skin collagen at 27°C and at neutral pH. Significant solubilisation of hydroxyproline occurred, half of the soluble hydroxyproline being in a dialysable form. Giffee et al. (1961) found that certain strains of Pseudomonas had a comparable effect to trypsin and would solubilise collagen in the presence of strong solutions of calcium chloride.

In addition to the proteolytic enzymes described above there are other enzymes which have an effect upon collagen fibres, although possibly not on the collagen molecule itself. The action of hyaluronidase probably falls into this category, since it was shown by Jackson (1953) that tendon treated with this enzyme could be almost completely dissolved in dilute acetic acid, whilst untreated tendon is soluble to a much smaller extent. Enzyme treated fibres also had a lower shrinkage temperature than the normal fibres. Another enzyme which possibly

falls into this category is the collagen/mucoproteinase of Banga and Balo (1956). This is not a proteolytic enzyme, but it dissolves a mucoid substance termed $mucoid_2$ from collagen fibres (Banga and Balo 1960). These workers report that 16% of the fibre is dissolved by this enzyme at 37°C. The extracted $mucoid_2$, which contains 10% of hydroxyproline, was suggested to be a stabilising factor in the fibres.

Soluble Collagens.

Nageotte (1927) was the first to discover that a part of the collagen of connective tissues could be dissolved in dilute solutions of weak acids. Orekhovitch et al. (1948) found that acid citrate buffers had the same effect as weak acids and termed the extracted material procollagen, believing it to be a precursor of the mature insoluble collagen. Later work showed that collagen could be solubilised in solutions other than those of weak acids or acid buffers. Harkness, Marko, Muir and Neuberger (1954) used saturated solutions of disodium phosphate (pH 9.0) to extract small amounts of an alkali soluble collagen from rabbit skin, and Gross, Highberger and Schmitt (1955) reported that dilute neutral salt solutions had a similar effect. These workers also found that increasing amounts of this neutral-salt-soluble

collagen could be extracted with salt solutions of increasing concentration.

Harkness et al. (1954) and Jackson (1957) have investigated the turnover rate of radioactive glycine in the various soluble collagen fractions of connective tissue, and have shown that the turnover is most rapid in the neutral salt-soluble fraction. It is now accepted that this neutral salt-soluble fraction is the precursor of both acid-soluble and insoluble collagens.

The mechanism of fibrogenesis has been clarified by Jackson and Bentley (1960). It is considered that native insoluble collagen fibres are derived from the newly-formed collagen units by the accretion of these units on to existing fibres, with the strength of the crosslinking within the fibre increasing with the age of the fibres. These workers consider that any tissue contains a continuous spectrum of collagen aggregates. Monomeric units will be extracted in very dilute salt solutions, while solubilisation of the larger aggregates requires more concentrated salt solutions and acid buffers.

Gelatines.

The formation of a gelatine requires a more extensive degradation of the collagen structure than is necessary for the production of a parent gelatin. In the commercial

preparation of a gelatine the necessary changes are brought about in a process which consists of two stages. The first, or pretreatment stage, usually consists of the prolonged treatment of the collagenous material (usually hide or bone collagen (ossein) with lime. The duration of this pretreatment is normally 6-8 weeks. An acid pretreatment is employed in some instances, the acid used normally being hydrochloric acid. This pretreatment stage renders the collagen more readily extractable as a gelatineunder reasonably mild conditions. The second stage of the process, termed the extraction stage, consists of heating the washed pretreated material in water, in which it dissolves as a gelatine. Extraction of the gelatine can also be achieved in the cold by the use of reagents which break hydrogen bonds (Courts, 1958). It is apparent therefore that the changes occurring in the extraction stage are analogous to those resulting in the formation of a parent gelatin, namely cleavage of hydrogen bonds. The more extensive changes which result in the production of gelatine thus occur in the pretreatment stage, and Courts (1958) has shown that there is some peptide bond cleavage in this stage, this being manifested by an increase in the number of free α-amino groups which are available for reaction with fluorodinitrobenzene. Ward (1960)

suggests that there is also some hydrolysis of lateral interchain bands in this stage, and in addition that the length of the polypeptide chains must be reduced to the order of a molecular weight of 70,000 for easy extraction of gelatine.

Protease of Streptomyces griseus.

Nomoto and Narahashi (1959a) found that the culture media of Streptomyces griseus contained large amounts of a very active protease. These workers succeeded in isolating this protease, which they termed Streptomyces protease G, and obtained the enzyme in a pure and apparently homogeneous form (Nomoto and Narahashi (1959(a), (b) and (c)). The same authors (1959d) examined the activity of the enzyme, using casein as substrate and showed that it had maximum proteolytic activity at 60°C. and that the optimum pH for enzyme activity was pH 8.0. They also reported that the enzyme was very active and digested almost all proteins, with the exception of the insoluble sclero-proteins. In further investigations Nomoto, Narahashi and Murakami (1960 (a) and (b)) examined the specificity of the protease, using both proteins and synthetic compounds as substrate, and found that the enzyme had an extremely broad specificity, being capable of hydrolysing almost all peptide bonds.

It was found that with casein or ovalbumin as substrate between 70 and 90 per cent of the total peptide bonds were hydrolysed. The protease was also found to be capable of hydrolysing synthetic dipeptides, and the authors consider that it is unique in that it belongs to neither of the two types of proteolytic enzymes (Endo- and exo-peptidases) of Bergman (1942), but exhibits both types of activity.

Sisal Enzyme.

The sisal enzyme used in this work was a crude extract of sisal juice prepared by acetone precipitation and supplied by Glaxo Laboratories Ltd.

INTRODUCTION TO THE PRESENT WORK

This work was undertaken to examine the action of Pronase on collagen since, as this enzyme has been reported to have a very high activity, it was considered that it might have a more extensive action than more commonly used proteolytic enzymes and thus might provide a useful tool for examining the structure of collagenous proteins. The sisal extract which became available was examined for proteolytic and collagenolytic activity since it has been found that extracts of certain plants do contain enzymes which have a limited action on collagen. The action of the carbohydrate-splitting enzymes was studied in the hope that they might give some information on the involvement of the carbohydrate components of collagen. The possibility was also examined that while these enzymes might not degrade the collagen molecule itself they might affect intermolecular links in collagenous tissues and in this way enable soluble material (either collagen or gelatine) to be obtained more readily from material which is normally in the main insoluble.

SECTION 2

E X P E R I M E N T A L

I. Preparation of collagen.

Collagen was prepared from both calf and ox skin. Two extraction procedures were employed.

(a) Extraction with acetic acid. (Steven and Tristram. 1962(a)).

The skin was freed from hair and subcutaneous fat, cut into small strips, and minced. The minced tissue was suspended in 5-7 volumes of 0.2M disodium hydrogen phosphate, and homogenised in a Waring Blender. A small amount of toluene was added, to inhibit bacterial growth, and the mixture was allowed to stand overnight at 4°C. After centrifugation at 4°C the supernatant, containing serum proteins and small amounts of neutral salt soluble collagen, was discarded, and the insoluble material rehomogenised in 0.2M Na_2HPO_4. Extraction with phosphate was repeated five times, or until the supernatant was practically clear. The residue was then homogenised in 0.1M acetic acid, allowed to stand overnight, and centrifuged in the cold at 2,500 r.p.m. The supernatant was collected, and the residue

re-extracted several times with 0.1M acetic acid, until the low viscosity of the supernatant revealed that little soluble collagen was being extracted.

(a)(i) Reconstitution and purification of acetic acid soluble collagen.

Soluble collagen was reconstituted from the pooled acetic acid extracts by dropwise addition of a solution of saturated sodium chloride, to a final concentration of approximately 10%. The solution was stirred continuously throughout the addition of sodium chloride. The reconstituted collagen was collected by centrifugation. Purification of the collagen was achieved by redissolving the collagen in 0.1M acetic acid, and reprecipitating with sodium chloride. This was repeated three times. A final reprecipitation was carried out by dialysing an acetic acid solution of the collagen against running tap water. The reconstituted collagen, collected by centrifugation, was stored at low temperature.

(a)(ii) Preparation of insoluble collagen.

The residual material from the above acetic acid extractions was resuspended in 0.1M acetic acid and centrifuged at 500 r.p.m. to remove the coarser particles. The finely divided material remaining in suspension was

centrifuged at 14,000 r.p.m. and the supernatant discarded. This material was extracted repeatedly with 0.1M acetic acid until the supernatant showed no trace of a precipitate on addition of saturated sodium chloride solution. The exhaustively extracted material was finally suspended in 0.1M acetic acid, dialysed against running water to remove all trace of acid, collected by centrifugation, and stored in the cold.

(b) Extraction with citrate buffer. (Harkness, R.D., Marko, A.M., Muir, H.M., Neuberger, A. (1954)).

The skin was freed from extraneous material as above and homogenised in a saturated solution of disodium hydrogen phosphate, pH 9.0. The mixture was allowed to stand overnight, centrifuged, and the supernatant discarded. This was repeated four times. Collagen was extracted by stirring the skin in 0.1M citrate buffer pH 3.8 overnight. After centrifugation the supernatant was dialysed against several changes of 0.01M Na_2HPO_4 (pH 8.6) to precipitate the collagen. The precipitated collagen was purified by repeatedly redissolving in citrate buffer and reprecipitating by dialysis. Repeated extraction of the skin with citrate buffer was carried out until negligible amounts of protein were extracted. The insoluble material was dialysed

against running water to remove all traces of citrate buffer, collected by centrifugation, and stored in the cold.

II. Fractionation of Pronase.

a) Preparation of cellulose phosphate.

Crude Pronase was fractionated into its components by chromatography on a column of cellulose phosphate (Whatman & Co., Ltd.). The cellulose phosphate was pretreated by heating at 100° for 2-3 hours in 2N sodium hydroxide in order to remove ammonia (this material is often marketed as the ammonium salt). The alkali was then removed from the cellulose by washing repeatedly with distilled water on a Buchner funnel, and the cellulose was finally equilibrated with 0.1M citrate-phosphate buffer pH 3.2

b) Fractionation procedure.

A suspension of cellulose phosphate in 0.1M citrate-phosphate-buffer (pH 3.2) was poured into a column of 2 cm. diameter, and the column packed by applying a slight pressure using a sphygmomanometer bulb, until the height of the resin in the column was approximately 30 cms.

250 mg. crude Pronase was dissolved in 5 ml. citrate-phosphate buffer (pH 3.2) and applied quantitatively to the top of the column, and elution commenced using the above buffer. 5 ml. fractions were collected, using a fraction

collector of the photo-electric drop counter type (Locarte, London). After elution of the first fraction, which could be seen on the column as a slightly yellow band, gradient elution was commenced. A pH gradient was employed, the increase in pH being brought about by addition of 0.05 N NaOH. Elution was continued until the eluate attained a pH of approximately 12-13, and the final fraction, which again was seen as a yellowish band, was eluted.

Individual fractions were analysed for protein content by measurement of their absorption at 280 mu, and an elution curve obtained. The bulked enzyme fractions were freed from salts by dialysing against running water for 48 hours. The fractions were concentrated by suspending the dialysis tube in the atmosphere and blowing a current of air over it.

Total nitrogen determinations were carried out on aliquots of each fraction, and the enzyme solutions were stored at 0°C. for subsequent examination for collagenolytic activity.

III. Separation of collagen sub-units.

a) Preparation of column.

Fractionations were carried out on columns of carboxymethyl cellulose (Whatman & Co., Ltd.), after pretreatment by the procedure described in II(a). The

cellulose was finally equilibrated with 0.01M acetate buffer (pH 4.8) and poured into a water-jacketed column 40 cm. long and 4 cm. in diameter. The column was packed by applying suction at the bottom.

b) Gradient.

The separation was affected by the use of an ionic gradient elution. The gradient was applied to the column from a 9-chambered Autograd (Technicon Instruments Ltd.) as described by Peterson and Sober (1960). The first chamber of the autograd was filled with the starting buffer, 0.01M acetate pH 4.8 while the remaining 8 chambers contained increasing concentrations of the same buffer viz:- 0.10M; 0.12M; 0.14M; 0.16M; 0.16M; 0.20M; 0.30M; 0.50M. 200 ml. of buffer were placed in each chamber.

c) Fractionation procedure.

The material to be fractionated was dissolved in 0.1M acetic acid, and dialysed for 24 hours against a large volume of the starting buffer (0.01M acetate pH 4.8). The collagen in solution was denatured in a water bath at 39°C for 15 minutes, and then pumped onto the column using a D.C.L. micro-pump (type S). Gradient elution was commenced, and continued until the first two fractions were eluted, when elution was continued with 0.5M acetate buffer. After elution of the third fraction with this buffer, the final component was eluted using a solution of 0.1M sodium

hydroxide containing 0.1M sodium acetate. Treatment with this solution also regenerates the column. The presence of the sodium acetate minimises the swelling of the carboxymethyl cellulose which is produced by the sodium hydroxide. Throughout the fractionation the column was maintained at 39°C. 10 ml. fractions of eluate were collected in a syphon-type fraction collector, and the eluate was examined for protein content by the colorimetric procedure of Folin-Lowry (1951) using an auto-analyser (Technicon Instruments Ltd.)

After elution of the final fraction the column was re-equilibrated with 0.01M acetate pH 4.8.

IV. Chromatography of amino acids and peptides.

(a) Desalting of solutions prior to chromatography.

Large amounts of inorganic ions interfere with the separation of compounds by paper chromatography and thus it is important to effect a removal of these ions prior to chromatography. Ion exchange resins have been widely used for the desalting of solutions of peptides and amino acids (Block, Durrum and Zweig, 1958).

(a)(i) Preparation and choice of resin.

The resins employed were all cation exchange resins of the sulphonated polystyrene type. Zeocarb 225; Dowex 50 and Amberlite CG 120 were used in this work. Steven and

Tristram (1962 a) reported that several commercial preparations of such resins contained trace amounts of free amino acids, and it is important therefore to examine all resins for the presence of amino acids before employing them for desalting. Of the three resins used in this work Amberlite CG 120 was found to be completely free of indigenous amino acids, and thus was used throughout for desalting procedures.

The resins were regenerated by the method of Hirs, Moore and Stein (1952). The resin was first heated on a boiling water bath for several hours with 2N sodium hydroxide, and then washed with water on a Buchner funnel until free of alkali. Conversion of the resin into the hydrogen form was effected by washing with 2N HCl, and excess acid was removed by washing with glass distilled water until the washings were neutral.

(a)(ii) Method of desalting.

Small columns of resin were prepared, approximately 2 cm. in diameter and 5 cms. long. The amino acid solution to be desalted, at a neutral or slightly acid pH, was then placed on the column, and the column washed with a large volume of glass distilled water, to remove inorganic ions. The amino acids were eluted from the resin with a solution

of N.NH_4OH, which was prepared from redistilled concentrated ammonia. After removal of ammonia, by concentration of the eluate to dryness in vacuo, the amino acids were dissolved in a small volume of water for application to a chromatogram.

(b) Chromatographic procedures and solvents.

(b)(i) Paper chromatography of amino acids was carried out using the system of Redfield (1953). Using this system two-dimensional separations were obtained on Whatman No.20 paper. The papers used were 20 x 20 cms. and several chromatograms were run at one time using the multisheet frames described by Datta, Dent and Harris (1950). Ascending chromatography was employed in both dimensions, and the solvent systems used were:-

1st dimension:- Methanol: Water: Pyridine. 80:20:4 (v/v).

2nd dimension:- tertiary Butanol: Methyl Ethyl Ketone: Water: Diethylamine. 40:40:20:4 (v/v).

Prior to staining, the chromatograms were treated with steam by autoclaving for 45 minutes at a pressure of 15 lbs./sq.in. to remove all traces of diethylamine.

(b)(ii) In some cases peptides and amino acids were chromatographed on larger sheets (30 x 30 cms.) of Whatman No.4 paper. Two-dimensional ascending chromatography was again employed, the solvent systems being:-

1st dimension:- Ethanol : Water : Ammonia. 80:20:1 (v/v).
2nd dimension:- n-Butanol : Acetic acid : Water. 3:1:1 (v/v).

(c) Detection of amino acids and peptides.

(c)(i) General stain. Chromatograms were dipped in a solution of 0.25% (w/v) ninhydrin in acetone (Toennies, 1951). After drying, the chromatograms were developed by warming to 60°C. for 5 - 10 minutes in a hot-air oven. Amino acids appear as blue spots except for proline and hydroxyproline which give yellow spots.

(c)(ii) Specific stain for proline and hydroxyproline.

Although proline and hydroxyproline produce distinctive yellow spots on staining with ninhydrin the spots produced were frequently very faint, and thus a more specific and sensitive method of detection was employed, viz:-

The reagent used was a solution of isatin (0.4% w/v) in n-butanol containing 4% v/v glacial acetic acid. This reagent produces a blue colour with the imino acids, the background colour of the paper being yellow. Roberts and Kolar (1957) recommend that the sprayed chromatogram be heated at 100°C. for 10 minutes, and then stored for 24 hours prior to inspection, and this procedure was followed.

V. Chromatography of sugars.

(a) Method.

Free sugars were identified by paper chromatography using single dimension descending chromatography on Whatman No.1 paper (45 x 50 cms.).

Solvent. The solvent system employed was

Pyridine: Ethyl acetate: Acetic acid: Water
5:5:1:3 (v/v)

and the chromatograms were developed in a tank saturated with the vapour of the following solvent system:-

Pyridine: Ethyl acetate: Water. 11:40:6 (v/v)

(Fischer et al. 1955).

(b) Detection of sugars.

Two methods were used for the detection of sugars on paper chromatograms.

(i)

Reagents:- (1) 0.5% (w/v) solution of 2:3:5 triphenyl-tetrazolium bromide in chloroform.

(2) 0.1M KOH in 99% ethanol.

Method:- The dried chromatograms were dipped in reagent (1) and dried. After dipping in reagent (2) and drying the chromatograms were heated at 100°C for 5 - 10 minutes in a hot air oven. Reducing sugars were detected by the presence of red spots.

(ii)

Reagents:- p.Anisidine reagent made up as follows:-

1.2 grams p.Anisidine
1.7 grams Phthalic acid
10 mg. $SnCl_2$
} made up to 100 ml. in ethanol.

Method:- The dried chromatogram was dipped in the above solution, and spots were developed by heating the chromatogram to 100°C for a few minutes.

VI. Estimation of enzyme activity.

a) Estimation of solubilised nitrogen.

The method was employed to determine the amount of collagen nitrogen which was brought into solution by the enzyme. The collagen was incubated at 20°C with a known quantity of enzyme, in buffer solution of required pH. At the termination of the reaction the mixture was centrifuged and total nitrogen determinations carried out on the supernatant.

b) Estimation of dialysable nitrogen.

To determine the amount of collagen nitrogen which was rendered dialysable by enzymic activity, digestions were carried out, at 20°C under appropriate pH conditions. After termination of the reaction, the mixture was placed in a dialysis tube, and suspended in a flask containing a large volume of glass distilled water. The water in the flask was constantly stirred by a magnetic stirrer, and was usually changed four times at 12 hour periods. The

bulked dialysates were reduced in volume in a rotary film evaporator, and total nitrogen determinations carried out.

c) Estimation of acetone soluble nitrogen.

To estimate collagen nitrogen which was soluble in acetone after enzymic digestion, the reaction mixtures were precipitated with 10 volumes of acetone. After centrifugation, total nitrogen determinations were carried out on the supernatant, after it had been reduced in volume.

d) Base of conversion to gelatin.

Collagen in the insoluble form can be converted to gelatin by heating in water to temperatures above the denaturation temperature, when the collagen dissolves as gelatin. Collagens which have been partially degraded by enzymes will be more readily solubilised than native protein. This provides a method of detecting enzymic activity. The following procedure was employed. A weighed amount of the material was suspended in water and warmed to 40°C for a given time. The suspension was centrifuged and the solubilised nitrogen determined by the microkjeldahl technique. The insoluble material can be re-suspended and the procedure repeated. Control experiments were carried out simultaneously.

e) Thermal gelation of collagen solutions.

Native collagen solutions will form a gel on warming to 37°C at neutral pH's. Collagen solutions were tested for gelling powers by dissolving in 0.05 M acetate buffer pH 5.5 approximately and warming to 37°C. After standing for 2-3 hours the solutions were examined for the presence of a gel.

f) The congo red dye method.

The congo red dye method, Roaf (1908), was also employed by Naughton and Sanger (1961) whose modified method was used in this work.

i) Preparation of congo red collagen. Finely divided insoluble collagen was suspended in a saturated aqueous solution of congo red and stirred or shaken for 24 hours. The suspension was centrifuged, the supernatant discarded, and the collagen resuspended in water. This was repeated until the washings were clear of dye. The collagen was then washed with acetone and ether and dried in a vacuum desiccator.

ii) Method. Equal weighed quantities of congo-red collagen were placed in a series of flasks containing buffer of required pH. Varying amounts of enzyme were added to the flasks, and one flask containing no enzyme was used as a blank. Buffer was then added so that each vessel

contained the same volume. The flasks were then shaken gently for 24 hours. The reaction mixtures were centrifuged, the supernatants filtered through glass wool, and the extinction measured at 495 mμ.

9) Measurement of viscosity changes.

Solutions of collagen exhibit a very high viscosity. This is a function mainly of the axial ratio of the molecules in solution, although interactions between charged groups on the protein has a slight effect (the electroviscous effect). This effect varies with the pH and ionic strength of the solution. Since any change in the size or degree of association of molecules in solution will be reflected in a change of viscosity, the measurement of the viscosity of solutions during the course of enzymic digestions provides an extremely useful method for the assessment of enzymic degradation.

Method. Viscosity measurements were carried out in Ostwald viscometers which had a flow time for water of 60 seconds at 20°C. The protein solution (10 mls.) was introduced into the viscometer immediately after the addition of an aliquot of enzyme solution, and the first reading was taken usually 2 minutes after the addition of the enzyme. Subsequent readings were taken at 5 or 10 minute intervals. An initial reading of the viscosity

was obtained using 10 ml. of the same protein solution containing an aliquot of buffer of equal volume to the aliquot of enzyme solution used.

η_o = viscosity of solution at zero time

η_T = viscosity of solution T minutes after adding enzyme

η_w = viscosity of water

Relative viscosity at zero time is $\frac{\eta_o}{\eta_w}$ and at time T is $\frac{\eta_T}{\eta_w}$, but since using dilute solutions the viscosity is proportional to the flow time t seconds, these relative viscosities may be given as $\frac{t_o}{t_w}$ and $\frac{t_T}{t_w}$.
Viscosity results throughout are expressed as a percentage of the initial relative viscosity, i.e. $\frac{t_T}{t_o} \times 100$ and since the ionic and pH conditions remain the same throughout the course of the reaction, the electroviscous effect can be ignored.

h) Measurement of optical rotation.

The high specific rotation of collagen solutions ($\alpha_D - 350^\circ$) is the result of the highly ordered helical configuration of the collagen molecules. Randomisation of the helical configuration, as in the thermal denaturation of collagen, results in a marked lowering of the specific

rotation (Doty and Nishihara 1958). Similarly, any destruction of the helical configuration as a result of proteolysis will be reflected in a lowering of the specific rotation.

Optical rotations were measured in an ETL-NPL Automatic polarimeter type 143 A. The polarimeter was fitted with a Honeywell chart recorder giving readings in millivolts, and in order to convert these readings into degrees of rotation the apparatus was calibrated using standard solutions of L(-)cystine which has a specific rotation of -214^{0} in N.HCl.

Solutions were introduced into the cell using an automatic pipetting syringe and the outflow from the cell could be returned to the original solution if desired. A blank reading was obtained using collagen alone, and after addition of enzyme, the solution was introduced into the cell as quickly as possible. At least 50 ml. of solution containing enzyme was washed through the cell in order to remove the original collagen and to ensure that the sample in the cell had the desired enzyme:collagen ratio.

i) The pH-Stat.

pH Stat titrations were carried out using an automatic titrator (Titrator TTTI, Radiometer, Copenhagen) fitted with a chart recorder. For constructional details of this apparatus see Jacobsen et al. (1957). The titration vessel

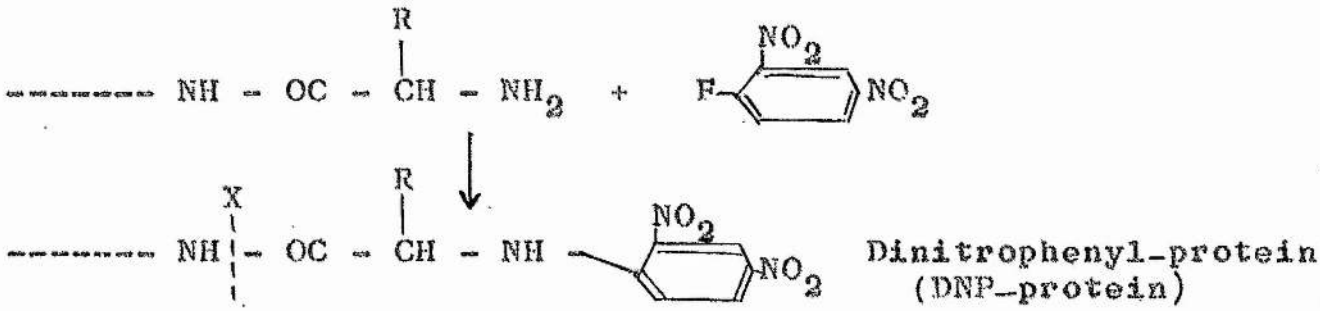
used had a capacity of 100 ml. and was closed to the atmosphere, the reaction mixture being agitated by means of a magnetic stirrer.

Acid-soluble collagen was dissolved in 0.01N HCl and 50 ml. aliquots (containing a known amount of nitrogen) were introduced into the reaction vessel, titrated to the required pH value (pH 3.5) and allowed to equilibrate at this pH for some time. 1 ml. of a solution of enzyme in distilled water was added to the collagen solution and the reaction allowed to proceed. Changes in pH as a result of liberation of carboxyl groups by enzymic cleavage of peptide bonds were corrected by automatic addition of acid (0.05N HCl). The volume of acid added to maintain the pH at 3.5 was automatically recorded.

VII. The fluorodinitrobenzene technique.

a) Introduction.

The examination of the free amino groups of proteins by reaction with 1-fluoro-2:4-dinitrobenzene (FDNB) was first used by Sanger (1945) in a study of the N-terminal amino acids of insulin. This method is now widely used for investigation of free amino groups in proteins and peptides, and has several advantages over other methods. The reaction of FDNB with the N-terminal amino acid of a protein may be represented thus:-



The reaction of a protein with FDNB can be carried out at room temperature, under conditions which minimise denaturation of the protein. Hydrolysis of the DNP-protein results in cleavage at the point X, releasing DNP-amino acids. The position is complicated somewhat as FDNB also reacts with the side-chain amino groups of lysine and arginine as well as with other groupings, e.g. -OH of tyrosine and -SH of cysteine. However such DNP-amino acids are not soluble in ether, as are the majority of the α-DNP amino acids (except α-mono-DNP-arginine and α-DNP-histidine), and thus the α-DNP-amino acids are readily separated from other amino acids by extraction into ether. The yellow colour of DNP-amino acids greatly facilitates their separation and estimation. The method does however possess some disadvantages, the greatest of these being that DNP-amino acids undergo degradation on hydrolysis. The extent of degradation varies for different DNP-amino acids. However, under standard hydrolysis conditions a correction can be made for this degradation. DNP-amino acids also undergo

some degradation in light, but this can be minimised by carrying out much of the procedure in the dark.

During hydrolysis of the DNP-protein some of the unreacted FDNB is converted to dinitrophenol (DNP.OH). The unreacted FDNB and DNP.OH (collectively termed artifact) are also ether-soluble and are much in excess of the α-DNP amino acids. They must therefore be removed prior to chromatographic examination of the α-DNP amino acids.

b) Reaction with FDNB.

The reaction conditions employed throughout were those of Sanger (1945). The material to be examined was dissolved in water (in the case of soluble collagen in 0.1M acetic acid) or in the case of insoluble collagen suspended in water. Two volumes of ethanol were added slowly to the solution, with continuous stirring to prevent precipitation of the protein, and the mixture was made alkaline (pH 8-9) by addition of sodium bicarbonate. 0.5 ml. of FDNB were then added. For reaction with collagen or gelatin the reaction mixture was shaken or stirred for 48 hours at room temperature, the pH of the reaction mixture being checked periodically to ensure that it remained alkaline. In the case of a mixture of free amino acids and small peptides a shorter reaction time (12 hours) was used.

c) Hydrolysis of DNP-protein.

After termination of the reaction by acidification with diluted hydrochloric acid, the mixture was evaporated to dryness by vacuum distillation. Constant boiling (5.7 N) glass distilled HCl was added to the DNP-protein which was hydrolysed in the dark for 18 hours, under reflux.

d) Extraction of DNP-amino acids.

The hydrolysis mixture was diluted with 2-3 volumes of water and extracted 5 times with 4 volumes of ether. [N.B. The ether had previously been freed from peroxides by standing over ferrous sulphate]. The bulked ether extracts were washed with water, the water washings being added to the aqueous phase.

e) Removal of artifact.

The ether extract was evaporated to dryness and the following procedure used to separate the artifact from the DNP-amino acids. The method is a modification of that of Li and Ash (1953), by Steven (1962).

8 grams silicic acid (Mallinckrodt 100 mesh) was thoroughly mixed with 4 ml. M/15 Na_2HPO_4. This was made into a slurry with chloroform previously buffered with M/15 phosphate, and poured into a small column of 1 cm. diameter with a cottom wool plug. The dried ether extract, dissolved in a small volume of buffered chloroform was applied to the

top of the column, which was then developed with buffered chloroform. This results in elution of the artifact from the column, leaving the DNP-amino acids firmly bound. The DNP-amino acids were then eluted from the column with chloroform containing 1% glacial acetic acid. Throughout this procedure the column was kept in the dark.

f) Chromatography of DNP-amino acids.

DNP-amino acids were separated by 2-dimensional paper chromatography on Whatman no.1 paper. The solvent systems used were -

1st dimension: (ascending) Toluene:Pyridine:2-Chloroethanol:0.8N Ammonia. 30:9:18:18 (v/v). (Biserte and Osteaux 1951: Levy 1954)

2nd dimension: (descending) 1.5M phosphate buffer, pH 6.1.

After application of the DNP-amino acids (in acetone) to the chromatogram, the paper was clipped into a cylinder and equilibrated for 2-3 hours in a tank saturated with 0.8N ammonia, before addition of the solvent. Development in the first dimension required 20 hours, after which time the chromatogram was dried prior to running the second dimension. All chromatographic and drying procedures were carried out in the dark.

g) Elution and estimation of DNP-amino acids.

After drying the chromatogram the yellow spots of the DNP-amino acids were carefully cut out and placed in test

tubes containing 4 ml. 1% sodium bicarbonate. Another small piece of paper of the same size was treated similarly to act as a blank. Elution was carried out in the dark at 37°C for 1 hour. After shaking the tubes the eluate was poured off and the optical density measured at 360 mμ.

h) Estimation of losses on hydrolysis.

A mixture of DNP-amino acids was chromatographed as above, and the quantities of the individual amino acids calculated from the optical density using the extinction coefficients given by Fraenkel-Conrat, Harris and Levy (1955). A similar amount of the DNP-amino acid mixture was hydrolysed under conditions identical to those used for hydrolysis of DNP-protein, and after extraction into ether were chromatographed and estimated as before. The degradation occurring on hydrolysis was then calculated.

i) Separation and chromatography of DNP-peptides.

Separation. Water soluble DNP-peptides were separated into two fractions on the basis of solubility in different solvents. The solutions of DNP-peptides, after removal of DNP-amino acids by ether extraction, were extracted three times with ethyl acetate, which extracted DNP-peptides termed the ethyl acetate soluble fraction. The DNP-peptides retained in the water phase constituted another fraction.

Chromatographic procedures.

Paper chromatography.

i) Ethyl acetate soluble DNP-peptides were separated by 2-dimensional paper chromatography (descending) on Whatman no.20 paper. The solvents used were:-

1st dimension: 3° Amyl alcohol: 3% Ammonia 1:1 (v/v)

2nd dimension: 0.8M phosphate pH 6.7.

ii) Water soluble DNP-peptides were separated by 2-dimensional descending chromatography on Whatman no.20 paper. The solvent systems being:-

1st dimension: 3° Amyl alcohol: IsoAmyl alcohol: 3% Ammonia 2:1:3 (v/v).

2nd dimension: 1.6M phosphate pH 7.0.

Column chromatography.

i) Water soluble DNP-peptides were separated into two fractions on columns of talc. The material, dissolved in N.HCl was applied to a column of talc equilibrated with acid. Elution with N.HCl removed peptides containing no ϵ-DNP lysine, and peptides containing ϵ-DNP-lysine were then eluted with ethanol:N.HCl 4:1 (v/v).

ii) DNP-peptides were fractionated on columns of DEAE cellulose. The starting buffer, with which the columns were equilibrated was 0.1M acetic acid, and ionic gradient elution was employed using 0.2M sodium acetate.

VIII. Quantitative estimation of hexose and hexosamine.

(a) Hydrolysis procedure.

Anastassiadis and Common (1958) reported that tissue samples could be hydrolysed with suspensions of a sulphonated polystyrene resin in dilute acid. The method has the advantage that the degradation of certain tissue components e.g. glucose, is very much less than that produced by normal hydrolytic procedures employing concentrated solutions of strong mineral acid (Haab and Anastassiadis, 1961). Resin hydrolysis was therefore used throughout this work where it was required to estimate the carbohydrate components of collagen. The procedure used was that described by Anastassiadis and Common (1958) and is outlined below.

The resin used throughout was Dowex 50, 200-400 mesh. It was prepared for use by the method of Hirs, Moore and Stein (1952) as described in section IV(a)(i), and then suspended in 0.05 N.HCl (2 volumes of acid per unit weight of resin). 10 ml. of this suspension was pipetted into glass tubes containing the material to be hydrolysed (normally approximately 250 mg. freeze dried material). In cases where the material to be hydrolysed was in solution, the solution was evaporated to dryness in vacuo and the material transferred quantitatively using 3 ml. 0.05N.HCl. In these instances 6 ml. of a 1:1 resin: acid suspension was

added to the tube. The tubes were then sealed and placed in an oven at 100°C for 36 hours.

(b) Elution from resin.

After cooling, the sealed tubes were opened, and the contents transferred to a column (25 cm. long and 1.5 cm. in diameter) with a glass wool plug. The tube was carefully washed out with 20 ml. distilled water, and the washings transferred quantitatively to the column. The water eluate from the column was collected in a 50 ml. conical flask. [Water eluate I].

20 ml. 2N.HCl was then added to the column and the eluate collected in a 50 ml. volumetric flask, together with 10 ml. distilled water which was passed through the column to wash out excess acid. This constitutes acid eluate I.

Water eluate I was once more added to the column and the eluate collected in a second 50 ml. volumetric flask, together with 20 ml. distilled water which was passed through the column to complete the water elution. The water eluate was then made up to 50 ml.

The volumetric flask containing acid eluate I was replaced under the column, and 20 ml. 2N.HCl washed through the column to elute reabsorbed hexosamine. The acid eluate was then made up to 50 ml.

The acid eluate contains the hexosamine and hydroxyproline, whilst the water eluate contains the hexose component, although in some instances a small percentage of the hexose was found in the acid eluate.

(c) Colorimetric determination of hexosamine.

The determination of hexosamine was carried out by the method of Cessi and Piliego (1960), this being a modification of the procedure of Elson and Morgan (1933). This method is advantageous in that it eliminates chromogens of other compounds which would otherwise interfere with the estimation. The separation of the chromogen of hexosamine from those of other compounds is achieved by the distillation stage of the procedure described below, since, unlike the other chromogens, that of hexosamine is volatile.

i) Reagents:-

a) Acetyl Acetone Reagent: This consists of 1 ml. colourless (redistilled) acetyl acetone reagent dissolved in 100 ml. 0.5N sodium carbonate-bicarbonate buffer containing 0.1M NaCl. The composition of the buffer is:-

23.02 g. sodium carbonate)
2.76 g. sodium bicarbonate) per litre
5.84 g. sodium chloride)

The final pH of the reagent, after addition of the acetyl acetone was 9.8, and the reagent was stable for one day at 0°C.

b) p-dimethylaminobenzaldehyde reagent: 80 mg. p-dimethylaminobenzaldehyde (A.R.) were dissolved in 100 ml. of absolute ethanol containing 3.5 ml. of concentrated HCl.

c) Glucosamine standards: Standard solutions of D-glucosaminehydrochloride were prepared, containing 25, 50, 75, and 100 micrograms per ml.

ii) Distillation apparatus:- Distillations were performed in an all-glass apparatus consisting of a round-bottomed 100 ml. flask, with a short neck, and a side arm connected to a 20 cm. condenser. The flask was heated directly with a bunsen burner.

iii) Method:-

2 ml. aliquots of test solution (or standard solution) were pipetted into test tubes, and 5.5 ml. acetyl acetone reagent was added. The tubes were capped with marbles, and heated in a boiling water bath for 20 minutes. The tubes were then cooled, and the contents transferred to the distillation apparatus, together with three 2 ml. washings of water. Distillation was carried out as described, and the first 2 ml. of the distillate collected in a 10 ml. volumetric flask containing 8 ml. p-dimethylaminobenzaldehyde reagent. After standing for 30 minutes, the extinction of the solutions was determined at 545 mμ.

(d) Colorimetric determination of hexose.

Determination of hexose was carried out using the method described by Winzler (1955).

i) Reagents:-

a) Standard glucose solutions containing 25; 50; 75 and 100 micrograms glucose per ml.

b) Orcinol reagent: 7.5 volumes of 60% sulphuric acid added to one volume 1.6% (w/v) orcinol in water.

ii) Method:-

1 ml. of test or standard solution was pipetted into a test tube together with 8.5 ml. orcinol reagent and the reagents throroughly mixed. A blank was also prepared containing 1 ml. distilled water. The tubes were capped with marbles and heated in a water bath at 80°C for 15 minutes. The tubes were then cooled, and the extinction read at 540 mμ against the blank.

IX. Determination of hydroxyproline.

The method used was that of Neuman and Logan (1950) as modified by Leach (1960). The experimental procedure is described below.

i) Reagents: 0.05 M copper sulphate solution.
2.5 N sodium hydroxide.
6% hydrogen peroxide.
3 N sulphuric acid.
5% p-dimethylaminobenzaldehyde in redistilled n-propanol.
Standard hydroxyproline solutions containing 5; 10; and 15 micrograms per ml.

ii) Method:-

1 ml. of each standard and test solution were pipetted into 1 x 6 inch pyrex test tubes. Duplicate samples of each were prepared, and a blank, containing 1 ml. distilled water was also made up.

Into each tube was pipetted 1 ml. of 0.05M $CuSO_4$ and 1 ml. of 2,5N NaOH. The reagents were thoroughly mixed, the tubes placed in a water bath at 40°C for 5 minutes, and 1 ml. 6% hydrogen peroxide added. The tubes were incubated at 40°C for a further 10 minutes, each tube being shaken occasionally. After removal from the water bath the tubes were cooled in iced water and 4 ml. of 3N H_2SO_4 added, followed by 2 ml. 5% p-dimethylaminobenzaldehyde reagent. The tubes were capped with glass beads and incubated for 16 minutes in a water bath at 70°C. After removal from the bath, the tubes were cooled and their extinction read at 555 mμ against the blank.

Hydroxyproline estimations were carried out on material hydrolysed by acid (5.7N HCl for 18 hours in sealed tubes), since the hydrolysis with resin, as used for estimation of hexoses and hexosamine, was found to give incomplete liberation of hydroxyproline. Acid hydrolysates were suitably diluted with distilled water prior to estimation.

X. Determination of total nitrogen.

Total nitrogen determinations were carried out using the method of Chibnall, Rees and Williams (1943). A weighed amount of the solid, or an aliquot of a solution, estimated to contain approximately 1 mg. of nitrogen, was placed in a microkjeldahl tube, and 2 ml. of nitrogen-free concentrated sulphuric acid were added. A small amount of a catalyst was then added. The catalyst was that described by Chibnall *et al.* *i.e.* 80 g. K_2SO_4: 20 g. $CuSO_4.3H_2O$ 0.34 g. sodium selenate. The mixture was then digested on an electric element for 12 hours. After cooling the contents of the flask were diluted with water and transferred quantitatively, with distilled water washings, to a standard microkjeldahl distillation apparatus. 10 ml. of a 40% solution of A.R. sodium hydroxide were added and the mixture steam distilled. The distillate was collected in a small conical flask containing a few ml. water and two drops of Tashiri indicator (Cole, 1933), the tip of the condenser being under the surface of the water. The solution was titrated with N/70 sulphuric acid.

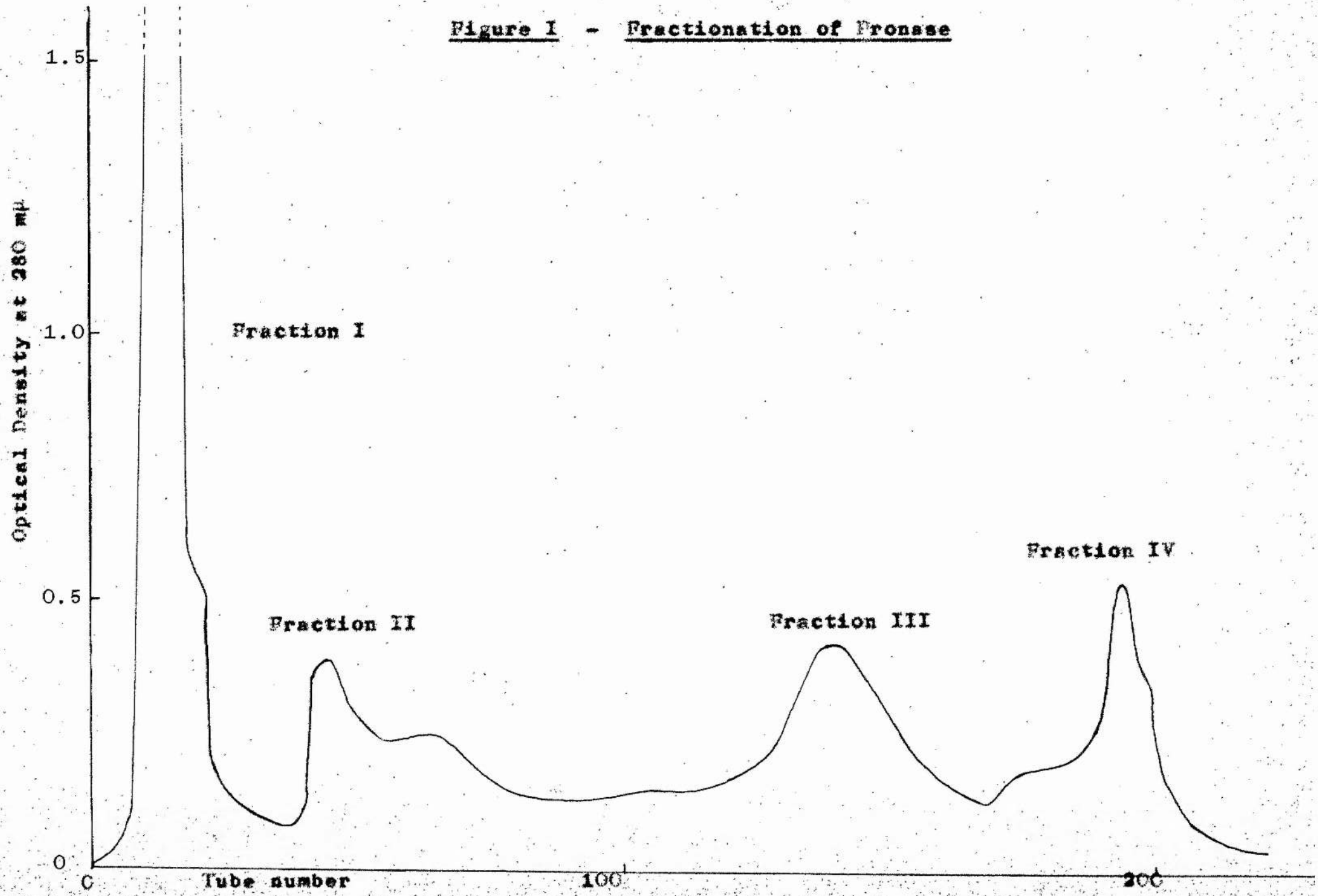
SECTION 3

R E S U L T S

I. Fractionation of Pronase.

Samples of commercial Pronase (Pronase P, Kaken Chemical Co. Ltd., Tokyo) were fractionated on cellulose phosphate columns as described in Section 2 II. 250 mg. samples of material, equivalent to 26.5 mg. of nitrogen, were used for each fractionation. A typical elution curve is shown in fig. I. Several identical fractionations were performed, the elution pattern being practically identical in each instance, although the degree of separation of the various fractions differed slightly with each fractionation. After dialysis and concentration of the individual fractions, white precipitates were observed in the dialysis tubes. These were removed by centrifugation and total nitrogen determinations carried out. In each case the nitrogen content of the precipitate was negligible, and it is probable that the material was cellulose phosphate which had been washed off the column during the fractionation procedure. It was observed that both the first and final (4th) fractions were yellow, although the 4th fraction did not appear as a yellow band until the pH of the column was

Figure I - Fractionation of Pronase



approximately 10. The yellow colour of both these fractions practically disappeared after dialysis. The total nitrogen content of each fraction and the pH at which it was eluted is given in Table I.

Table I

Fraction	pH eluted	Total Nitrogen(mg.)	% of Initial Total Nitrogen
1	3.2	6.3	23.7
2	4.2	3.3	12.5
3	7.0	3.8	14.3
4	10.0	1.6	6.0
	Total	15.0	56.5

A recovery for total nitrogen of 56.5% appeared rather low, but continuation of the fractionation after removal of the 4th fraction resulted in no further elution of nitrogenous material, and thus it was considered probable that the losses occurred at the dialysis stage. In order to investigate this possibility 45 ml. of a solution of the crude Pronase (0.115 mg. N per ml.) was dialysed for 48 hours against a large excess of distilled water. Total nitrogen determinations were then carried out on the material remaining in the dialysis tube. The results obtained are given below.

	Initial total nitrogen	5.175 mg.
	Non-dialysable nitrogen	2.70 mg. (53% of total)
∴	Dialysable nitrogen	2.475 mg. (47% of total).

II. Characteristics of soluble collagen.

The acetic acid soluble collagen used in this work had the following characteristics:

Ash content	1.13%	
Nitrogen	18.1%	On ash and moisture free basis.
Hydroxyproline	13.0%	
Hexose	2.5%	
Hexosamine	0.12%	

The values found are comparable with those of other workers, e.g. Bowes, Elliot and Moss (1955) found that citrate soluble collagen of calf skin had a nitrogen content of 17.7% and a hydroxyproline content of 13.6%. The value for hexose is slightly higher than reported values, e.g. Grassmann et al. (1957) found 0.73% hexose in twice recrystallized collagen, and Gross (1957) reported a hexose content of 1.0%. The hexosamine content is within the limits of values of other workers, which range from 0.01% (Grassmann et al. 1957) to 0.40% (Dresner and Schubert, 1955).

III. Viscosity Studies.

Investigations of the effect of Pronase on soluble

collagen were initially carried out using the crude enzyme preparation. Experiments were performed with collagen solutions at two pH values (pH 3.5 and pH 6.5). For a solution at pH 3.5 the collagen was dissolved in 0.1M acetic acid and to obtain a solution at pH 6.5 the acetic acid solution was made 0.2M with $CaCl_2$ and the pH adjusted with 0.1N NaOH. For the purpose of comparison the effect of crystalline trypsin on the viscosity of a solution of collagen at pH 6.5 was also examined. Experiments were also carried out at the above two pH values using a solution of commercial gelatine. The results of these studies are shown in fig. II.

Since it was shown that a large amount of the total nitrogen of crude Pronase is in a dialysable form experiments were carried out to determine the effect of dialysis on the activity of the enzyme. A solution of the crude enzyme was prepared containing 1 mg./ml. of enzyme. This was divided into two portions, one of which was dialysed against a large excess of distilled water at 4°C, the other being simply allowed to stand at this temperature. Viscosity studies were carried out with both solutions after one and five days. Since dialysis appeared to effect a marked reduction in activity the dialysate was reduced <u>in vacuo</u> to a small volume and added to the non-dialysable portion and the activity

Figure II

Effect of Pronase on the viscosity of solutions of soluble collagen and gelatine

Collagen solutions 0.1%
Gelatine solutions 0.5%

▲–▲–▲–▲ Pronase + collagen solution pH 6.5 in 0.2M $CaCl_2$. E/S ratio approx. 1/100.

△–△–△–△ Pronase + collagen solution pH 3.5 in 0.1M acetic acid. E/S ratio approx. 1/100.

●–●–●–● Pronase + gelatine solution pH 6.5. E/S ratio approx. 1/300.

○–○–○–○ Pronase + gelatine solution pH 3.5. E/S ratio approx. 1/300.

■–■–■–■ Trypsin + collagen solution pH 6.5 in 0.2M $CaCl_2$. E/S ratio approx. 1/100.

Figure III

Effect of time and dialysis on the activity of Pronase solutions.

Collagen solution 0.1% in 0.1M acetic acid.
E/S ratio approx. 1/100.

●–●–●–● Pronase after dialysis overnight.

▲–▲–▲–▲ Pronase after standing overnight.

○–○–○–○ Pronase after dialysis for 5 days.

△–△–△–△ Pronase after standing for 5 days.

□–□–□–□ Dialysed pronase + added dialysate.

Figure II

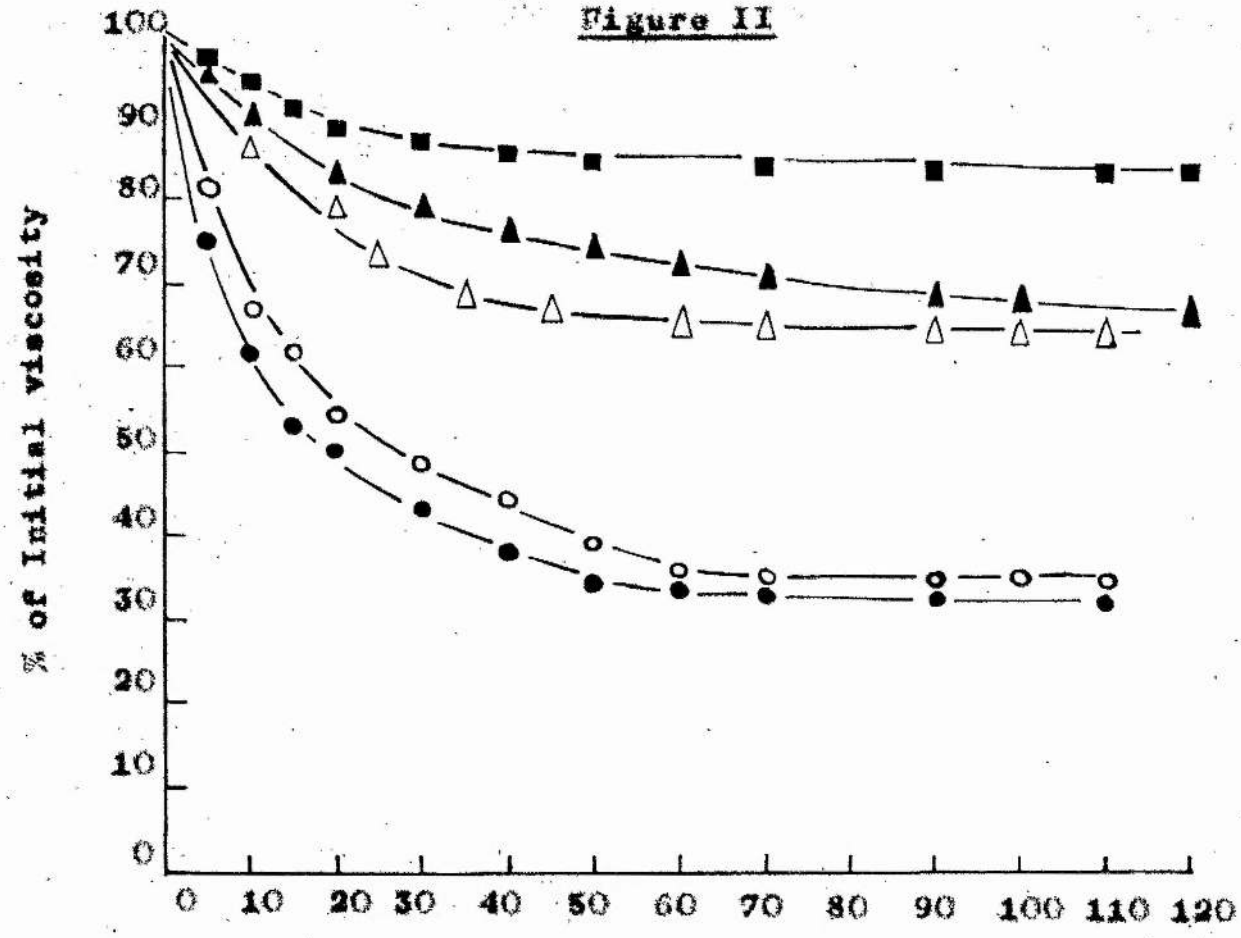
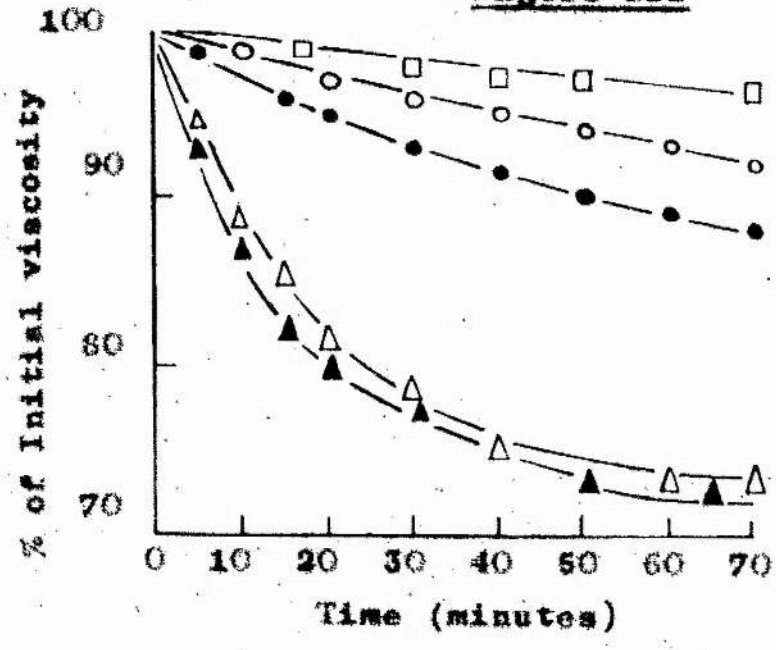


Figure III



again determined. Fig. III summarises the results of these experiments.

The effect of the Pronase fractions on the viscosity of collagen solutions, at both pH 3.5 and pH 6.5, was also examined and the results of these investigations are given in figs. IV and V.

Extracts of the sisal enzyme (See section I) were also examined for collagenolytic activity on collagen solutions using both neutral and acid collagen solutions, and the effect of these extracts on the viscosity of gelatin solutions was similarly investigated. The results are once more expressed graphically in figs. VI and VII.

Other enzymes examined for collagenolytic activity, as manifested by a decrease in viscosity, were hyaluronidase and pectinesterase. As both of these enzymes have an optimum activity at neutral pH values, the collagen solutions used were at pH 6.5 in 0.2M $CaCl_2$. The effect of these enzymes on collagen solutions, compared with the action of trypsin under similar conditions is shown in fig. VIII.

IV. Effect of enzymes upon optical rotation of collagen solutions.

The effect of enzymes upon the optical rotation of collagen solutions was examined as described in Section 2 VI h.

Figure IV

Effect of Pronase fractions on the viscosity of solutions of soluble collagen at pH 3.5.

Collagen solution approx. 0.1% in 0.1M acetic acid.
E/S ratio approx. 1/100.

▲-▲-▲-▲ Pronase fraction I

△-△-△-△ Pronase fraction II

○-○-○-○ Pronase fraction III

●-●-●-● Pronase fraction IV

Figure V

Effect of Pronase fractions on the viscosity of solutions of soluble collagen at pH 6.5.

Collagen solution approx. 0.1% in 0.2M Calcium chloride
E/S ratio approx. 1/100.

▲-▲-▲-▲ Pronase fraction I

△-△-△-△ Pronase fraction II

○-○-○-○ Pronase fraction III

●-●-●-● Pronase fraction IV

Figure IV

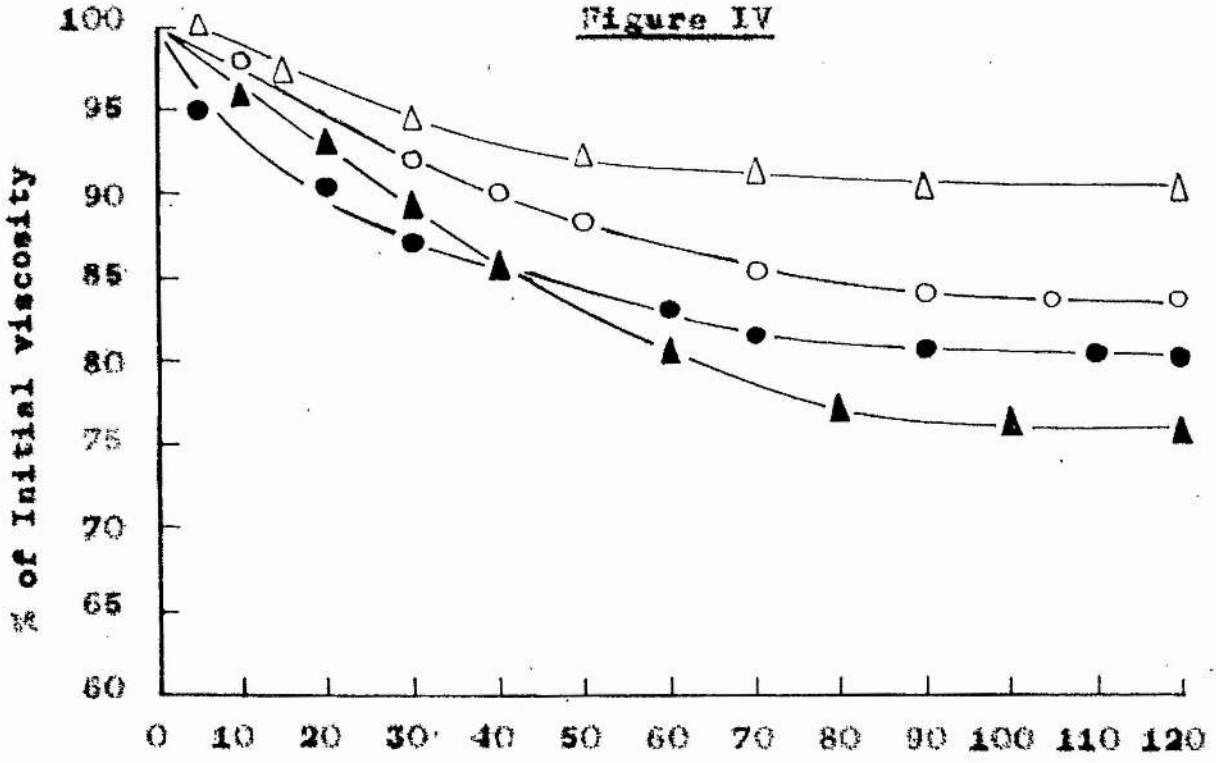


Figure V

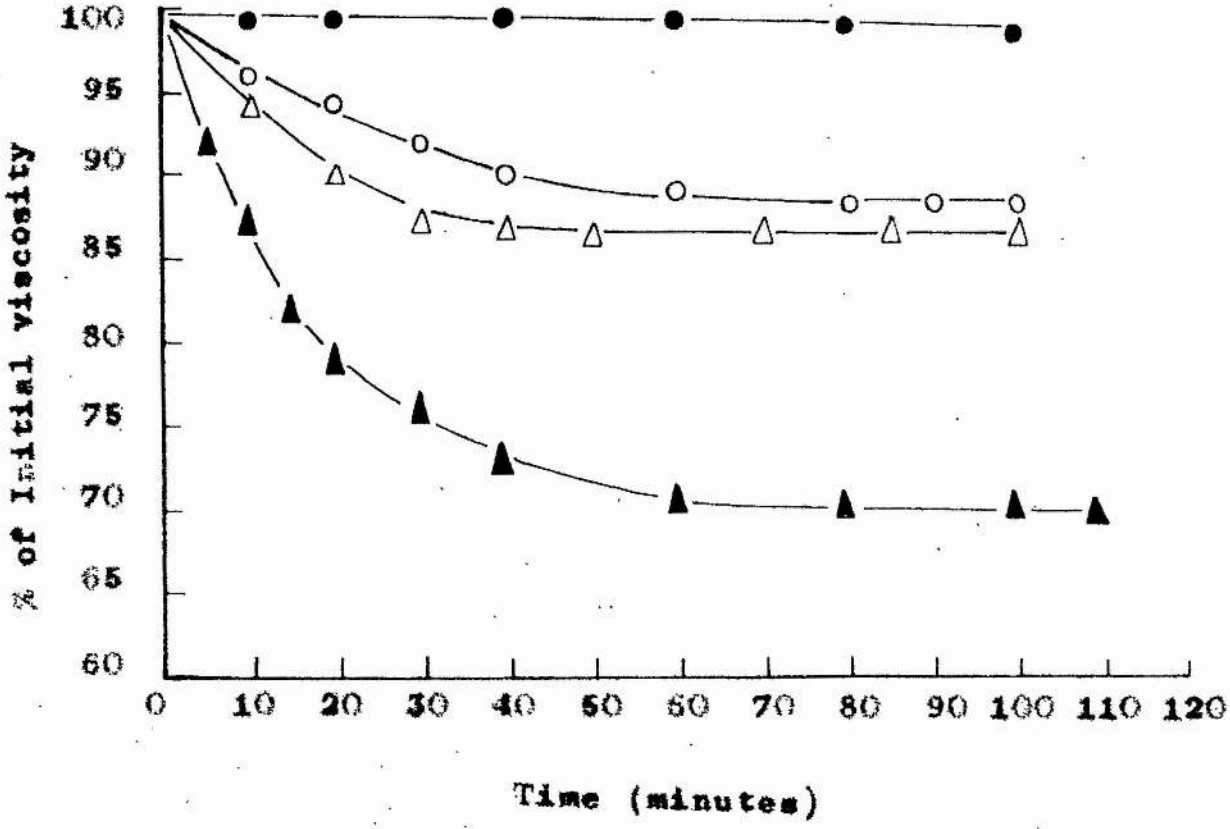


Figure VI

Effect of sisal enzyme on the viscosity of solutions of soluble collagen and gelatine at pH 3.5.

Collagen solution approx. 0.1%

Gelatine solution 0.5%

△-△-△-△ Collagen solution + acetate extract (pH 3.5) of sisal enzyme.

○-○-○-○ Collagen solution + phosphate extract (pH 6.5) of sisal enzyme.

● ● ● ● Gelatine solution + phosphate extract of sisal enzyme.

Figure VII

Effect of sisal enzyme on the viscosity of solutions of soluble collagen and gelatine at pH 6.5.

Collagen solution approx. 0.1% in 0.2M Calcium chloride.

Gelatine solution 0.5%

△-△-△-△ Collagen solution + acetate extract of sisal enzyme.

○-○-○-○ Collagen solution + phosphate extract of sisal enzyme.

● ● ● ● Gelatine solution + acetate extract of sisal enzyme.

Figure VI

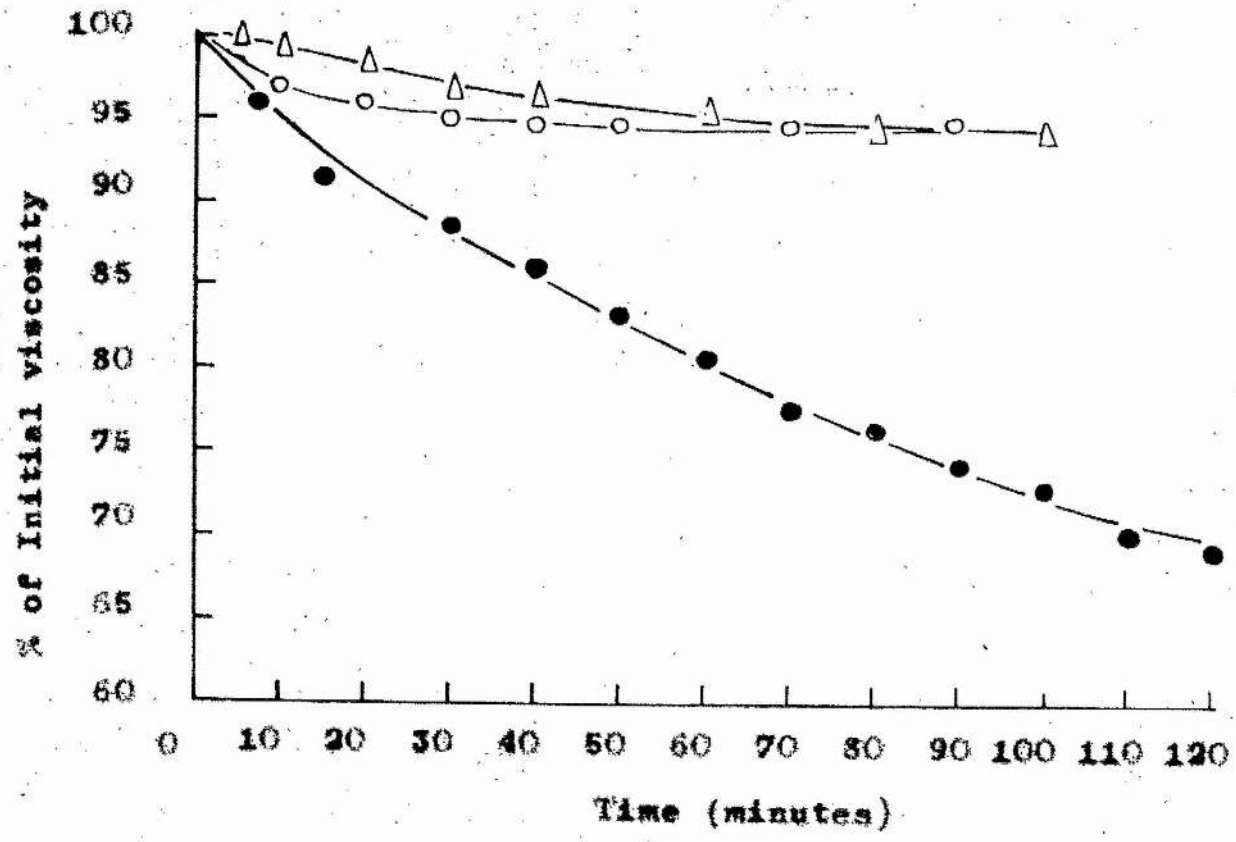


Figure VII

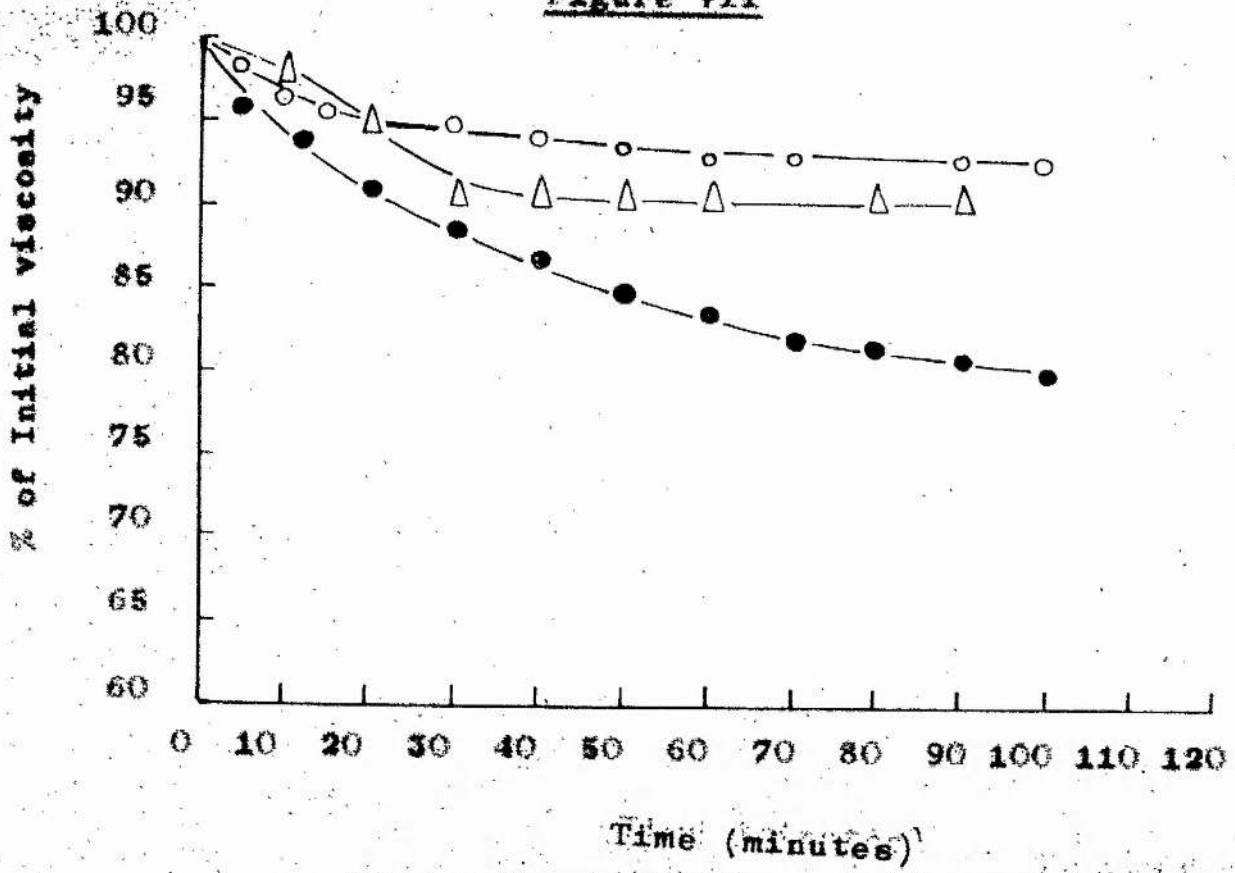


Figure VIII

The effect of enzymes on the viscosity of solutions of soluble collagen at pH 6.5

(Collagen solutions approx. 0.1% in 0.2M Calcium chloride)

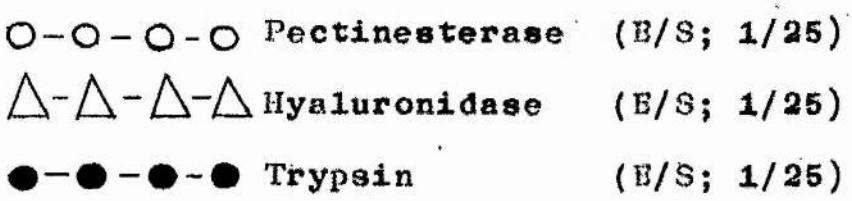
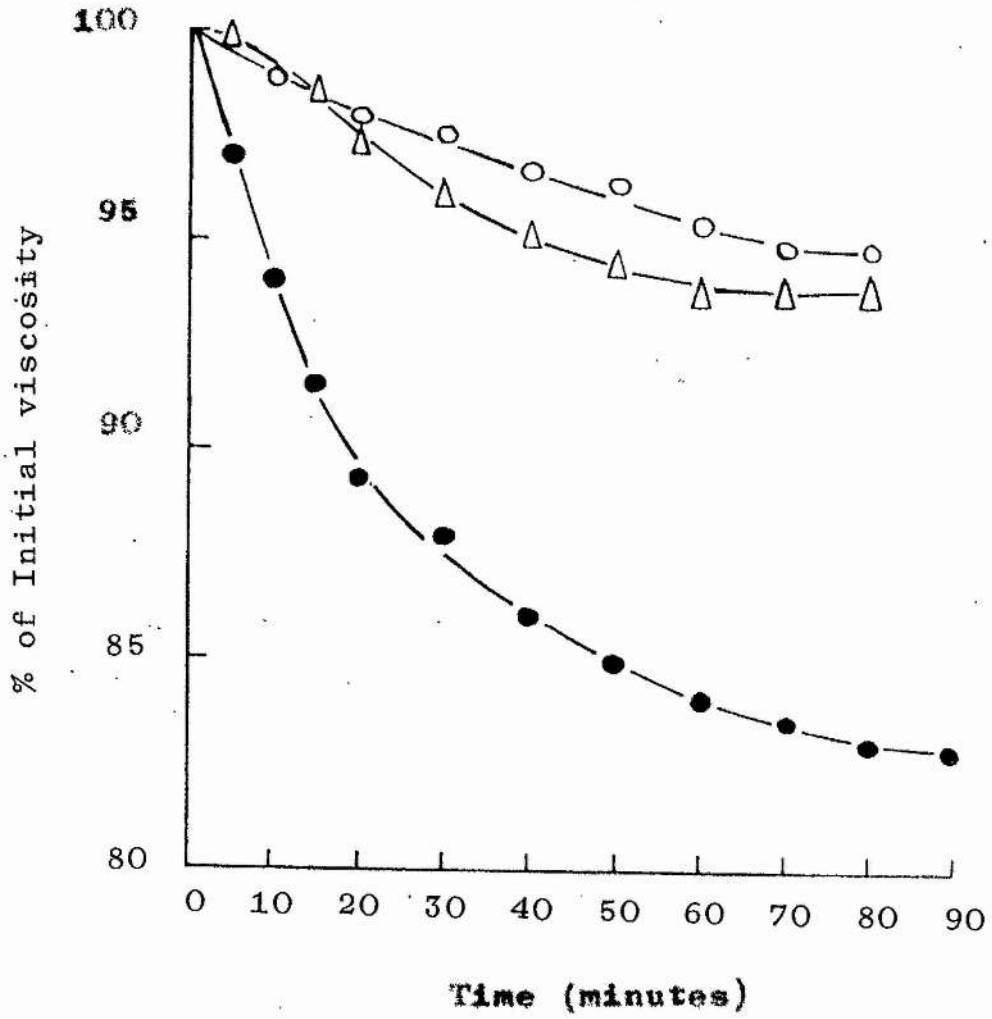


Figure VIII


100
95
90
85
80
% of Initial viscosity
0
10
20
30
40
50
60
70
80
90
Time (minutes)

The collagen solution used contained 30.8 mg. of nitrogen per 100 ml. 50 ml. of this solution were digested with 2 ml. enzyme solution (enzyme:substrate approximately 1:100). Results are shown in fig. IX.
(The dilution effect caused by addition of enzyme solution has been corrected for)

The initial value for the optical rotation of the collagen solution (α_D = -423) compares reasonably with that of other workers, e.g. Doty and Nishihara (1958) reported α_D = -415 for calf skin citrate soluble collagen. Results show that neither hyaluronidase nor sisal enzyme have any effect upon the optical rotation, but that Pronase, under both acid and neutral pH conditions, produces approximately a 4% decrease in rotation. This decrease is probably not significant, since it represents a reduction in α_D only to -407, a value well within the range of literature values for native collagen.

V. pH-Stat Studies.

The proteolytic degradation of collagen in solution was also followed by use of the pH stat. For reactions at pH 3.5 the collagen was dissolved in 0.01N.HCl and titrated to the required pH. The solution was first allowed to equilibrate at this pH, until a steady trace was obtained,

Figure IX

The effect of enzymes on the optical rotation of solutions of soluble collagen.

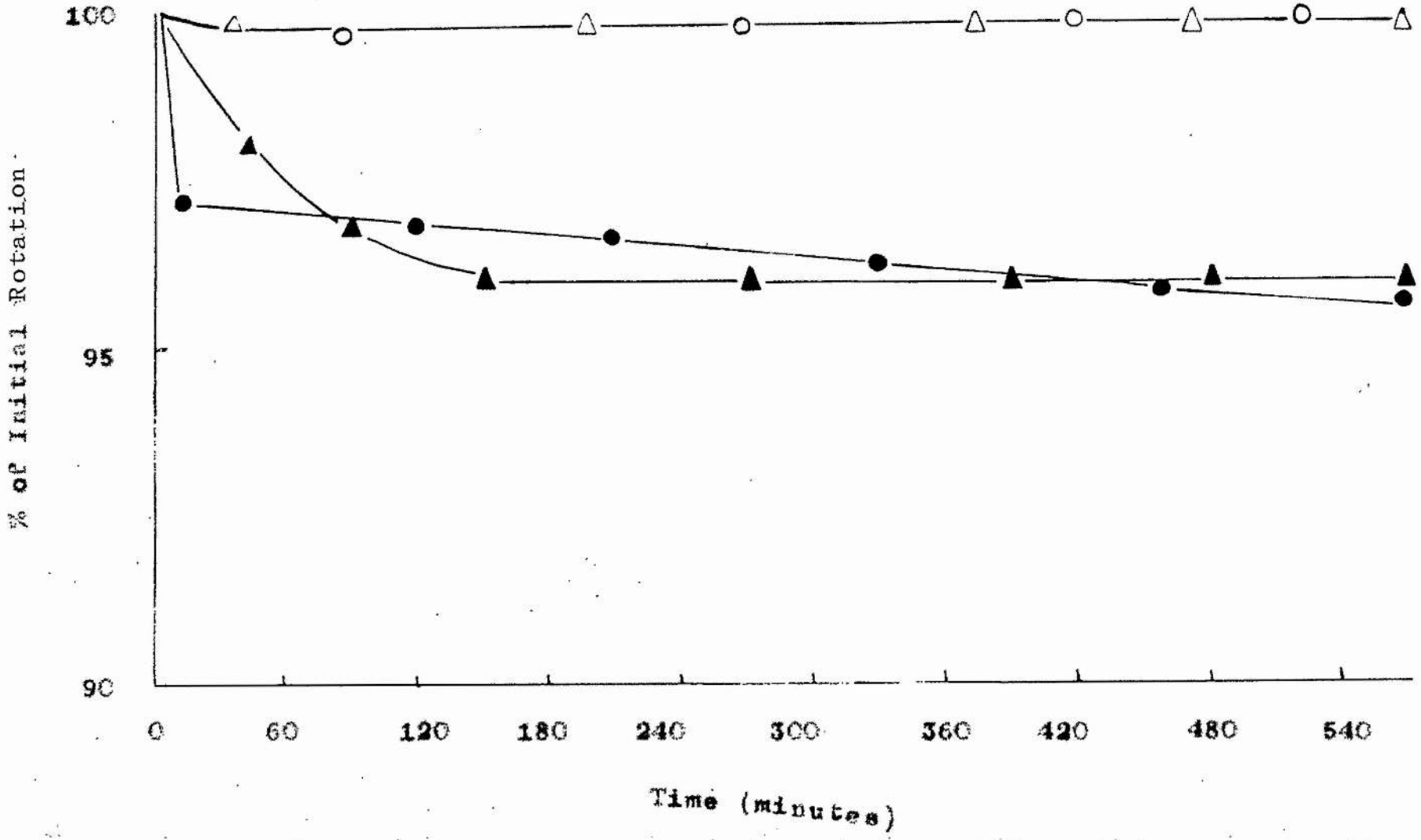
○-○-○-○ Sisal Enzyme : collagen solution pH 6.5 in 0.2M $CaCl_2$

△-△-△-△ Hyaluronidase : collagen solution pH 6.5 in 0.2M $CaCl_2$

● ● ● ● Pronase : collagen solution pH 6.5 in 0.2M $CaCl_2$

▲-▲-▲-▲ Pronase : collagen solution pH 3.5.

Figure IX


100
95
90
% of Initial Rotation
0
60
120
180
240
300
360
420
480
540
Time (minutes)

before addition of the enzyme. The results of these titrations are shown in fig. X. The pH of 3.5 was chosen as it is approximately the pK of the carboxyl group of collagen, and simplifies calculation of results, since 1 meq. of acid required to maintain the pH at 3.5 is equivalent to a cleavage of two peptide bonds.

Attempts were also made to follow proteolysis at pH 6.5, but since collagen cannot be maintained in solution at this pH without the presence of $CaCl_2$ no satisfactory results could be obtained. Further attempts were made to obtain a value for the number of bonds hydrolysed at this pH by titrating the collagen, before addition of enzyme, from pH 6.5 to pH 8.0 using 0.1N KOH. The collagen was then titrated back to pH 6.5, using 0.1N HCl, enzyme added, and the reaction allowed to proceed for several hours. The system was again titrated to pH 8.0, and the additional amount of alkali required for this titration calculated. The results of these experiments, expressed as the percentage of the total collagen bonds hydrolysed are given in Table II. N.B. Results were calculated based on a collagen molecule of molecular weight 360,000, and containing 3000 amino acid residues.

Figure X

Cleavage of the peptide bonds of collagen by Pronase fractions.

(Collagen solutions at pH 3.5)

▲-▲-▲-▲ Pronase fraction IV

○-○-○-○ Pronase fraction I

●-●-●-● Pronase fraction III

Figure X

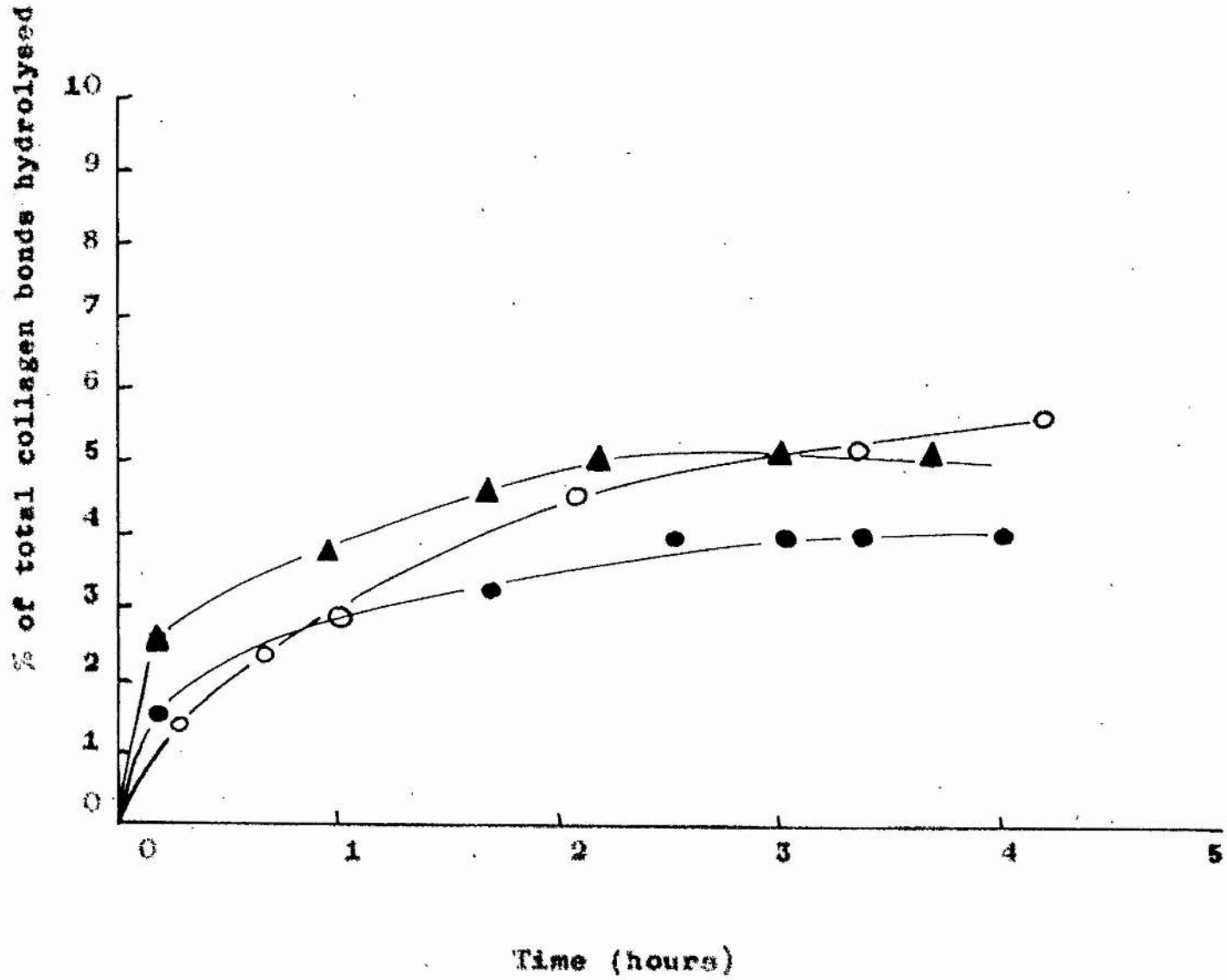

% of total collagen bonds hydrolysed
10
9
8
7
6
5
4
3
2
1
0
0
1
2
3
4
5
Time (hours)

Table II

Reaction	Collagen T.N.	% of total bonds cleaved
1	12.0	7.6
2	12.0	6.1
3	11.2	5.0
4	7.3	10.7
5	8.7	7.5

The above experiments were all performed using the crude enzyme, and in view of the variation in the results, comparable experiments using enzyme fractions were not carried out.

Preliminary chromatographic examination of the acetone-soluble material from the above experiments, by both the Redfield system (section 2 IV) and by the FDNB method (section 2 VII) revealed that the following were present as free amino acids,- glutamic acid; aspartic acid; glycine; alanine; threonine; serine; valine; leucine; phenylalanine and lysine.

VI. Acetone soluble nitrogen.

(i) Phosphate extract of skin.

Experiments were carried out to determine the amount of the phosphate extract which was soluble in 80% acetone before and after enzyme treatment. The phosphate soluble material was prepared from calf-skin using 0.2M disodium hydrogen phosphate. Enzymic digestions were performed at 20°C for 24 hours, the digestions being carried out at two pH's, (pH 3.5 and pH 6.5). The phosphate extract was adjusted to these pHs by appropriate addition of 0.1N.HCl. The results of these experiments are given in Table III.

Table III

Acetone soluble nitrogen of phosphate extract before and after enzyme treatment.

	pH	E : S ratio	Initial Total Nitrogen (mg.)	T.N. soluble in acetone	
				mg.	% of initial T.N.
Phosphate Extract	3.5	-	44.7	5.0	11.2
	6.5	-	44.7	3.7	8.4
Phosphate Extract and sisal enzyme	3.5	1:25	44.7	6.2	13.8
	6.5	1:25	44.7	3.8	8.5
Phosphate Extract and Pronase	3.5	1:25	44.7	7.2	16.1
	6.5	1:25	44.7	7.1	15.9

Thus it can be seen that the sisal enzyme has very little effect upon the phosphate extract, in so far as the

additional amount of nitrogen which is soluble in 80% acetone, is scarcely increased by enzyme treatment, whereas Pronase degrades the protein of the extract to the extent that a further 5% - 7.5% of the nitrogen is soluble in acetone.

(ii) Acetic acid-soluble collagen.

Similar experiments to those described in VI(i) were carried out using acid-soluble collagen under various conditions. Since the sisal enzyme had a negligible effect upon the phosphate extract the experiments were carried out using only Pronase. Experimental conditions and results are shown below (Table IV).

Table IV

Acetone-soluble-nitrogen of acid soluble collagen after enzyme treatment.

System	pH	E : S ratio	Initial Total Nitrogen (mg.)	Acetone-Soluble-Nitrogen	
				mg.	% of initial T.N.
Collagen	3.5	-	34.0	0.2	0.6
Collagen + Pronase	3.5	1:25	34.0	2.5	7.1
Collagen + Pronase + 0.2M $CaCl_2$	6.5	1:25	34.0	2.2	6.5
Collagen + Pronase	6.5	1:25	27.2	1.2	4.4

Pronase therefore degrades the collagen in solution to the extent that 6 - 7% of the total nitrogen is soluble in 80% acetone, Where the collagen is not in solution i.e. at pH 6.5 in the absence of calcium chloride, the degradation is less, only 4.4% of the nitrogen being soluble.

VII. Base of conversion to gelatin.

500 ml. of a solution of acid soluble collagen (total nitrogen 117 mg.[illegible]) at pH 3.5 in acetic acid, was digested with 10 mg. of crude Pronase for 48 hours at 20°C. After precipitation by dialysis, and centrifugation, the collagen was dried by washing in acetone and ether. 150 mg. of the dried material was homogenised in 25 ml. distilled water and warmed to 40°C. to test for ease of conversion to gelatin. (Section 2 VI(d)). This test was carried out consecutively several times, and a control experiment using untreated collagen was also performed. Results are given below.

Table V

Ease of conversion to gelatin of collagen treated with Pronase and normal untreated collagen.

(The times in the first column are times of individual incubations carried out successively).

Time of Incubation at 40°C. (mins.)	Total Nitrogen of Supernatant (mg.)	
	Untreated	Enz. treated
15	2.0	3.25
30	1.5	3.25
60	1.25	3.25
90	0.0	2.0
120	0.5	3.75
180	0.75	0.35
495	6.0	15.85

The results show that collagen after treatment with enzyme is much more readily solubilised under the conditions of the experiment than is the untreated material.

VIII. Digestion of acid soluble collagen with Pronase: Examination of N-Terminal residues.

(i) Determination of losses of DNP-amino acids on hydrolysis.

Losses of α-DNP-amino acids during hydrolysis were determined as described in 2 VII (h). The values used throughout this work were average values from several determinations performed by the author and Steven, F.S. and are reported by Steven and Tristram (1962b).

(ii) N-terminal analysis of collagen and enzyme treated collagen.

The N-terminal amino acids of collagen were examined by the methods described in Section 2 VII. Analyses were performed on untreated acetic acid soluble-collagen and also on collagen treated with crude Pronase. For enzyme treated material a solution of collagen in acetate at pH 3.5 was digested for 48 hours with Pronase. (Enzyme : substrate; 1:50). The reaction mixture was dialysed exhaustively against running water, centrifuged, and the precipitated collagen washed three times with water. The material was then redissolved, nitrogen determinations carried out, and reaction with FDNB and subsequent analysis performed as described. The results of these analyses are given in Table VI below.

Table VI

N terminal amino acids of collagen and Pronase-treated collagen.

Amino Acid Residue	nM residue/10^6 grams material	
	Untreated Collagen	Enz.-treated
Glutamic acid	212.0	5140
Aspartic acid	446.5	
Serine	616.5	1490
Threonine	260.5	960
Glycine	369.0	7240
Alanine	215.5	740
Valine	132.0	408
Leucine/Isoleucine	145.0	990
Phenylalanine	131.0	704
Lysine	24.5	790
Total	2552	36.700
Cn	3.92×10^5	2.73×10^4

The results reveal that there is a great increase in the N-terminal residues of collagen after treatment with Pronase.

There is an increase in the amount of all acids found as N-terminal acids, although the increase varies greatly for different amino acids, e.g. Valine shows only a threefold increase whilst glycine shows a 20-fold increase. It is of interest to note at this point that trace amount of tyrosine, proline and hydroxyproline were observed on chromatograms of the N-terminal amino acids of both treated and untreated collagen. These were visible to the naked eye as extremely faint yellowish spots, which could also be detected by slight fluorescence in ultra-violet light. The optical densities of these spots after elution were so low as to be unreadable.

IX.(i) Solubilisation of insoluble collagen: congo red method.

This was carried out as described in Section 2 VI(f). 50 mg. quantities of Congo Red-insoluble collagen were used, and the total volume of each reaction mixture was 50 ml. The digestions were carried out in 0.2M phosphate buffer pH 6.5 and the enzyme solutions used were 1% throughout. The results of these experiments, using Pronase, sisal enzyme and trypsin, are shown in fig. X(a).

Figure X(a)

Enzymic solubilisation of congo-red collagen.

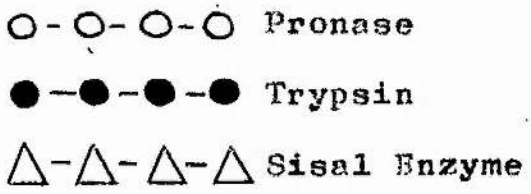
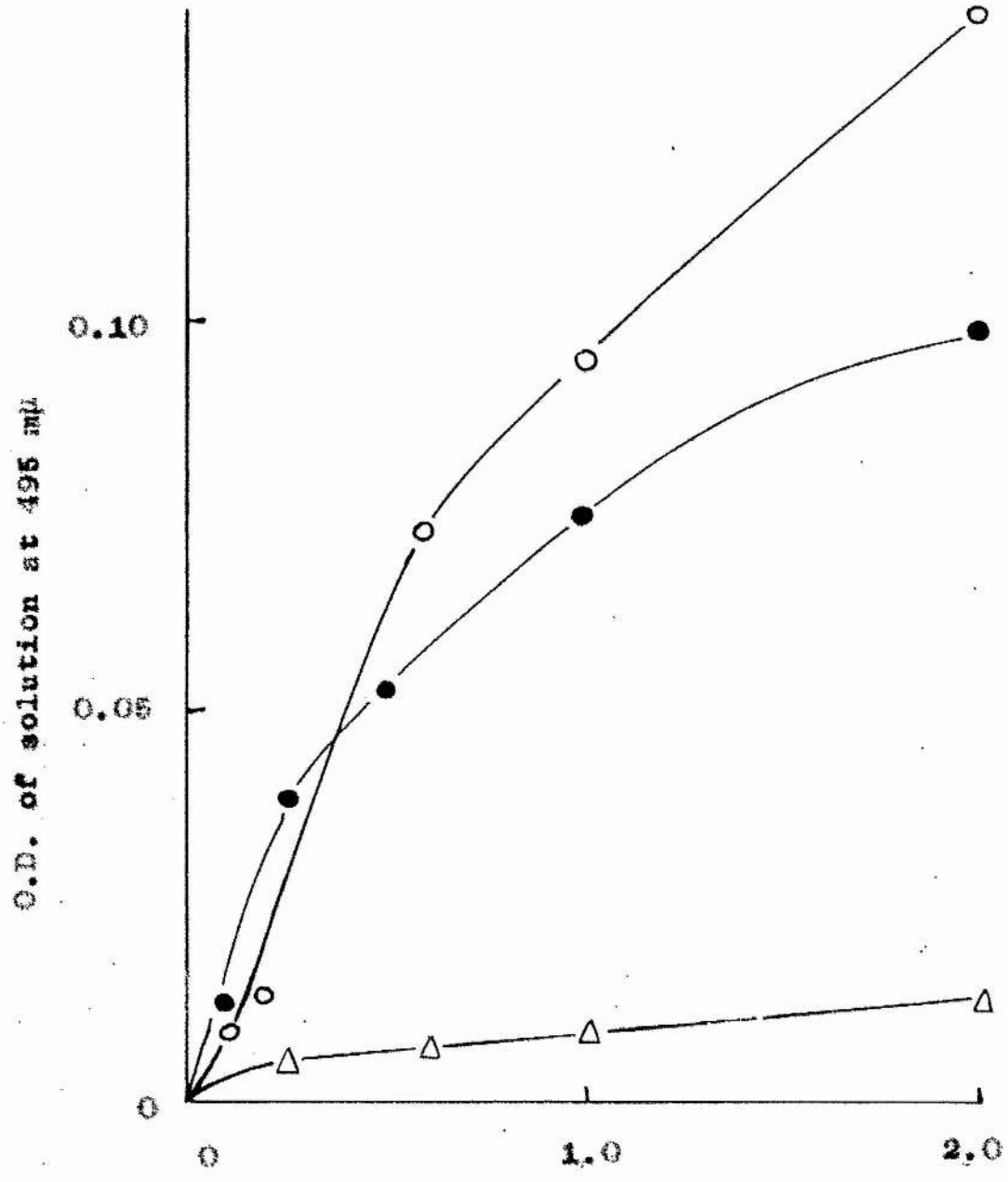


Figure X (a)


0.10
0.05
0
O.D. of solution at 495 mμ
0
1.0
2.0
ml. 0.1% enzyme solution added

IX.(ii) Solubilisation of insoluble collagen.

(a) By Pronase.

500 mg. of lyophilised insoluble collagen (≡ 86 mg.N) were homogenised in 120 ml. 0.2M phosphate buffer pH 6.5, and digested with crude Pronase for 48 hours at 20°C. The reaction was carried out in duplicate, using different enzyme concentrations, and a control reaction using no enzyme was also performed. The mixture was centrifuged and the soluble nitrogen determined. Hydroxyproline and viscosity measurements were also carried out on the supernatant. The Table below summarises the results.

Table VII

Solubilised nitrogen and hydroxyproline after Pronase treatment.

	Enzyme: substrate	Collagen Nitrogen solubilised		Hydroxy-proline solubilised		Relative viscosity
		mg.	% of total.	mg.	% of total	
Control	-	2.0	2.3	0.4	0.7	1.01
Digest (1)	1:100	6.6	7.7	2.1	3.5	1.04
Digest (2)	1:20	9.2	10.6	3.0	5.0	1.05

Attempts to determine the optical rotation of the solubilised material proved unsuccessful as the solutions were too dilute to give an adequate reading on the polarimeter. The solutions were also tested for fibre or gel formation at 37°C (Section 2 VI(e)), but no fibres or gel were formed, probably for the reason stated above.

(ii)(a)(i). Base of conversion to gelatin of insoluble collagen before and after Pronase treatment.

The insoluble material from these experiments was examined for ease of conversion to gelatin. The precipitates were washed three times with water, to effect complete removal of enzyme, suspended in 100 ml. distilled water, and incubated at 40°C. After determining the gelatin formed after 90 minutes incubation, the material was resuspended in water and incubated for a further five hours, and soluble nitrogen again determined.

Table VIII

Collagen nitrogen solubilised by heating, before and after Pronase treatment.

	mg. nitrogen solubilised			% of total nitrogen
	incubated 90 minutes	incubated 5 hrs.	Total	
Control	1.3	2.4	3.7	4.4
Digest(1)	3.0	4.9	7.9	9.9
Digest(2)	3.6	5.8	9.4	12.2

It is apparent therefore that treatment of insoluble collagen with Pronase enhances the solubilisation of the material when it is gently warmed in water, as is also the case with soluble collagen described previously.

(ii)(a)(ii). Solubilisation of insoluble collagen in dilute acetic acid, before and after Pronase treatment.

In a further experiment 250 mg. insoluble collagen (≡ 43 mg.N) was digested with Pronase as above (enzyme : substrate 1:100) and after removal of enzyme the insoluble material was stirred in 100 ml. 0.1M acetic acid at room temperature. After centrifugation the soluble nitrogen was determined. A control reaction was also carried out.

Control: Soluble Nitrogen 4.0 mg. (9.2% of total N)

Digest: Soluble Nitrogen 20.8 mg. (48.4% of total N).

Thus the enzyme has a profound effect upon the acid solubilisation properties of insoluble collagen.

(b) By sisal enzyme.

500 mg. lyophilised insoluble collagen (≡ 86 mg.N) were digested, with the phosphate extract of sisal enzyme, in 0.2M phosphate buffer pH 6.5 for 48 hours at 20°C. Total nitrogen, hydroxyproline and viscosity measurements were performed on the soluble material, and the residues from the digests were warmed to 40°C in water for 2 hours to determine the nitrogen solubilised as gelatin. Table IX below gives the results.

Table IX

Effect of sisal extract on insoluble collagen.

	Enzyme: Substrate	Collagen Nitrogen (mg.)sol-ubilised	Hydroxy-proline solubil-ised(mg.)	Relative Viscosity	Nitrogen Soluble at 40°C.
Control	-	1.32	0.25	1.00	1.6
Digest(1)	1:75	1.40	0.25	1.01	2.0
Digest(2)	1:30	1.40	0.25	1.00	2.2

Solubilisation of insoluble collagen in dilute acetic acid before and after treatment with sisal enzyme.

A further experiment was carried out to examine the effect of this enzyme on the solubilisation of the collagen in acetic acid:

Control: Soluble Nitrogen 5.0 mg. (5.8% of total N)

Digest: Soluble Nitrogen 7.5 mg. (8.7% of total N).

X. Digestion of acid-soluble collagen with Pronase and sisal enzyme.

(i) Nitrogen; hydroxyproline and carbohydrate in diffusible and non-diffusible fractions.

Acid soluble collagen (Total Nitrogen 172 mg.) in acetate buffer pH 4.0 was digested for 48 hours with dialysed

Pronase (enzyme:substrate 1:100). The digestion mixture was then transferred to dialysis tubing and dialysed against several changes of distilled water, until precipitation of the collagen inside the dialysis tube occurred. The dialysate was reduced in volume *in vacuo*, total nitrogen determinations carried out, and the remainder of the solution divided into two portions, one for hydroxyproline determination, the other for determination of hexose and hexosamine. The precipitated collagen was centrifuged, washed with water, and the above estimates performed. A similar digestion using sisal enzyme (enzyme:substrate 1:100) was also carried out, as was a control reaction containing no enzyme. Results are summarized in Table X.

Table X

Hexose, hexosamine and hydroxyproline in diffusible and non-diffusible fractions of soluble collagen and enzyme digests of soluble collagen.

	Control				Pronase Digestion				Sisal Digestion			
	Diffusate		Precipitate		Diffusate		Precipitate		Diffusate		Precipitate	
	mg.	% of Total	mg.	% of Total	mg.	% of Total	mg.	% of Total	mg.	% of Total	mg.	% of Total
Nitrogen	1.6	0.9	174.0	99.1	15.4	8.7	160.0	91.3	4.4	3.9	169.2	96.1
Hydroxy-proline	Trace	-	128.0	100.0	3.2	2.6	120.0	97.4	1.4	1.0	122.0	99.0
Hexose	11.5	48.0	12.4	52.0	12.0	47.6	13.2	52.4	17.5	43.6	22.6	56.4
Hexo-samine	0.25	21.7	0.9	78.3	0.9	66.7	0.45	33.3	0.65	52.0	0.60	48.0

The results thus reveal that Pronase converts 8.7% of the nitrogen of soluble collagen into a diffusible form, and that sisal enzyme has a similar but smaller effect. The same is true of the hydroxyproline, although the amount of total hydroxyproline diffusible in each case is very low. The results for hexose reveal that collagen hexose can be removed in part by dialysis (approximately 50%) and treatment with Pronase does not increase the diffusible hexose. The hexose results for the sisal digestion reveal inconsistencies in that the total hexose is well above the collagen hexose content. This is probably due to the fact that the sisal preparation used was impure and contained carbohydrate material, and some co-precipitation of this material with the collagen occurred during dialysis. One-fifth of the hexosamine of the collagen is diffusible, but considerably more is rendered diffusible by enzyme treatment.

(ii) Amino acid analysis of enzyme-treated collagens.

The precipitated collagens from the above experiments were subjected to amino acid analysis after hydrolysis with constant boiling (5.7 N) hydrochloric acid.

Table XI

Amino acid analyses of collagen and enzyme treated collagens. Results are expressed as moles of amino acid residue per 1000 mols.

Amino Acid Residue	Collagen	Pronase treated Collagen	Sisal * treated Collagen
Hydroxyproline	105	128	112
Aspartic acid	30	39	39
Serine and Threonine	47	30	27
Glutamic acid	60	40	66
Proline	120	137	140
Glycine	326	332	286
Alanine	108	115	118
Valine	35	43	44
Methionine	2	4	5
Isoleucine	10	11	13
Leucine	30	26	30
Tyrosine	2	-	2
Phenylalanine	12	12	14
Hydroxylysine	4	6	8
Lysine	28	26	44
Histidine	5	3	3
Arginine	44	46	38

* The values for some amino acids in the sisal-treated

material are not consistent with the low activity of the enzyme, e.g. the low glycine content and the increase in other amino acids, e.g. lysine, methionine, glutamic acid. These inconsistencies can probably be explained in terms of co-precipitation of material from the crude sisal extract with the collagen, as in the case of the increased hexose content in table X.

The values given for collagen agree reasonably well with those reported by other workers (see Table A Section 1). The hydroxyproline figure is slightly high, as is the figure for valine. The values for the dicarboxylic amino acids are low and the value of valine is high. Treatment with enzymes has no highly significant effect on the amino acid composition, within the experimental errors of the analysis procedure, with the possible exception that treatment with Pronase eliminates the tyrosine. The significance of this will be discussed later.

XI. The effect of enzymes on soluble collagen.

(i) Effect upon thermal gelation of collagen solutions.

Solutions of acetic acid soluble collagen were digested with the following enzymes:- Pronase; sisal enzyme; Hyaluronidase; trypsin; and pectinesterase. Digestion with Pronase was carried out at pH 4.0 in acetate buffer, while digestion with other enzymes were carried out on collagen dissolved in 0.2M Calcium chloride at pH 6.5. All digestions were performed at 20°C, for 24 hours. Digestion mixtures were dialysed exhaustively against running water, and the precipitated material removed by centrifugation. The precipitate in each case was washed three times with distilled water, redissolved in 0.1M acetic acid, and adjusted to pH 5.5 - 6.0 by addition of sodium acetate. The solutions were incubated at 37°C and inspected after 3 hours for gel formation. The table below summarises the results.

Table XII

Effect of enzymes on thermal gelation of collagen.

Material	Treatment	Incubation at 37°C.
Soluble collagen	-	Gelation
Soluble collagen	Pronase	Gelation
Soluble collagen	Sisal enzyme	Gelation
Soluble collagen	Trypsin	Gelation
Soluble collagen	Hyaluronidase	Gelation
Soluble collagen	Pectinesterase	Gelation

In each case after the formation of the gel the tubes were shaken, this giving rise to fibres which were visible to the naked eye.

(ii) Effect upon the fractionation pattern of denatured collagens.

Solutions of acid soluble collagen were digested with various enzymes under the conditions described above, and the reconstituted material redissolved in 0.1M acetic acid. The sub-units of these materials were then fractionated as described in Section 2 III after denaturation at 39°C. The fractionation patterns obtained are shown in fig. XI.

Figure XI

Fractionation patterns of denatured collagen and collagen denatured after enzyme treatment.

Fig. XI(a) Collagen

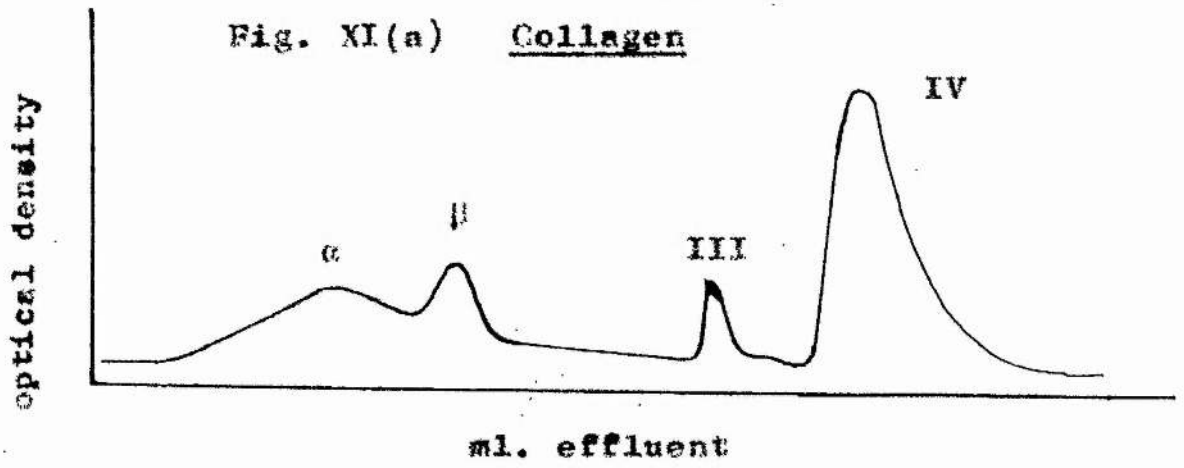


Fig. XI(b) Collagen after treatment with Pronase.

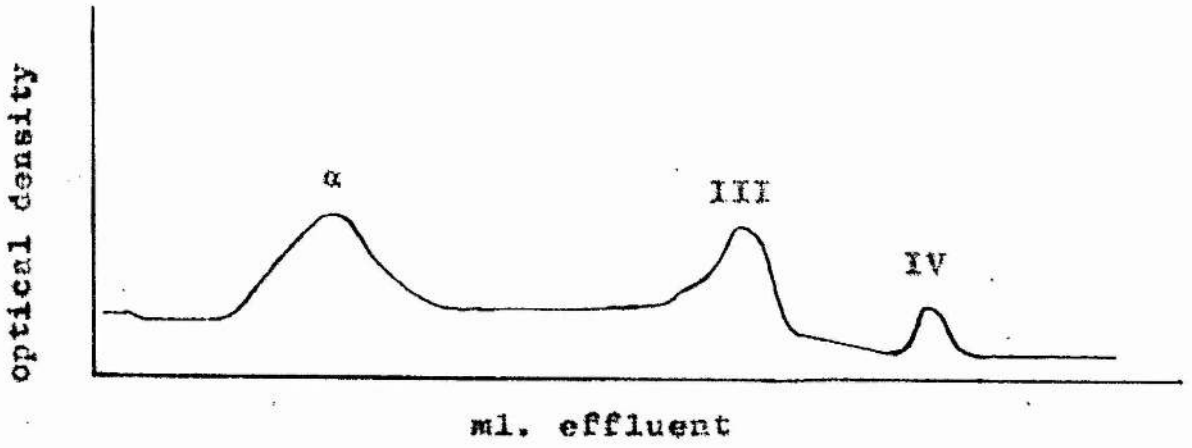


Fig. XI(c) Collagen after treatment with trypsin

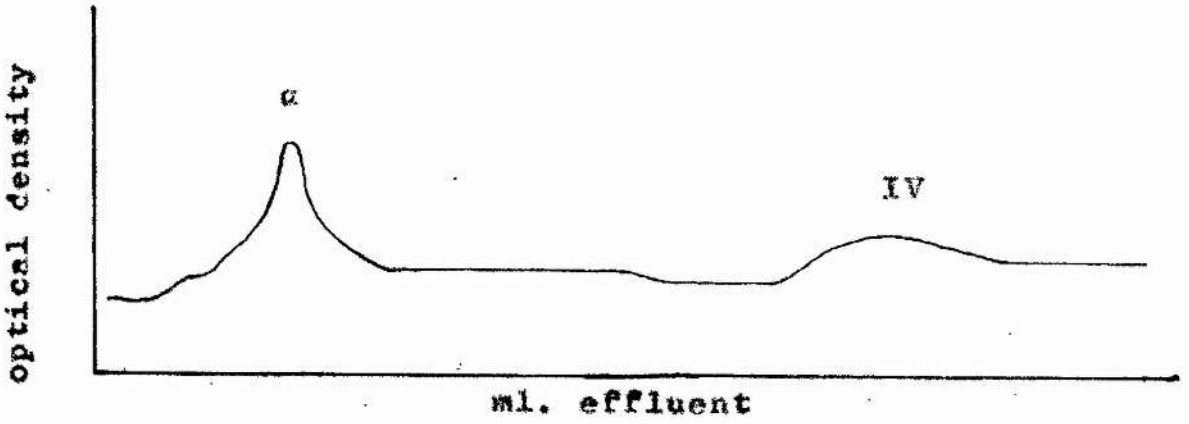


Fig. XI(d) Collagen after treatment with sisal enzyme

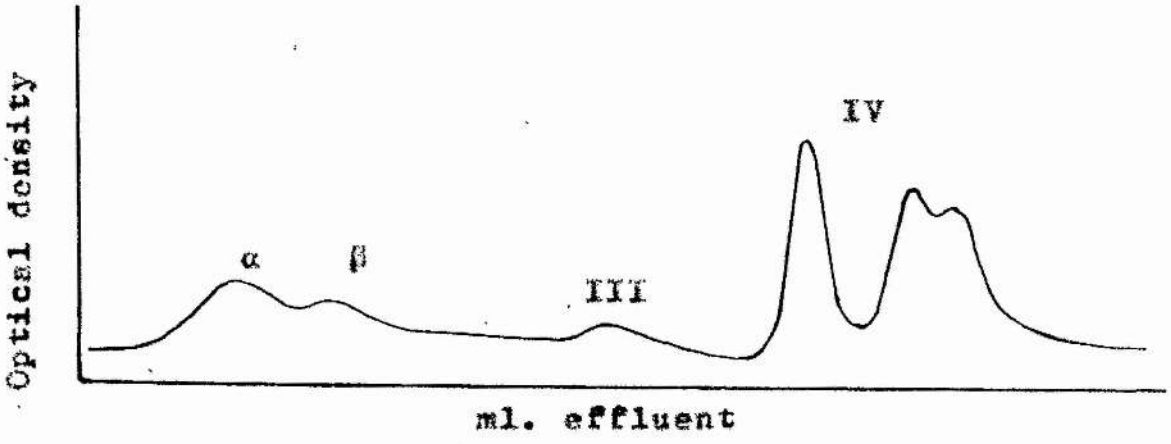


Fig. XI(e) Collagen after treatment with hyaluronidase

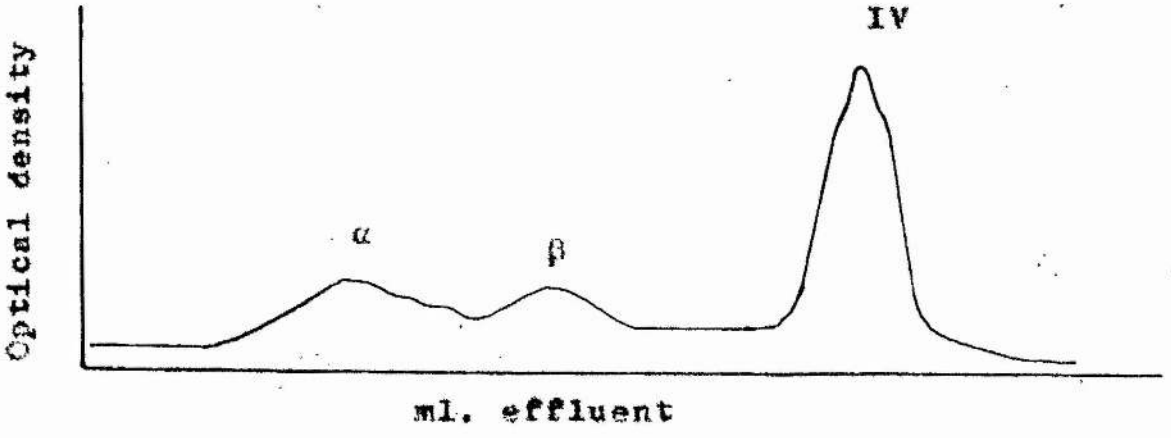
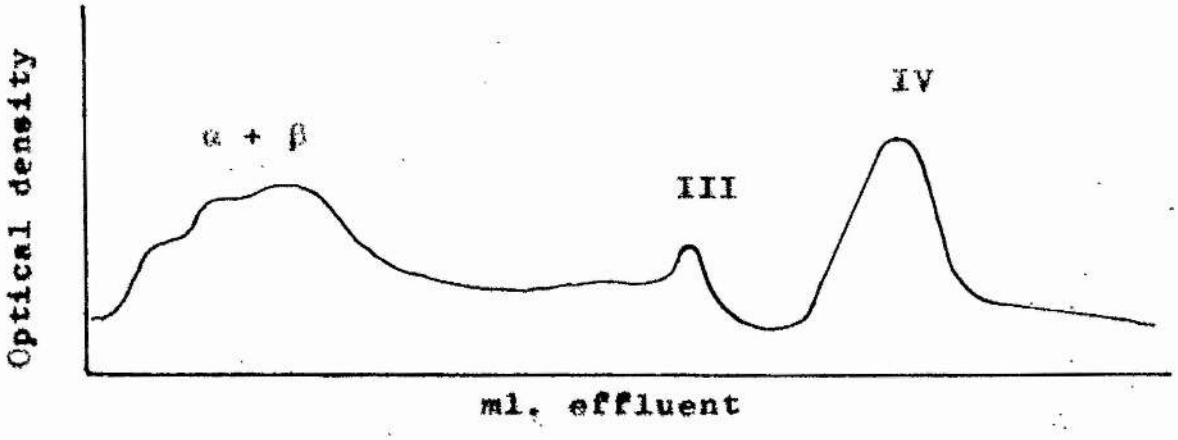


Fig. XI(f) Collagen after treatment with pectinesterase



The pattern for untreated collagen is similar to that found by Piez, Weiss and Lewis (1960), in that there appear to be only two sub-units (α and β) and not the more complex pattern of four sub-units reported by Piez, Eigner and Lewis (1963).* The fourth fraction, eluted with alkaline buffer, was not reported by Piez *et al.*, but has been reported by Worrall, Tristram and Steer (1963) and suggested to be identical to the sodium hydroxide fraction of Kulonen *et al.* (1962).

The proportions of the various components present are tabulated below.

---

* Recent work by Tristram *et al.* (1963) has accounted for this apparent discrepancy. These workers have shown that separation of denatured collagen into the four sub-units (α_1, α_2, β_1, and β_2) can only be achieved on certain types of CM-Cellulose, while apparently similar material produced by other manufacturers results in the separation of only the two sub-units reported here.

Table XIII

Sub-unit composition of enzyme treated collagens.

Material	α- + β- (% of total)	IIIrd component (% of total)	IVth component (% of total)	α : β (approx.)
Untreated collagen	24	11	65	2:1
Collagen treated with Pronase	62	27	11	-
Collagen treated with sisal enzyme	27	13	60	1:1
Collagen treated with trypsin	52	-	48	-
Collagen treated with hyaluronidase.	37	-	63	2:1
Collagen treated with pectinesterase	27	7	69	?

The values for untreated collagen agree reasonably well with those of Worrall, Tristram et al. (1963). Pectinesterase and sisal enzyme appear to have no effect upon the collagen, since the slight differences found are within the limits of accuracy of the experimental procedure. In the case of hyaluronidase treated material the increase in (α + β) and absence of IIIrd component is probably due to the IIIrd component being insufficiently separated from the first two.

The effect of Pronase and trypsin is more pronounced. The amount of IVth component is reduced in each case, although the reduction is much greater in the case of Pronase treated collagen, and there is a twofold increase in IIIrd component in this case, whereas in trypsin treated collagen no IIIrd component was found.

Although from the above table pectinesterase has no apparent effect, the separation of α and β sub-units was not achieved, and the fractionation pattern suggests that there may be three components present, although the separation of these components is very poorly defined.

XII. Effect of Pronase on skin and insoluble collagen.

(i) Nitrogen, hydroxyproline and carbohydrate content of materials.

The materials used as substrate in these experiments were:-

i) Skin extracted five times with 0.2M Na_2HPO_4. This material is referred to hereafter simply as skin.

ii) Skin exhaustively extracted with 0.1M acetic acid (referred to as insoluble collagen).

These materials were obtained in a freeze-dried form, and total nitrogen, hydroxyproline, hexose and hexosamine determinations carried out. The content of

these components in the two materials is given below.

Material	Nitrogen %	Hydroxyproline %	Hexose* %	Hexosamine %
Skin	16.9	12.7	1.5	0.33
Insoluble Collagen	17.2	15.0	1.6	0.43

* Hexose values are expressed as the glucose equivalent, since all hexose determinations were based on glucose standards. The values found are within the range of literature values. Gross, Dumsha and Glazer (1958) reported 1.5% hexose and 0.4% hexosamine in calf corium, and a hexosamine content of 0.42% for calf skin after phosphate extraction was reported by Bowes, Elliot and Moss (1955). The lower values of nitrogen and hydroxyproline of skin are probably due to non-collagenous material still present in the material. Repeated extraction with acetic acid will remove this material and thus increase the above values.

(ii) Digestion of insoluble collagen: examination of N-terminal amino acids of digests.

Since Pronase was found to effect only slight digestion of insoluble collagen at neutral pH (Section 3 IX(b))

digestions here were carried out at pH 4.0 in acetate buffer. Insoluble collagen was digested at this pH with Pronase which had been previously dialysed against distilled water. Digestions were carried out at room temperature (20°C) for 48 hours. At the end of this time the reaction mixture was reacted with FDNB, and α-DNP-amino acids estimated as described in section 2 VII. Similar digestions were carried out using Fraction 1 (FI) and Fraction 3 (FIII) of Pronase, and a control experiment, in which no enzyme was added, was also performed. Since freeze dried collagen was not used as substrate in these digestions the amount of collagen used for the digestion was determined from hydroxyproline estimations carried out after hydrolysis of the DNP-protein. Results of these experiments are recorded in Table XIV.

Table XIV

DNP-amino acids of insoluble collagen and Pronase-insoluble collagen digestions.

Results are expressed as moles of DNP-amino acid residue isolated per 10^6 grams of collagen. All results are corrected for losses of DNP-amino acid occurring on hydrolysis.

DNP Amino acid	Digestion with Pronase	Digestion with FI	Digestion with FIII	Insoluble Collagen
Glutamic	6.4	8.4	4.4	0.48
Aspartic	6.0	12.1	7.6	0.34
Serine	4.9	10.7	5.9	0.48
Threonine	4.6	18.0	17.9	0.79
Glycine	5.7	14.6	3.4	0.52
Alanine	5.1	17.7	4.9	0.75
Valine	4.2	9.2	3.4	0.39
Leucine/ Isoleucine	5.8	13.0	6.8	0.51
Phenylalanine	5.7	9.6	5.4	0.21
Lysine	2.5	8.7	2.4	Trace
Total	50.9	122.0	68.1	4.47

The results shown above reveal that there is a large increase in free α-amino groups after reaction with Pronase and Pronase fractions. The free α-amino groups may be either the N-terminal amino acids of peptides or free amino acids. The significance of this will be discussed later.

(iii) Digestion of insoluble collagen with Pronase : Fate of hydroxyproline and carbohydrate components.

Insoluble collagen, suspended in acetate buffer pH 4.0, was digested with Pronase FI, Pronase FIII and crude dialysed Pronase. Digestions were carried out at 20°C and allowed to proceed for 48 hours. Digestion mixtures were centrifuged at 14,000 r.p.m. and the supernatants transferred to dialysis tubing and dialysed against 6 changes of 4 volumes of distilled water. The insoluble material was solubilised by warming in water to 100°C, and nitrogen determinations were carried out on the three fractions, i.e. insoluble, soluble and diffusible fractions. Each fraction was then divided into two, one aliquot for hydrolysis with 5.7 N HCl for determination of hydroxyproline, the other for resin hydrolysis prior to estimation of hexose and hexosamine. A control reaction was also carried out. Results are tabulated below.

Table XV

Fate of hydroxyproline and carbohydrate components.

i) Control : insoluble collagen.

Fraction	Total Nitrogen		Hydroxy-proline		Hexose		Hexo-samine		Hexo-samine
	mg.	% of total	mg.	% of total	mg.	% of total	mg.	% of total	Hexose
Insoluble	160.0	93.8	117.5	95.0	17.0	77.3	3.5	100.0	0.20
Soluble	9.4	5.5	5.5	4.4	2.5	11.3	-	0.0	-
Dialysable	1.1	0.7	0.2	0.6	2.5	11.3	-	0.0	-

ii) Digestion with Pronase PI

Fraction	Total Nitrogen		Hydroxyproline		Hexose	
	mg.	% of total	mg.	% of total	mg.	% of total
Insoluble	6.8	7.7	4.3	8.1	1.05	8.1
Soluble	75.8	86.0	48.8	91.8	7.0	53.7
Dialysable	5.6	6.4	0.1	0.2	5.0	38.2

iii) Digestion with Pronase PIII

Fraction	Total Nitrogen		Hydroxyproline		Hexose	
	mg.	% of total	mg.	% of total	mg.	% of total
Insoluble	29.6	43.0	21.2	41.1	4.2	42.3
Soluble	36.0	52.0	29.6	57.4	3.6	36.3
Dialysable	3.2	4.6	0.7	1.4	2.1	21.2

iv) Digestion with dialysed Pronase (crude)

Fraction	Total Nitrogen		Hydroxy-proline		Hexose		Hexosamine		Hexose
	mgs.	% of total	mgs.	% of total	mgs.	% of total	mgs.	% of total	Hexo-samine
Insoluble	21.2	13.2	14.0	11.1	1.25	13.8	0.5	16.1	0.40
Soluble	130.0	80.7	110.0	86.9	6.4	65.6	2.3	74.1	0.36
Dialys-able	9.6	6.0	2.5	2.0	2.1	21.5	0.3	9.7	0.14

N.B. It is important to note that the hexose content of the insoluble collagen used in these experiments, as calculated from the total hexose of the three fractions, exhibits considerable variations. Thus values vary from 2.4% to 1.2% as against the 1.6% hexose content found for the freeze-dried material. There are several possible explanations for this variation:-

i) It was impracticable to prepare sufficient material at one time for all experiments, thus several preparations were made as required, which may account for slight differences in hexose content.

ii) There may have been some losses as a result of transference of small volumes quantitatively to sealed tubes.

iii) Resin hydrolysis, in an oven at 100°C, could not be continuously automatically shaken as suggested by Anastassiadis and Common (1958) and were thus shaken at intervals by hand,

which is not so satisfactory. Variations in oven temperature might also have an effect.

(iv) Chromatography.

The soluble and diffusible fractions from digestions with crude Pronase were subjected to paper chromatography prior to hydrolysis, and water eluates from resin hydrolysates of these two fractions and from the insoluble fraction were also chromatographed. (Section 2 V). Typical chromatograms are shown in Figs. XII and XIII. No spots were visible in the diffusible fraction prior to hydrolysis, indicating that hexoses, if present, were bound in some form. After hydrolysis each of the three fractions showed spots corresponding to glucose and galactose, and the soluble fraction possibly contains mannose. A spot corresponding to that of fucose appears only in the diffusible fraction.

Chromatograms were also run to detect amino acids and peptides in the diffusible fraction, which was made 90% v/v with acetone. The acetone soluble and insoluble fractions were chromatographed after desalting. Diagrams of the chromatograms are shown in Figs. XIV and XV. Only free amino acids and very small peptides are acetone soluble, and the chromatogram of this fraction reveals the same spots as for a chromatogram of amino acids. Larger peptides are insoluble in acetone, and the chromatogram reveals a complex

Figure XII

Chromatography of insoluble, soluble and diffusible fractions of Pronase-insoluble collagen digest.

Paper : Whatman no.1.

Solvent : Pyridine; ethyl acetate; acetic acid; water : 5 : 5: 1 : 3 (v/v)

Spray : p-Anisidine reagent.

R - Rhamnose

Fu. - Fucose

Man. - Mannose

Gal. - Galactose

Glu. - Glucose

D - Diffusible fraction
N - Soluble fraction
I - Insoluble fraction
} After resin hydrolysis

d - Diffusible fraction
n - Soluble fraction
} Prior to resin hydrolysis

◯ - Spots readily visible

◌ - Very faint spots

(w) - Bleached spots : pale blue under u.v. light.

Figure XII

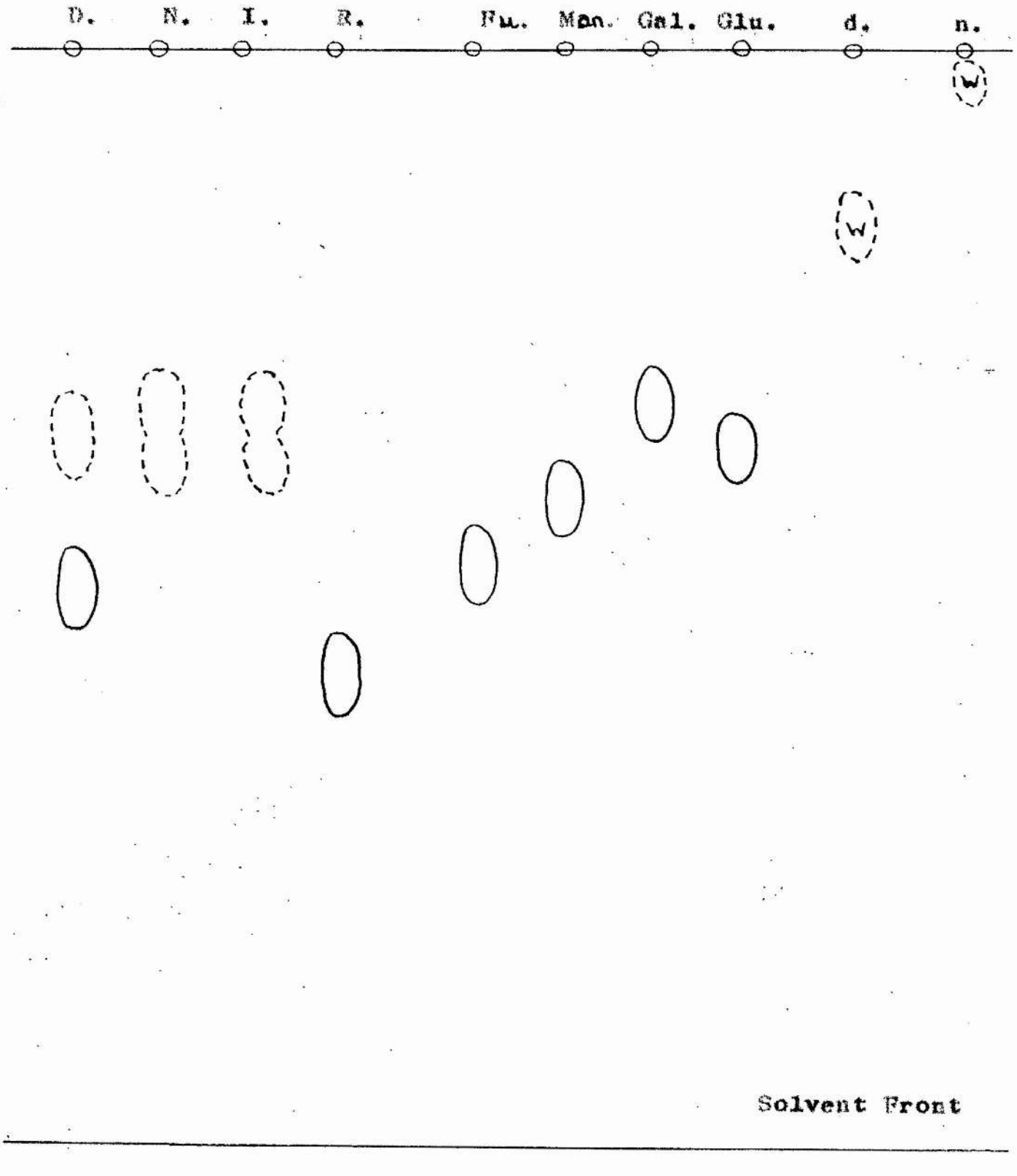


Figure XIII

Chromatography of insoluble, soluble and diffusible fractions of Pronase-insoluble collagen digests.

Paper : Whatman no.1.

Solvent : Pyridine; Ethyl acetate; Acetic acid; Water : 5: 5 : 1 : 3 (v/v).

Spray : Tetrazolium bromide.

Glu. - Glucose

Gal. - Galactose

Man. - Mannose

Fu. - Fucose

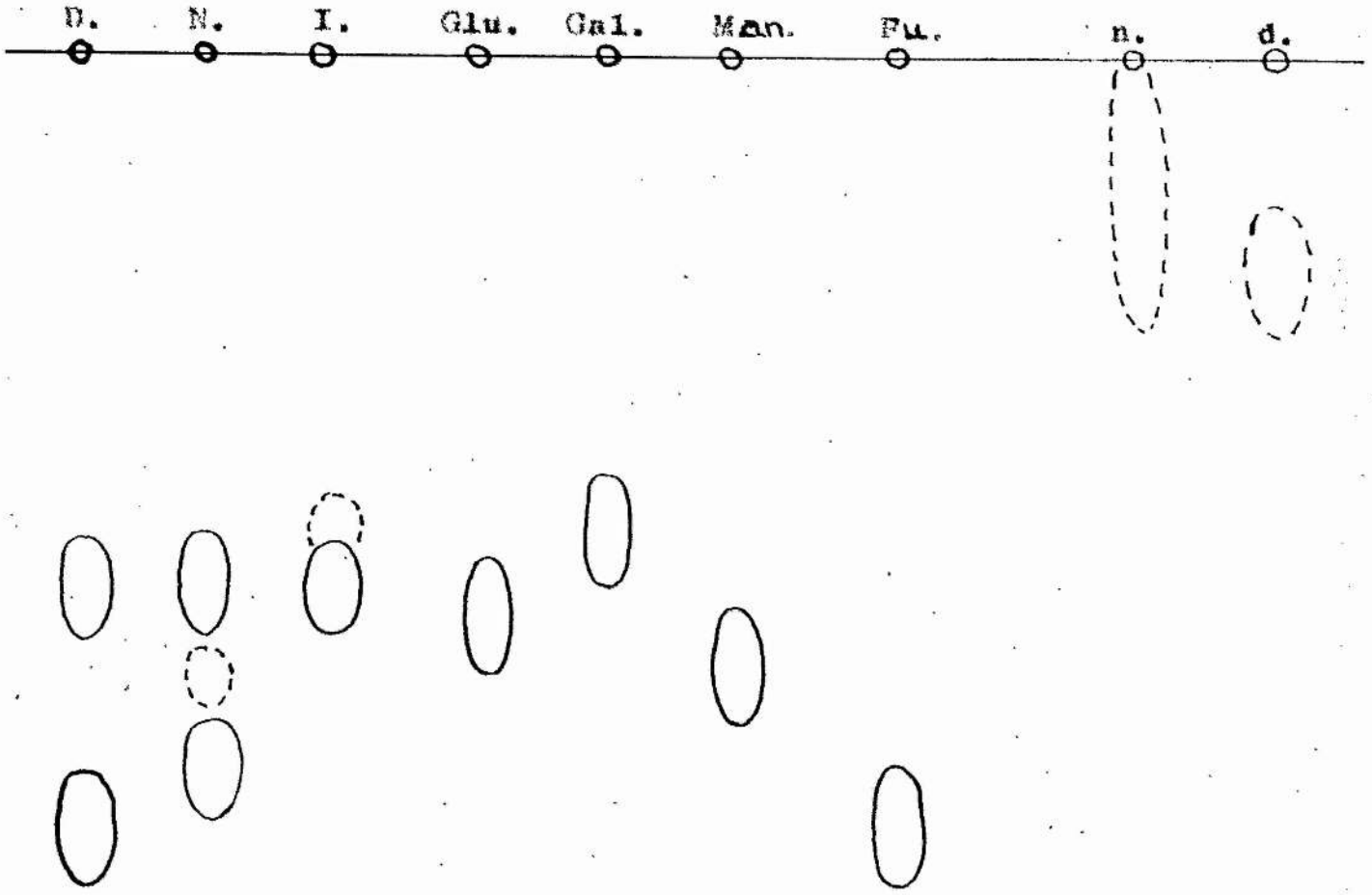
D - Diffusible fraction
N - Soluble fraction
I - Insoluble fraction
} After resin hydrolysis

n - Soluble fraction
d - Diffusible fraction
} Prior to resin hydrolysis

◯ - Spots readily visible

◌ - Faint spots appearing after standing.

Figure XIII



Solvent Front

Figure XIV

Chromatogram of acetone-soluble material of diffusible fraction of Pronase-insoluble collagen digests.

Figure XV

Chromatogram of acetone-insoluble material of diffusible fraction of Pronase-insoluble collagen digests.

Paper : Whatman no.4 (30 x 30 cms.)

Solvent : (1) Ethanol; Water; Ammonia. 80:20:1 (v/v)

(2) n-Butanol; Acetic acid; Water. 3:1:1 (v/v)

Spray : 0.2% ninhydrin in acetone.

Figure XIV

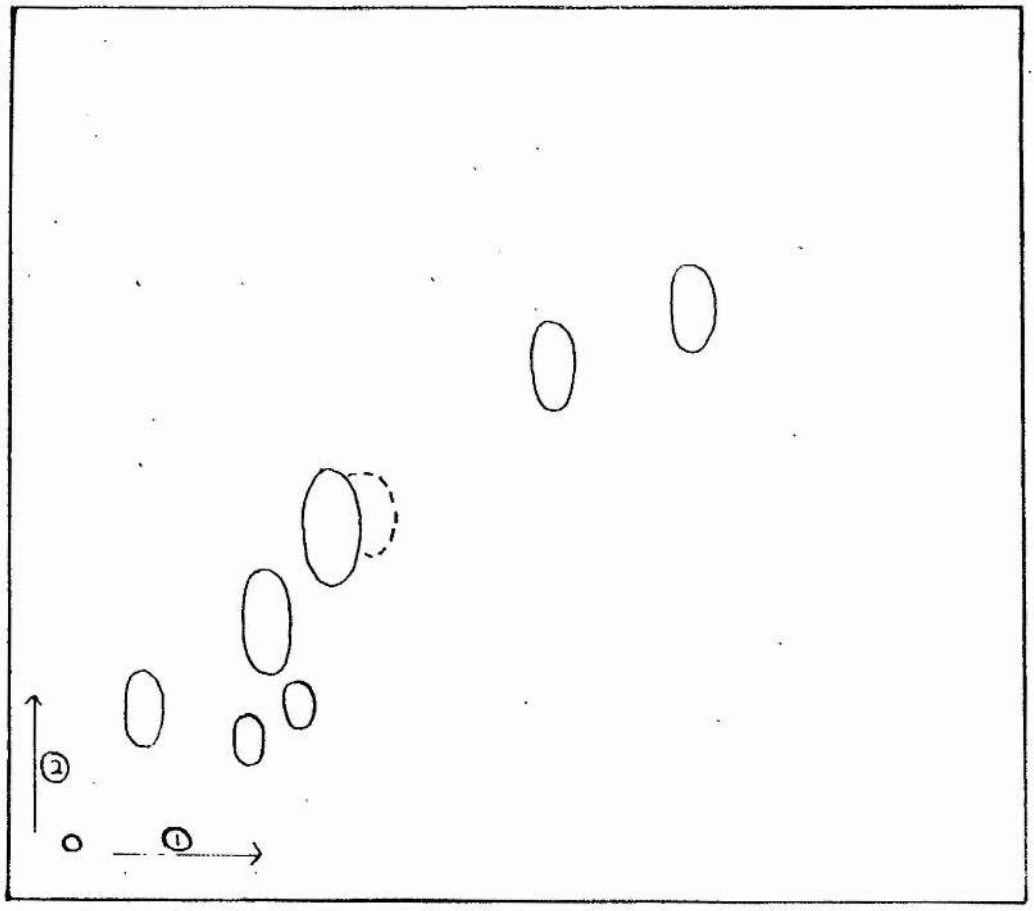
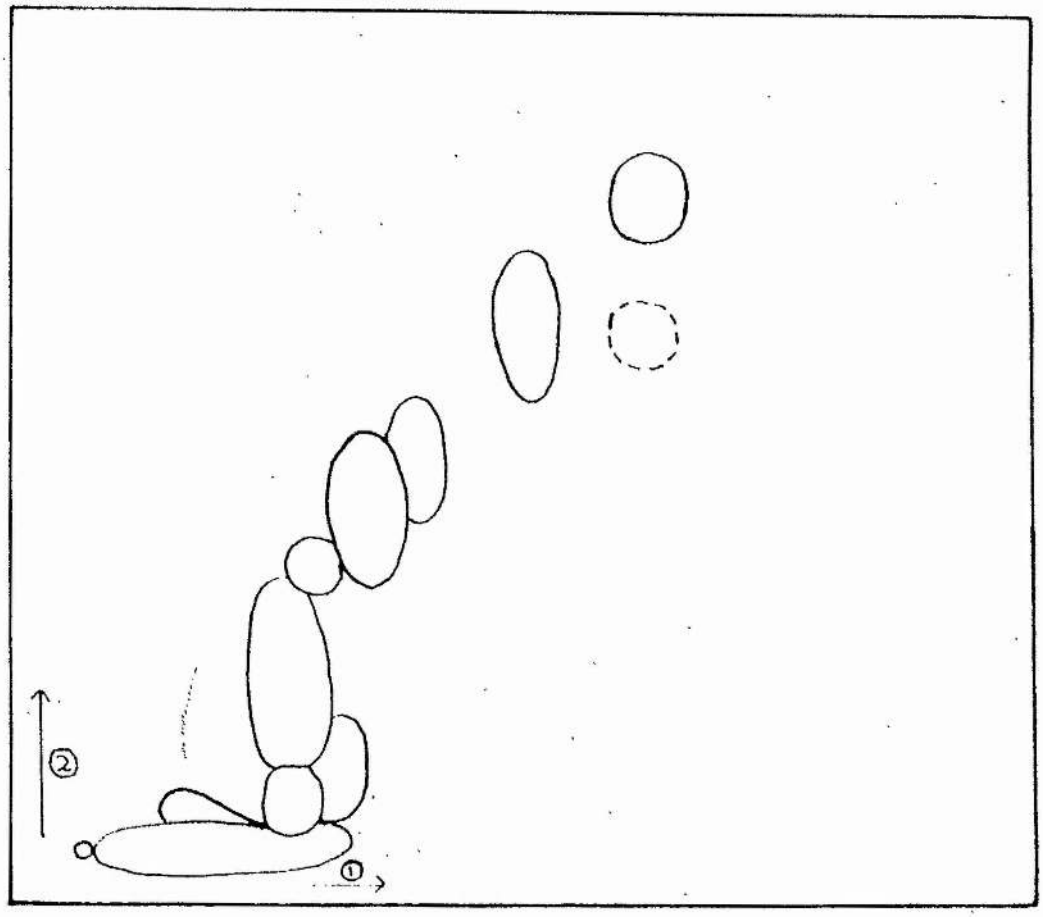


Figure XV



mixture of such peptides.

(v) Amino acid analyses of fractions.

Aliquots of the three fractions, after hydrolysis with 5.7 N HCl. were subjected to amino acid analysis, the results of which are shown in Table XVI below.

Table XVI

Amino acid analyses of insoluble, soluble and diffusible fractions of insoluble collagen after Pronase digestion.

Results shown as moles of amino acid residue per 1000 moles.

Amino acid Residue	Insoluble Fraction	Soluble Fraction	Dialysable Fraction
Hydroxyproline	87.7	113.0	-
Aspartic acid	50.6	31.3	65.5
Serine & Threonine	31.7	25.4	83.4
Glutamic acid	74.2	64.4	59.4
Proline	115.0	170.0	101.2
Glycine	239.0	297.0	230.3
Alanine	121.0	133.0	101.2
Valine	97.0	52.7	71.4
Methionine	4.7	13.7	11.9
Isoleucine	22.6	17.6	23.7
Leucine	43.8	27.3	71.4
Tyrosine	13.1	3.9	41.6
Phenylalanine	21.7	15.6	29.7
Lysine	50.6	7.8	71.4
Histidine	6.1	5.8	35.7
Arginine	29.0	39.1	11.9

The most significant features of the preceding results are the absence of hydroxyproline and the high tyrosine content of the diffusible fraction. The lowered glycine content, and comparatively high tyrosine content of the insoluble fraction is probably due to the presence of elastin in this fraction. This is considered likely in view of the fact that a small proportion of the insoluble fraction could not be solubilised even in boiling water.

(vi)a) Digestion of skin with Pronase.

It was considered possible that, in the preparation of insoluble collagen, the prolonged treatment with acetic acid had in some way rendered the material more susceptible to enzymic degradation. To examine this possibility similar experiments to the above were carried out using skin untreated with acetic acid. Results of these experiments are tabulated below.

Table XVII

Fate of hydroxyproline and carbohydrate components.

i) Control:- Skin.

Fraction	Total Nitrogen		Hydroxy-proline		Hexose		Hexosamine		Hexosamine
	mgm.	% of Total	mgm.	% of Total	mgm.	% of Total	mgm.	% of Total	Hexose
Insoluble	988.0	90.2	760.0	83.9	35.9	66.0	1.96	40.3	0.06
Soluble	202.0	18.4	145.0	15.9	13.0	24.5	2.6	53.5	0.20
Dialysable	3.7	1.4	1.1	0.2	4.5	9.5	0.3	6.2	0.07

ii) Digestion with sisal enzyme.

Fraction	Total Nitrogen		Hydroxy-proline		Hexose		Hexosamine		Hexosamine
	mgm.	% of Total	mgm.	% of Total	mgm.	% of Total	mgm.	% of Total	Hexose
Insoluble	49.6	16.0	31.2	14.9	5.1	18.7	1.25	22.7	0.24
Soluble	242.0	78.5	176.0	84.1	14.9	54.7	3.35	63.2	0.22
Dialys-able	16.8	5.5	2.0	0.9	7.2	26.5	0.75	14.0	0.12

It can thus be seen that Pronase has a comparable solubilising effect upon skin to that upon insoluble collagen, indicating that prolonged acetic acid treatment does not render the material more readily digestible. A considerable amount of collagen is solubilised in the control, this being the normal acetic acid-extractable soluble collagen, but the solubilisation is greatly enhanced by enzyme treatment.

XIII. Digestion of soluble collagen with Pronase Fraction I.

Acetic acid soluble collagen, dissolved in 0.5M calcium chloride at pH 7.0 was digested for 48 hours with Fraction I of Pronase (Enzyme : substrate approximately 1:100). At the end of the reaction the digestion mixture was acidified by the addition of N.HCl, resulting in the precipitation of the collagen. The precipitate was removed by centrifugation and the supernatant, after reduction in volume in vacuo at 40°C, was dialysed against several changes of distilled water. Nitrogen determinations were carried out on the precipitated material, and on the diffusible and non-diffusible fractions of the supernatant. A control reaction was also carried out. Results are shown in Table XVIII below.

Table XVIII

Nitrogen contents of precipitated collagen, soluble and diffusible fractions.

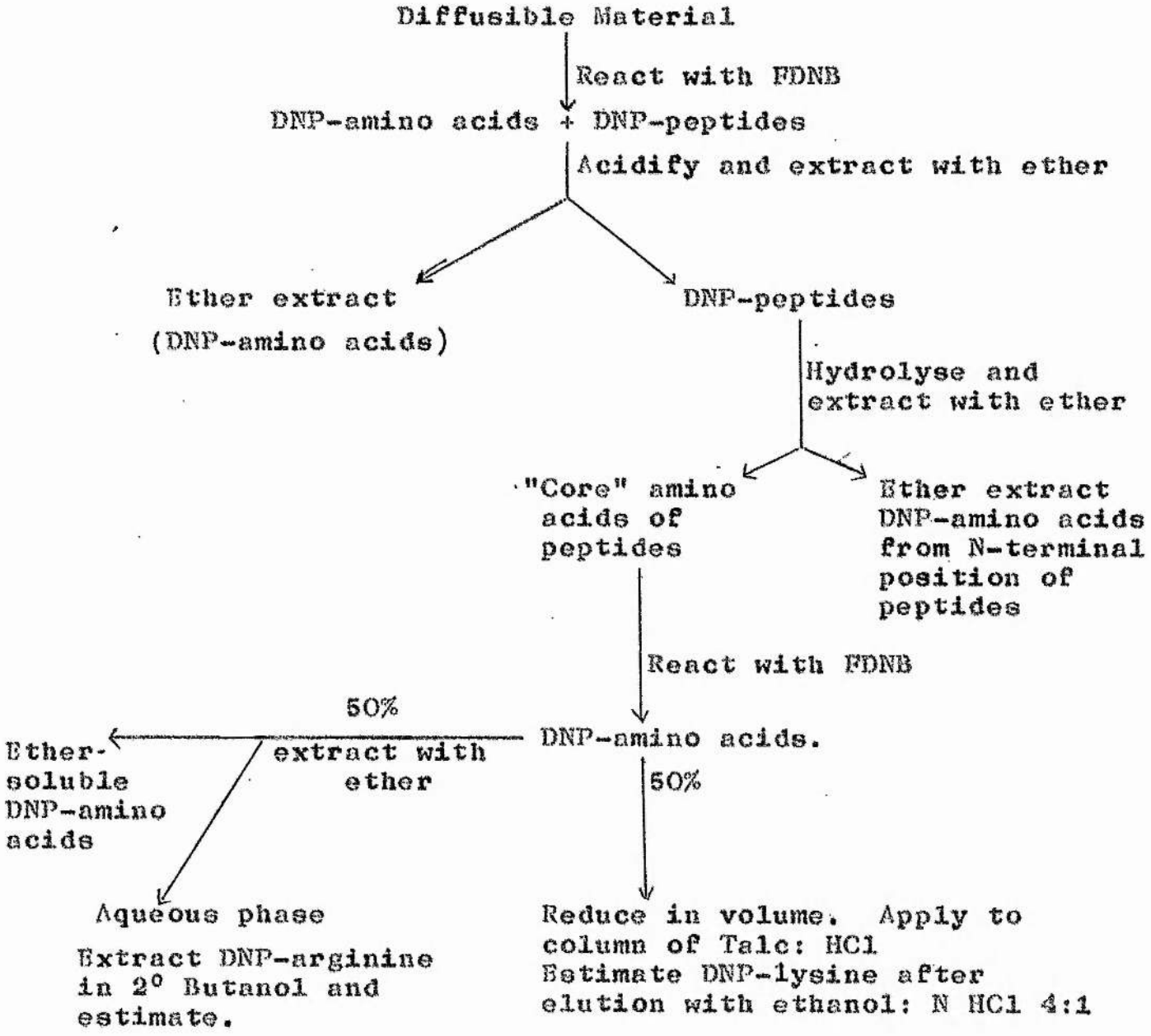
	Total Nitrogen			
	Enzyme Digestion		Control	
	mg.	% of Total	mg.	% of Total
Precipitate	112.4	75.0	137.0	93.0
Non-dialysable	23.6	16.0	8.0	5.4
Dialysable	12.0	8.2	2.0	1.3

The enzyme thus renders 8.2% of the total collagen nitrogen into a diffusible form. (cf. 8.2% diffusible nitrogen from digestion with crude enzyme at pH 4.0). The 1.3% of the total nitrogen of the control which is diffusible will be considered later in the discussion and the section dealing with non-protein nitrogen. The fact that only 75% of the total nitrogen of the digested material is precipitated as against 93% in the control experiment, may be of significance in a consideration of the aggregation properties of the collagen.

Examination of diffusible material.

The diffusible material, consisting probably of free

amino acids and peptides, was reacted with FDNB by the usual method (Section 2 VII). The following procedure was then carried out.



The results of the above studies are tabulated below. Free amino acids are expressed as mM α-DNP amino acid per

10^6 grams collagen. N-terminal amino acids of peptides are expressed in similar fashion, these values being corrected for losses on hydrolysis. The remaining amino acids of the peptides are expressed in their molar ratios.

Table XIX

Diffusible material from Pronase Fraction I digestion of collagen.

Amino acid Residue	Free Amino acids	N-Terminal from peptides	Core Amino acids of peptides
Glycine	251.0	1.46	6.0
Alanine	74.0	0.13	4.5
Serine	19.0	0.13	2.5
Threonine	25.0	0.19	1.5
Valine	131.0	0.14	3.0
Leucine	293.0	0.07	3.0
Phenylalanine	Trace	-	2.0
Proline	53.0	-	120.0
Hydroxyproline	Trace	-	42.6
Glutamic	Trace	-	2.5
Aspartic	Trace	-	1.0
Lysine	-	-	5.0
Arginine	-	-	11.0

Digestion of soluble collagen with Pronase Fraction I : Examination of some acetone soluble peptides.

Acid soluble collagen (Total nitrogen 117 mg.) dissolved in 0.5M calcium chloride pH 7.0 was digested with Pronase Fraction I for 48 hours at 20°C. The reaction mixture was precipitated by addition of acetone, and the precipitated material removed by centrifugation. Nitrogen determinations were carried out on the supernatant. A control reaction without enzymes was performed simultaneously.

	Control	Enzymic Digestion
Initial Total N. mg.	117.0	117.0
Soluble Nitrogen mg.	1.8	11.6
% of total N. which is soluble	1.5	9.8

The supernatant from the enzymic digest was desalted on Amberlite CG 120. Since the ammonia eluate from the column contained only 30% of the nitrogen put on the column the water washings were also retained for examination. Both the ammonia eluate and the water washings were reacted

with FDNB, and free α-DNP-amino acids extracted with ether and estimated. The free α-DNP-amino acids found are shown in Table XX.

Table XX

Free amino acids from Pronase Fraction I digest of soluble collagen (mM)

Glutamic acid	14.0	Alanine	20.0
Aspartic acid	28.0	Valine	10.0
Serine	18.0	Leucine	45.2
Threonine	40.0	Phenylalanine	68.1
Glycine	62.0	Lysine	110.3

Examination of ethyl acetate soluble peptides of ammonia eluate.

The solution of DNP-peptides from this eluate, after extraction of free α-DNP-amino acids, was extracted with ethyl acetate. The ethyl acetate extract was divided into two portions, one of which was hydrolysed with acid. The acid hydrolysate was extracted with ether, and the α-DNP amino acids chromatographed (Section 2 VII f). The following DNP-amino acids were detected, in the molar

proportions indicated.

Glycine	2
Alanine	1
Serine	1
Phenylalanine	2

This suggests the presence of at least six peptides in the ethyl acetate solution.

The aqueous phase of the acid hydrolysate was chromatographed by the system of Redfield (Section 2 IV), the following amino acids being identified:- aspartic, glutamic, alanine, serine, proline and valine.

The second portion of the ethyl acetate extract was chromatographed in an attempt to separate the DNP-peptides; fig. XVI shows the result of this chromatogram. Two of these spots, peptides 6 and 7, after elution in N HCl, were hydrolysed, and the N-terminal α-DNP-amino acids extracted into ether and chromatographed. The remaining amino acids of the peptides were then converted to the DNP-amino acids, and estimated after chromatography.

Peptide	6	7
N-terminal Residue	-	-
other amino acids (molar proportions)	Glycine 2 Alanine 3.5 Serine 1 Proline 3 Valine 4.5	Glycine 2 Alanine 3.5 Serine 1 Valine + (Leucine) 6

Figure XVI

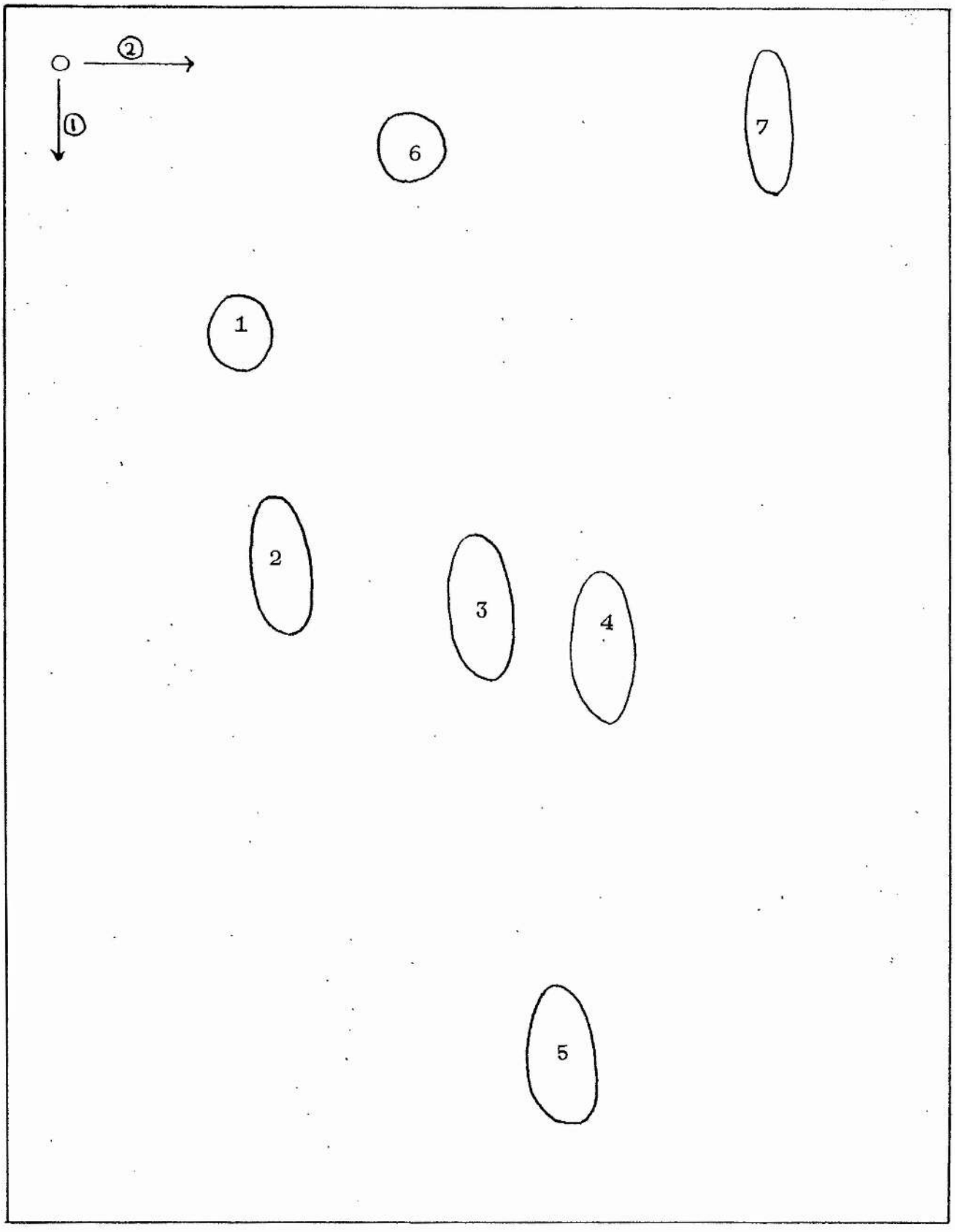
Chromatogram of ethyl acetate soluble DNP-peptides from Pronase P_I digest of soluble collagen.

Paper : Whatman no.20.

Solvents : (1) Tert. Amyl alcohol: 3% Ammonia. 1:1(v/v)

(2) 0.8M. phosphate pH 6.7.

Figure XVI



The absence of N-terminal amino acids may indicate that the α-DNP-amino acid in each instance was glycine, since α-DNP glycine is most readily destroyed on hydrolysis.

Examination of water soluble peptides of ammonia eluate.

A preliminary examination of the contents of this solution was performed. The α-DNP-amino acids N-terminal in these peptides were released by hydrolysis, extracted into ether and chromatographed. Trace amounts of the following DNP-amino acids were found:- glycine, serine and possibly glutamic (and/or aspartic) acid.

Examination of ethyl acetate soluble peptides from water eluate of column.

A two-dimensional chromatogram was run of the DNP peptides soluble in ethyl acetate. The chromatogram is shown in fig. XVII. The presence of a spot at position (1) was extremely doubtful, and after elution in N HCl no reading of the optical density at 360 mμ was found. The other spots were also eluted in N HCl and optical densities measured. Spot (2) was found to be bleached in acid and thus was probably artefact. The optical densities of the other spots were:-

Peptide (3) 0.060

Peptide (4) 0.025

No further examination of these peptides was attempted.

Figure XVII

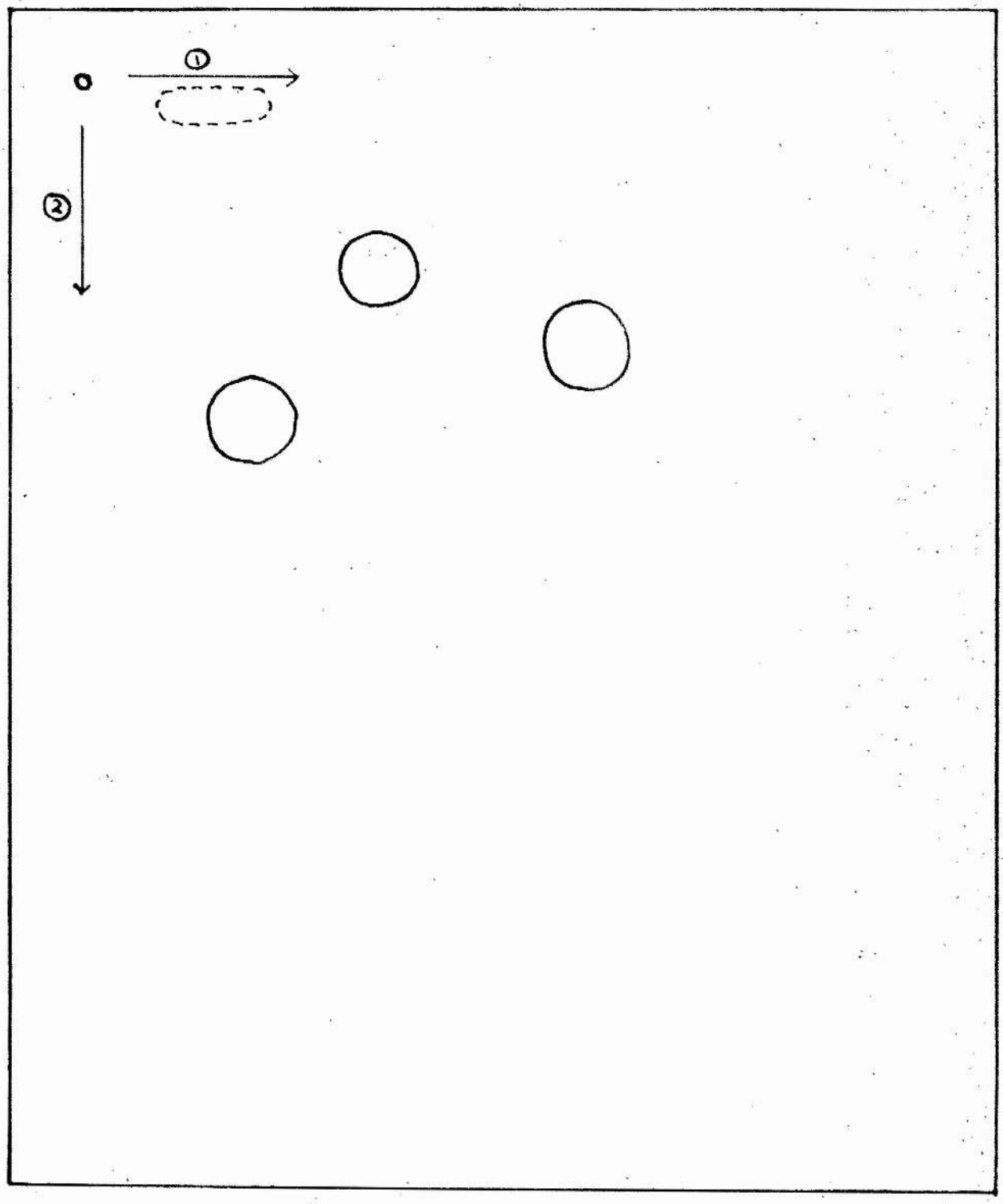
Chromatogram of ethyl acetate soluble DNP-peptides from Pronase F_I-soluble collagen digest.

Paper : Whatman no.20

Solvents : (1) Tert.amyl alcohol: 3% ammonia. 1:1(v/v)

: (2) 0.8M phosphate pH 6.7.

Figure XVII



XIV. The non-protein nitrogen of collagenous proteins.

During the course of investigations on small peptides produced as a result of enzymic hydrolysis of collagen it has been found that small amounts of non-protein nitrogen were consistently found in control experiments. (See section 3 Tables IV, VII, IX etc.). This was first reported in 1961 by Steven, Tristram and Tyson, and the non-protein nitrogen of acetic acid soluble collagen has been extensively examined by Steven and Tristram (1962(a) and (b)). The non-protein nitrogen of insoluble collagen has also been further examined as detailed below.

Non-protein nitrogen was prepared from insoluble collagen by acetone precipitation or by dialysis. The insoluble collagen used in these experiments was calf-skin exhaustively extracted with citrate buffer (Section 2 I(b)).

Table XXI

(i) Removal of non-protein nitrogen from insoluble collagen at various pH's.

Method of Preparation.	pH	N.P.N. as % of total collagen N.
Acetone precipitation	3.5	0.1)
followed by		) 0.6%
Acetone precipitation	3.5	0.5)
Dialysis	3.5	0.43)
followed by		) 0.68%
Dialysis	3.5	0.25)
Dialysis	7.0	0.21)
followed by		) 0.31%
Dialysis	3.5	0.10)
Dialysis	12.0	0.15)
followed by		) 0.28%
Dialysis	3.5	0.13)
Dialysis	7.0	0.27)
followed by		) 0.54%
Dialysis	12.0	0.17)
followed by		)
Dialysis	3.5	0.10)

It can be seen therefore that non-protein nitrogen can be removed from insoluble collagen over a wide range of pH's. The percentage of the collagen nitrogen which can be removed

as non-protein nitrogen varies slightly from preparation to preparation, but in no case does it exceed 1% of the total nitrogen.

(ii) Chromatography of non-protein nitrogen.

Non-protein nitrogen samples as prepared above were chromatographed on paper using the two systems described in Section 2 IV(b). Specimen chromatograms are shown in fig. XVIII, and reveal the presence of several free amino acids, viz.- aspartic acid; glutamic acid; glycine; alanine; serine; valine; leucine and threonine. The spots of lysine and phenylalanine were very faint and were not detected on all chromatograms. Similarly the spot P was not found on all chromatograms, and when present was usually extremely faint.

Non-protein nitrogen was also chromatographed after reaction with FDNB and extraction of α-DNP-amino acids into ether. Fig. XIX shows a typical chromatogram of free α-DNP-amino acids detected in this manner. Trace amounts of tyrosine, proline and hydroxyproline were frequently, although not invariably, found on such chromatograms. The detection of these amino acids in the DNP form, although not as free amino acids, is a result of the greater sensitivity of this method.

Figure XVIII

Chromatograms of N.P.N. of insoluble collagen.

(a) Paper : Whatman no.4 (30 x 30 cms.)

Solvents:

(1) Ethanol: Water: ammonia 80:20:1 (v/v)

(2) n-Butanol: acetic acid: water 3:1:1 (v/v)

Spray : 0.25% ninhydrin in acetone.

(b) Paper : Whatman no.20 (20 x 20 cms.)

Solvents:

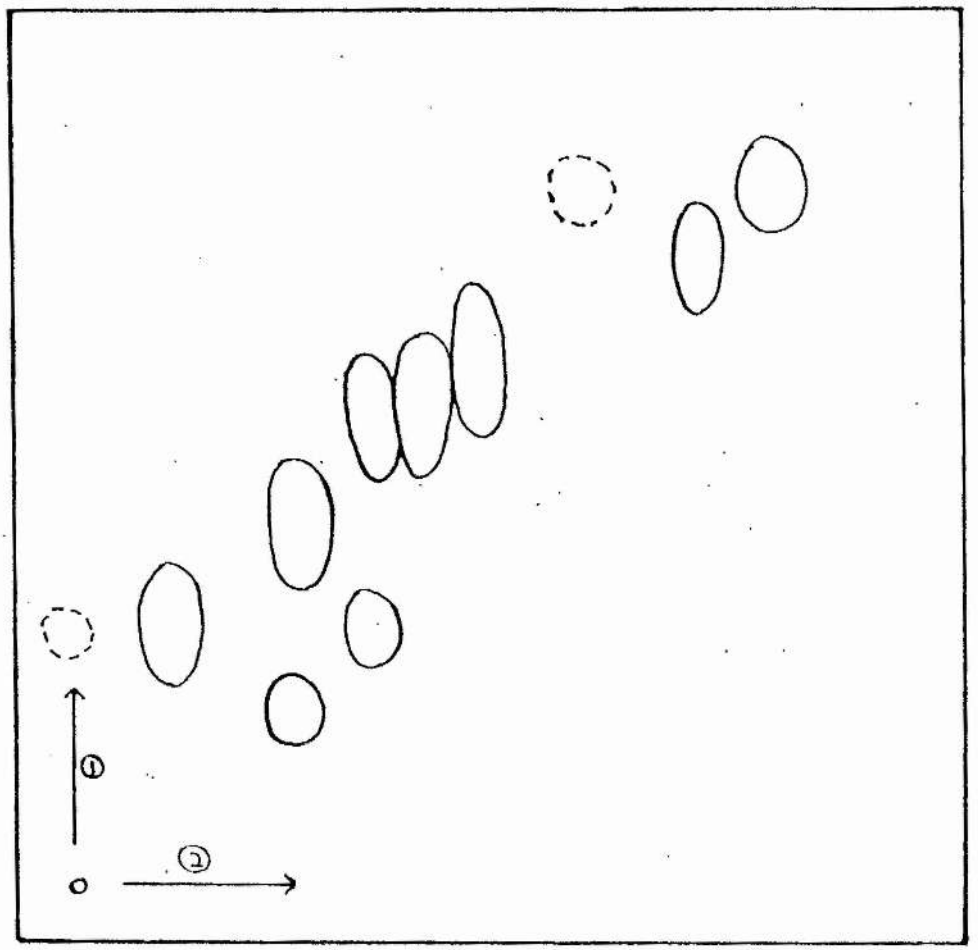
(1) Methanol: water: pyridine 80:20:4 (v/v)

(2) Tert.butanol: Methylethyl ketone: water: diethylamine. 40:40:20:4 (v/v)

Spray : 0.25% ninhydrin in acetone.

Figure XVIII

(a)



(b)

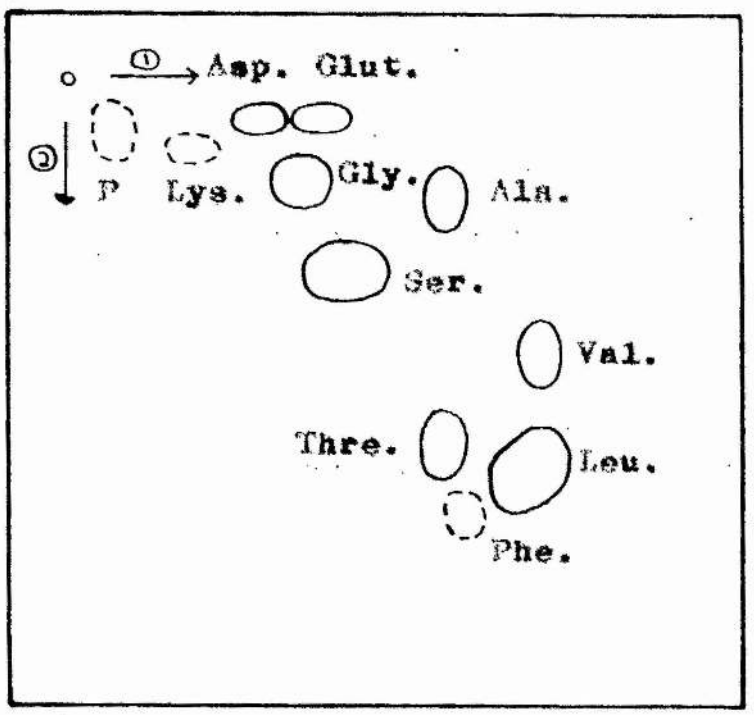


Figure XIX

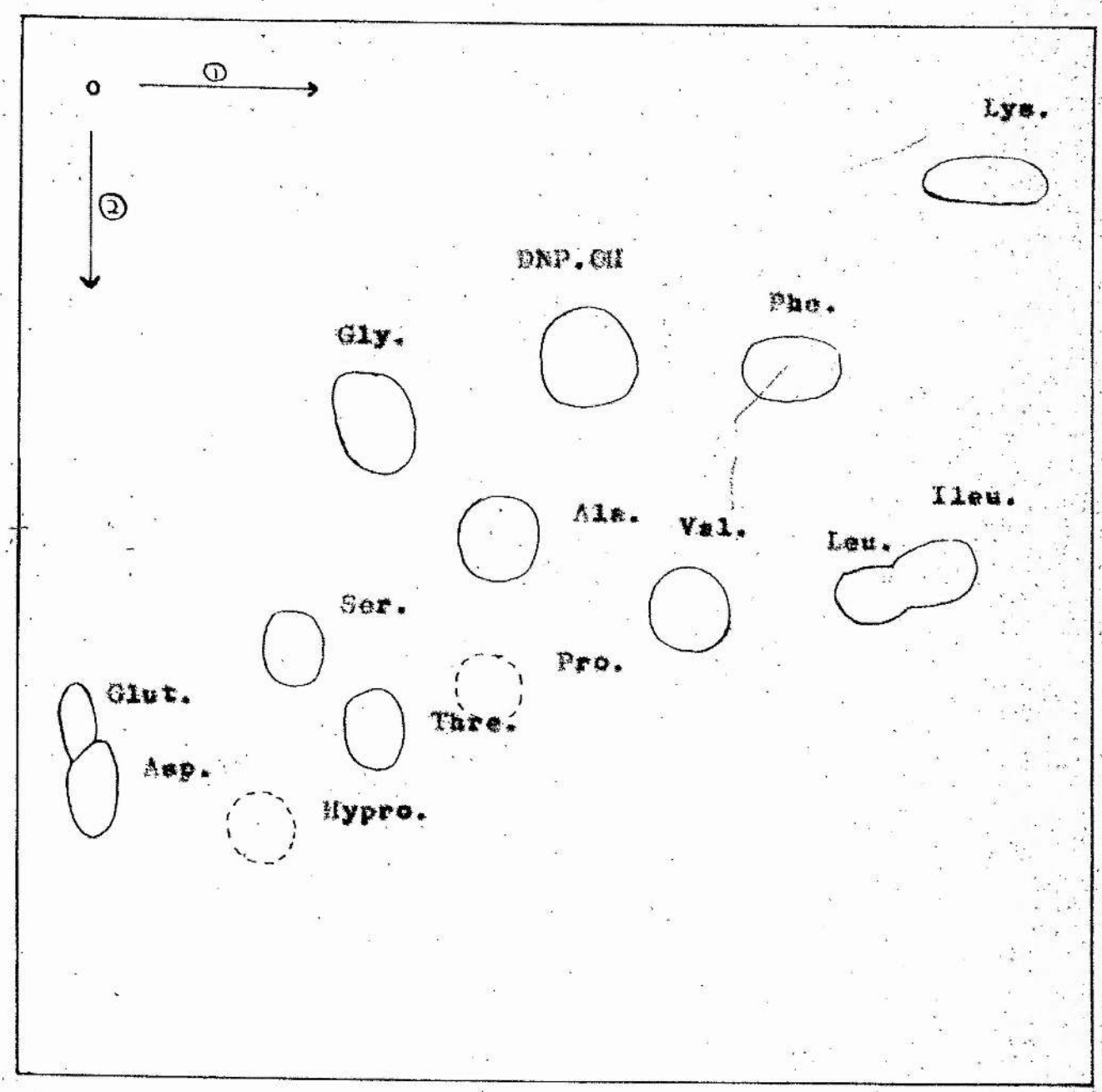
Chromatogram of α-DNP-amino acids of N.P.N. of insoluble collagen.

Paper : Whatman no.1.

Solvents: (1) Toluene: pyridine: 2-chloroethanol: 0.8N ammonia. 30:9:18:18 (v/v)

(2) 1.5M phosphate pH 6.1.

Figure XIX


o
①
②
Lys.
DNP.Gl
Phe.
Gly.
Ile.
Ala.
Val.
Leu.
Ser.
Pro.
Glut.
Thre.
Asp.
Hypro.

(iii) Peptide material of non-protein nitrogen.

Although little, if any, peptide material could be detected on chromatograms of non-protein nitrogen the presence of peptide material was confirmed by the following method. Non-protein nitrogen was reacted with FDNB, free α-DNP-amino acids extracted into ether, and the residual aqueous phase hydrolysed with acid. The hydrolysate was also extracted with ether, and the ether extract chromatographed. No α-DNP-amino acids were detected in these extracts. The hydrolysate, after reduction in volume and chromatography on the system of Redfield (Section 2 IV(b)) revealed the presence of the following amino acids:- glutamic; aspartic; glycine; alanine; serine; valine; leucine; lysine and arginine. The lysine and arginine were not present as free amino acids, but as the DNP derivatives, but their identification was confirmed by comparison with chromatograms of standard ε-DNP-lysine and DNP-arginine.

Peptide material in non-protein nitrogen was also chromatographed as DNP-peptides. DNP-non-protein nitrogen, after extraction of free α-DNP-amino acids, was extracted with ethyl acetate. The ethyl acetate soluble material and the residual aqueous phase were then subjected to two-dimensional paper chromatography as described in Section 2 VII(f). The chromatograms of the ethyl acetate extract

revealed a strong yellow spot (1) with another very faint spot at (2), and the aqueous phase showed only one peptide spot on chromatography. Fig. XX (a) and (b).

(iv) Carbohydrates in N.P.N. fraction.

1 gram of lyophilised ox insoluble collagen was suspended in 0.1M acetic acid, and 10 volumes of acetone were added. The mixture was centrifuged, and the supernatant filtered and reduced to a small volume. After removal of aliquots for nitrogen determinations, the remaining material was hydrolysed and hexose and hexosamine determinations carried out. The results are given below.

Total nitrogen of N.P.N. = 1.6 mg.

Total hexose in N.P.N. = 1.8 mg.

Total hexosamine in N.P.N. = Trace ?

(v) N-terminal analyses of non-protein nitrogen fractions.

a) N-terminal analysis of ox-skin insoluble collagen.

The N-terminal residues of ox-skin insoluble collagen were examined by the FDNB technique of Sanger (Section 2 VII). The quantities of collagen used in each estimation were calculated from hydroxyproline determinations carried out after hydrolysis of the DNP-protein. Results are tabulated below.

Figure XX

Chromatograms of DNP-peptides of N.P.N. of insoluble collagen.

(i) Ethyl acetate soluble DNP-peptides.

Paper : Whatman no.20.

Solvents: (1) Tert.amyl alcohol: 3% ammonia. 1:1 (v/v).

(2) 0.8M phosphate pH 6.7.

(ii) Water-soluble DNP-peptides.

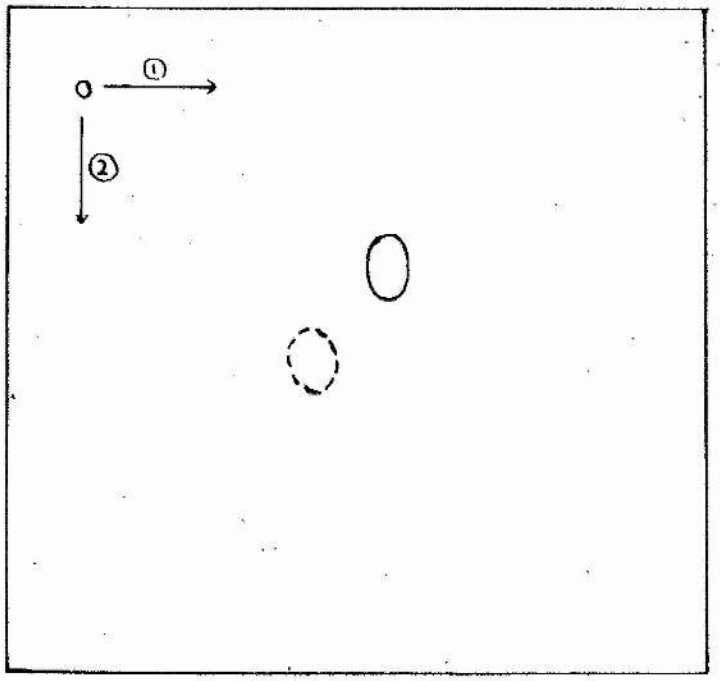
Paper : Whatman no.20.

Solvents : (1) Tert. amyl alcohol: isoamyl alcohol: 3% ammonia. 2:1:3 (v/v)

(2) 1.6M phosphate pH 7.0.

Figure XX

(i)



(ii)

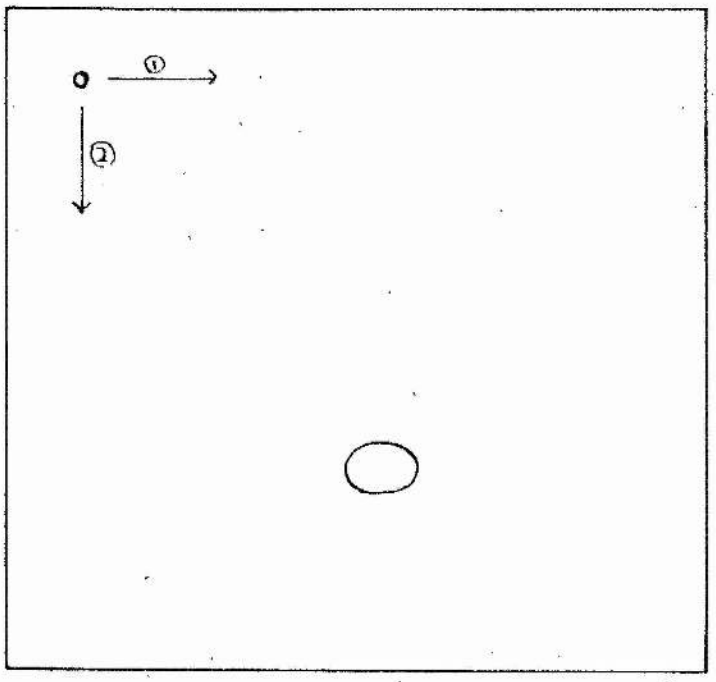


Table XXII

N-terminal residues of ox-skin insoluble collagen.

Results are expressed as moles of N-terminal residue per 10^6 grams of collagen, after correction for losses on hydrolysis.

Amino acid Residue	Moles Residue per 10^6 g.
Glutamic / Aspartic	0.23
Serine	0.10
Threonine	0.22
Glycine	0.36
Alanine	0.24
Valine / Leucine/Isoleucine	0.28
Phenylalanine	0.18
Lysine	0.04
Total	1.67
C_n	6.0×10^5

Complete separations of the dicarboxylic amino acids, and of valine and the leucines were not obtained, and a

combined figure is given. The same N-terminal amino acids were found as were present in soluble collagen (Table VI) and calf-skin insoluble collagen (Table XIV), but the molar proportions of the amino acids vary in each case. The value for C_n is higher than that for either soluble collagen or calf-skin insoluble collagen, although all values are of approximately the same order. Trace amounts of tyrosine, proline and hydroxyproline were observed on some, but not all, of the chromatograms.

b) N-terminal analyses of non-protein nitrogen.

Non-protein nitrogen fractions were obtained from insoluble collagen by six serial acetone precipitations of acetic acid suspensions of the collagen. N-terminal analyses were then performed on the non-protein nitrogen fractions. A typical chromatogram of α-DNP amino acids present in such non-protein nitrogen is shown in fig. XIX, and the results of N-terminal estimations are shown in Table XXIII.

Table XXIII

α-DNP-amino acids of non-protein nitrogen. Results are shown as moles of α-DNP-amino acid per 10^6 grams of insoluble collagen. Results are corrected for losses on hydrolysis.

α-DNP-amino acid	Moles of Residue per 10^6 gr. collagen					
	I	II	III	IV	V	VI
Aspartic) Glutamic)	0.26	0.15	0.29	0.13	0.13	0.15
Serine	0.34	0.32	0.56	0.23	0.23	0.32
Threonine	0.65	0.36	0.32	0.20	0.26	0.12
Glycine	0.85	0.75	0.47	0.91	0.54	0.25
Alanine	0.55	0.53	0.53	0.31	0.31	0.43
Valine) Leucine/Isoleucine)	0.83	0.25	0.16	0.26	0.13	0.14
Phenylalanine	0.008	Trace	0.003	0.006	Trace	Trace
Lysine	0.18	0.009	0.002	0.008	0.002	0.006
Total	0.374	0.24	0.235	0.218	0.162	0.147

The results tabulated above reveal that non-protein nitrogen fractions contain the same α-DNP amino acids as those found as apparent N-terminal residues in insoluble collagen. The molar proportions of the various DNP-amino

acids appear to vary for individual non-protein nitrogen fractions. The non-protein nitrogen removed by six serial acetone precipitations, although it represents only approximately 1% of the total collagen nitrogen, contains material reacting with FDNB to the extent of 1.37 moles of α-DNP amino acid per 10^6 grams of original collagen. This would account for 80% of the apparent N-terminal residues of the original collagen.

c) Removal of N.P.N. by dialysis: N-terminal analysis of N.P.N. and residual collagen.

Two non-protein nitrogen fractions were obtained from insoluble collagen by serial dialysis at pH 3.5 against distilled water. Each fraction was obtained by dialysing the collagen against four changes of 5 volumes of distilled water. The non-protein nitrogen fractions and the residual collagen were examined by the FDNB technique. Results of these investigations are tabulated below.

Table XXIV

α-DNP-amino acids of non-protein nitrogen fractions and residual collagen expressed as moles of α-DNP-amino acid per 10^6 grams of collagen. (Results are corrected for losses on hydrolysis).

α-DNP-amino Acid	Moles of α-DNP-amino acid per 10^6 gr. collagen		
	Dialysable N.P.N. I	Dialysable N.P.N. II	Residual Collagen
Glutamic / Aspartic	0.12 (0.40)	0.06 (0.21)	0.24 (0.16)
Serine	0.17 (0.11)	0.54 (0.52)	0.20 (0.17)
Threonine	0.17 (0.54)	0.37 (0.32)	0.12 (0.14)
Glycine	0.18 (0.20)	0.48 (0.57)	0.28 (0.25)
Alanine	0.06 (0.12)	0.34 (0.69)	0.15 (0.14)
Valine / Leucine/ Isoleucine	0.09 (0.99)	0.17 (0.39)	0.27 (0.22)
Phenylalanine	Trace -	Trace (Trace)	0.13 (0.24)
Lysine	0.02 (0.21)	0.06 (0.10)	0.03)Trace)
Total	0.79 (2.57)	2.02 (2.80)	1.42 (1.32)

The values given in brackets represent results from a similar experiment carried out under identical conditions,

and are included to show the variations which occur in the composition of non-protein nitrogen fractions. The results show that free α-amino groups present in non-protein nitrogen are considerably greater than those which are available for reaction in the insoluble collagen. (cf. Table XXII). The apparent N-terminal residues of the collagen are only slightly decreased by the removal of the non-protein nitrogen.

SECTION 4

D I S C U S S I O N

As reported in section 1, the apparent inertness of native collagens towards proteolytic enzymes has been recognised for a long time, and although the Clostridial collagenases extensively degrade native collagens, no other enzyme had been found which was capable of such a degradation. However it was known that fibrous collagen was extensively destroyed in certain physiological conditions, e.g. in the uterus after parturition (Harkness and Moralee 1956). This suggested that enzymic processes were involved, although until fairly recently no mammalian enzymes had been found which had any effect upon native collagen. However more recent work (reviewed in section 1) has revealed that proteolytic enzymes, of both mammalian and non-mammalian origin, other than Clostridial enzymes, do in fact have an effect upon native fibrous collagens. The effect of these enzymes, although slight, and producing very little change in the basic structure of the collagen macromolecule, produces large changes in the aggregation and solubility properties of collagen, and has been used as evidence for the existence of end chains in the collagen molecule, as postulated by Boedkter and Doty (1955). (Schmitt (1963) in a recent review has proposed the name

telopeptides for these end-chains).

The effect of proteolytic enzymes on collagen is of interest from several different aspects:-

(i) Enzymes form useful tools in the examination of protein structure. This aspect of enzymic degradations has been reported by Harrington, von Hippel and Mihalyi (1959).

(ii) Studies of the effect of enzymes on collagen may provide further knowledge of the processes involved in the *in vivo* metabolism of collagen.

(iii) A possible industrial aspect in the production of gelatines. The present commercial production requires a prolonged chemical pretreatment of collagenous material, prior to thermal extraction of gelatin. Although chemical pretreatment has been employed for a long time, the actual chemical changes involved cannot be very readily controlled. An enzymic process, in which non-collagenous proteins were completely degraded, and the collagenous proteins attacked to a limited extent, but to such a degree that the material is more readily extracted as gelatine, might be of great use. An enzymic process of the type described would have the advantage that it would take much less time than the 6 - 8 weeks required for chemical pretreatment. In addition to this, two other considerations are of importance. The

source of enzyme must be inexpensive and readily available, and the gelatine produced must be of a sufficiently high grade.

In the work reported in this thesis a few enzymes, in particular the protease of Streptomyces griseus (PRONASE) have been examined for collagenolytic activity, and their effect upon collagen has been examined by a variety of means, both chemical and physical.

The enzyme Pronase was examined initially since reports of the activity of this enzyme suggest that, in addition to being extremely unspecific, it is one of the most powerful proteolytic enzymes so far reported. (Nomoto and Narahashi 1959; 1960). In view of this it was considered possible that Pronase might degrade collagen, where enzymes having less powerful proteolytic activity were inactive.

Although Pronase as used in this work was reported to be purified, chromatography (fig. I) revealed that the product was not a single compound, but consisted of at least four components. In view of this, each of the four fractions was examined initially for proteolytic activity. The significance of the four fractions will be discussed later.

Viscosity studies (fig. II) reveal that crude Pronase effects a profound reduction in the viscosity of gelatine solutions, which is to be expected in view of the reported activity of the enzyme. It also produces a smaller, although still extensive reduction (approximately 35%) in the viscosity of solutions of undenatured collagen, at both acid and neutral pH values. Similarly the isolated enzyme fractions (fig. IV and fig. V) reduce the viscosity of collagen solutions, although, as the graphs show, to a slightly less extent than the crude enzyme. The viscosity reduction differs for the different enzyme fractions, fraction I having the greatest effect, at both acid and neutral pH's. Since viscosity is a function of the axial ratio of the solute particle, the reduction in viscosity represents a decrease in axial ratio, i.e. a decrease in the length of the particle. This has been widely used as a criterion of enzymic hydrolysis (e.g. by Seifter, Gallop and Meilman (1958). Hodge et al. (1960) have shown that trypsin will reduce the viscosity of collagen solutions, and this reduction in viscosity has been attributable to cleavage of interlinking end-chains, resulting in the depolymerisation of linear polymers present in solution. The presence of polymers of the tropocollagen molecule in solutions has been reported by Hodge et al. (1960) and by

Boedkter and Doty (1955). Pepsin is reported to have the same effect as trypsin, and Nishihara and Miyata (1962) have shown that pepsin reduces the viscosity of collagen solutions by 15%. In this work the effect of enzymes on viscosity has been compared with the effect of trypsin. Figs. II and VIII show trypsin produces a 15 - 17% viscosity reduction, a value which compares with that of Nishihara. The greater viscosity reduction produced by Pronase would suggest that this enzyme causes more extensive hydrolysis than either pepsin or trypsin.

The effect of sisal enzymes, hyaluronidase, and pectinesterase on the viscosity of collagen solutions is much smaller than that produced by trypsin (figs. VI, VII and VIII), and it would seem therefore that these enzymes do not produce depolymerisation. Gross (1957) also reported a small reduction in viscosity using hyaluronidase, but did not comment on its significance. Although the reduction reported here was extremely small, it was consistent and repeatable. It is possible that these enzymes do in fact attack the end-chains, but only those end-chains which are free and not involved in inter-molecular links, and are therefore probably more susceptible to enzymic attack. Cleavage of such free end-chains might account for the small viscosity drop observed.

Other physical studies were carried out on the enzymic hydrolysis of soluble collagen to give an indication of the extent and nature of the attack. pH-Stat investigations using Pronase (Table II, fig. X) show that 5 - 10% of the total peptide bonds of collagen are hydrolysed, which might suggest fairly extensive breakdown of the collagen structure. However, such experiments give no indication of the location of the hydrolysed bonds within the collagen molecule.

The high optical rotation of collagen solutions is a manifestation of the intact triple helix configuration. Fig. IX shows that the enzymes examined have no effect upon optical rotation, indicating that the helical portion of the molecule is not degraded by the action of these enzymes. In the case of sisal enzyme and hyaluronidase this is to be expected in view of the very slight viscosity reductions produced. The fact that no degradation of the helix occurs with Pronase is highly indicative of peptide bond hydrolysis solely in the end-chain regions of the molecule.

Table VI shows the free α-amino groups of acid-soluble collagen and of collagen after reaction with Pronase. Bowes, Elliot and Moss (1955) detected only trace amounts of two N-terminal amino acids (aspartic acid and alanine) in acid-soluble collagen. In this work 10 amino acids were

found as apparent N-terminal groups in significant amounts, and a further three N-terminal amino acids were detected in trace amounts. This large number of apparent N-terminal amino acids has previously been reported by Steven, Tristram and Tyson (1961), and Steven and Tristram (1962), and an explanation has been put forward that at least some of these apparent N-terminal amino acids may be due to physically associated non-protein-nitrogen. As is shown in Table VI treatment with Pronase increases greatly the number of free α-amino groups available for reaction with FDNB, this being further evidence that cleavage of peptide bonds is brought about by the enzyme. The absence of proline and hydroxyproline as N-terminal amino acids after enzyme treatment reveals that bonds involving these amino acids in the helical portion of the molecule are not attacked. The value for the average chain length reported for the products of hydrolysis is misleading since further results, VII(iv) and Table XIXa show that substantial amounts of free amino acids are produced as a result of the action of Pronase on collagen.

Table III reveals that 5 - 8% of the total nitrogen of a phosphate extract of skin is reduced to a non-protein nitrogen form (soluble in 90% acetone) by treatment with Pronase. The origin of this non-protein nitrogen, however,

is uncertain, since the phosphate extract contains blood proteins and other tissue proteins in addition to neutral salt soluble collagen. Tables IV, X and XVIII show that a similar amount (6-7%) of the total nitrogen of acid soluble collagen is reduced to a non-protein nitrogen form (dialysable or acetone-soluble). In view of the results discussed so far which suggest that Pronase is merely cleaving in the region of the end-chains the percentage of non-protein nitrogen produced is rather high, since single end-chains at each end of the postulated dimensions (100-200Å at each end; Hodge and Schmitt, 1958, 1960) if completely removed would account for only about 3% of the total nitrogen. These results would therefore suggest that there is more than a single end-chain, at least at one end of the tropocollagen molecule, and that probably complete removal of these is effected by Pronase. The amount of NPN produced by enzyme autodigestion may be ignored since the ratio of enzyme to substrate was small, and the enzyme solution was dialysed previously, thus removing most autodigestion products. The amount of nitrogen removed by sisal enzyme (Tables III and X) is smaller, suggesting incomplete cleavage of end-chains.

The results reported in Table X show the percentage of some of the constituents of soluble collagen released

as dialysable non-protein nitrogen by treatment with enzymes. It is seen that although Pronase reduces a significant percentage of the total nitrogen to a dialysable form, only a very small percentage of the total hydroxyproline of the collagen is rendered dialysable. The low hydroxyproline:nitrogen ratio of the dialysable fraction, compared with that of whole collagen, constitutes further evidence that only the extrahelical regions of the tropocollagen molecule are attacked by Pronase. The presence of small amounts of hydroxyproline in the end-chains has also been reported by Hodge et al. (1960), but Rubin et al. (1963) found no hydroxyproline in the end-chain material liberated by pepsin. The dialysable peptides from Pronase digestions also contained hexose and hexosamine. The presence of hexose is not of significance in this context since a similar percentage of the total hexose is found in the dialysable fraction from collagen which had not been treated with enzyme. The presence of hexosamine however is of greater significance, since, although hexosamine is found in the dialysable fraction of the control experiment, additional hexosamine is liberated by enzyme treatment. The presence of hexosamine has not been reported in analyses of end-chain material released by trypsin and pepsin (Hodge et al. (1960); Rubin et al.

(1963)), but Kühn, Kühn and Hannig (1961) have shown that trypsin reduces the hexosamine content of collagen, although these authors do not consider that trypsin attacks any part of the collagen molecule itself, but merely acts upon associated proteins.

The dialysable products of sisal enzyme digestion are similar to those of the Pronase digestion, but the degradation produced by sisal enzyme is shown to be less extensive, as smaller percentages of nitrogen, hydroxyproline and hexosamine are released.

The results of amino acid analyses carried out on soluble collagen and collagen treated with enzymes and precipitated by dialysis are recorded in Table XI. These results reveal that digestion with Pronase completely eliminates tyrosine from the collagen molecule, which indicates that tyrosine residues are found in the end-chain regions of the collagen molecule. The presence of tyrosine in the end-chains has also been indicated by the work of Hodge et al. (1960) and Rubin et al. (1963). Kühn, Kühn and Hannig (1961) have shown that treatment with trypsin reduces the tyrosine content of collagen, but unlike Hodge et al. they do not consider the tyrosine as being present in end-chains or any part of the collagen molecule, but simply as part of an associated non-collagenous protein, as in the case of the hexosamine. In addition to eliminating

tyrosine from collagen digestion with Pronase significantly reduces the content of glutamic acid, which is in accordance with the work of Hodge et al. (1960), who showed that trypsin released an acidic peptide which contained glutamic acid. The presence of glutamic acid in the end-chains has also been shown by Rubin et al.(1963). Digestion with Pronase also reduces the collagen content of serine and threonine. The peptide material produced by treatment with trypsin (Hodge et al.) also appears to contain these amino acids in fairly large amounts, but the peptide fraction released by pepsin (Rubin et al.) does not contain very large amounts of these amino acids. This would suggest that pepsin and trypsin to some extent might cleave different end-chains, although the presence of tyrosine in both peptide fractions suggests overlapping of the sites of attack of these two enzymes. Pronase would then hydrolyse the end-chains in the same regions as both pepsin and trypsin, and since the amounts of serine and threonine released is very high it seems probable that further cleavage at yet another site is occurring - possibly hydrolysis of bonds in an end-chain which is not attacked by pepsin or trypsin. In view of the very wide specificity reported for Pronase (Nomoto, Narahashi, Murakami, 1960) compared with the specificities of pepsin and trypsin, such additional hydrolysis is not unlikely.

The most significant changes in the amino acid composition of collagen resulting from treatment with sisal enzyme are a reduction in the content of serine and threonine, as in the case of Pronase treated collagen. However, unlike Pronase, sisal enzyme does not effect a reduction in the glutamic acid or tyrosine content of the collagen. This is suggestive that the site of attack of the sisal enzyme is only partly the same as the sites attacked by Pronase. However, in addition, sisal enzyme also appears to attack a part of the molecule which is not attacked by Pronase, since sisal enzyme effects a reduction in arginine which is not the case with Pronase. The reduction in arginine suggests that sisal enzyme attacks the end-chain containing basic residues, which would also seem to be attacked by trypsin, since Hodge et al. (1960) have shown that trypsin splits off significant amounts of arginine. However, since tyrosine is not released by sisal enzyme it is obvious that the extent of hydrolysis produced by sisal enzyme is less than that produced by trypsin. This is in accordance with the results of viscosity studies.

Treatment of soluble collagen with Pronase facilitates the ease with which collagen can be converted to gelatin on warming in water (Table V). The conversion of collagen

to gelatin has been shown to require cleavage of peptide bonds and some lateral bonds, in addition to rupture of the hydrogen-bond system. (Ward, 1960). Since the action of Pronase does not result in rupture of the hydrogen bonding, as revealed by the lack of change in optical rotation on enzyme treatment, it is apparent that the peptide bonds cleaved by Pronase, i.e. those in the end-chain regions, are important in the maintenance of the structural integrity of the collagen molecule. This does not eliminate the possibility that some lateral bonds are cleaved and the results reported in section XI reveal that this is indeed the case. It is probable that these bonds are normally cleaved in the pretreatment stage of gelatin production.

At this juncture it is convenient to discuss the sub-units of the collagen molecule. The fractionation pattern of thermally denatured collagen, shown in fig.XI(a), reveals four components, and the relative proportions of these four components (Table XIII) correspond closely to those reported by Worrall, Tristram and Steer (1963). Fractions I and II correspond to the α-component and β-component respectively of Piez, Weiss and Lewis (1960). Worrall, et al. (1963) consider that the IIIrd component consists of three α-components linked together by hydrogen

bonds, and that the IVth component, at the denaturation temperature employed here, is heterogeneous, consisting of (a) an α-component and a β-component covalently linked together, i.e. the γ-component (Grassmann, Hannig and Engel (1961)) and (b) an α-component and a β-component weakly linked together.

Piez, Weiss and Lewis (1960) have suggested that the separation of collagen sub-units on carboxy-methyl cellulose is dependent upon the different molecular sizes of these units; the components of lower molecular weight being eluted first. However, subsequent work, (e.g. Piez, Eigner and Lewis (1963)) where the β-components are eluted prior to the smaller α_2-component, is not in accord with a separation based solely on molecular size. In addition the structures proposed by Worrall et al.(1963) for components III and IV indicates similar molecular sizes for these components, and separation of such components would not be achieved by a fractionation dependent only on size. The additional factor involved in the fractionation is presumably ionic binding, due to interaction of charged groups of the protein sub-units with the CM-Cellulose.

According to Worrall et al. (1963), component IV contains covalent links between its constituent α-chains,

whereas component III does not. The formation of such covalent links between charged groups would result in components III and IV having different ionic binding capacities, and account for their separation.

Treatment with enzymes, which would result in the liberation of charged groups, would alter the binding capacity of a component and consequently result in a change in the fractionation pattern. In addition, enzymic cleavage of a link binding sub-units together would result in the production of components of smaller size and this could also result in an alteration of the fractionation pattern.

As may be seen from the results (fig.XI; Table XIII), treatment of collagen with Pronase or trypsin produces similar changes in the fractionation pattern. Both enzymes effect a reduction in the amount of component IV, the reduction being most marked in the case of the Pronase-treated material. This reduction in fraction IV results in an increase in the initial fraction which, unlike that formed by denaturation of untreated collagen, consists only of a single component, the β-component apparently being broken down to produce α-component. This indicates that the bonds linking two α-components as a β-component lie in the region of the molecule which forms the telopeptides of the tropocollagen molecule, since the enzymes attack

tropocollagen only in this region. This has also been shown by Rubin *et al.* (1963) who found that treatment of collagen with pepsin increased the ratio of α-component to β-component on denaturation.

In the case of the Pronase-treated material however some of the original IVth component appears as IIIrd component, which does not occur in the case of trypsin-treated material. This could be the result of differences in the proteolytic attack of the two enzymes, such that hydrogen-bonding between the products can occur in the case of the Pronase-treated material (resulting in the formation of additional IIIrd component of the structure proposed by Worrall *et al.* (1963)) but not in the case of trypsin-treated material. This however would seem unlikely since results, e.g. viscosity studies, show that Pronase produces more extensive degradation than trypsin.

However, since there is no molecular weight data available for components III and IV, it cannot be discounted that they may differ in size, probably with component IV being larger than component III. Component III could be three α-units linked together by covalent bonds, i.e. γ-component. This would seem possible in view of the fact that Piez, Eigner and Lewis (1963) have reported the presence of small amounts of γ-component eluted at the

end of their α_2-component, i.e. in a comparable position to that of the IIIrd component in the separation reported here. This does not exclude the possibility that the IIIrd component might consist, at least in part, of three α-units linked by hydrogen bonds, since in a separation based mainly on molecular size (which cannot be excluded in the present work) such a component would be eluted together with a γ-component.

Component IV, being larger than component III could then be visualised as consisting of aggregates of tropocollagen monomers. Collagen solutions have been shown to contain such aggregates (Boedkter and Doty (1955); Hodge et al. (1960)) and Jackson and Bentley (1960) have suggested that solutions of collagen will contain a spectrum of such aggregates of increasing age and strength of interlinking. The mild heating employed as a means of denaturation in this work would probably depolymerise those aggregates which are only weakly linked, leaving intact those aggregates which are more strongly interlinked, which would then appear as component IV.

The reduction in the amount of component IV brought about by treatment with Pronase and trypsin is envisaged as the result of enzymic depolymerisation of these aggregates. This would seem to be in accordance with the reduction in

viscosity of collagen solutions brought about by these enzymes, which is also the result of depolymerisation of aggregates of tropocollagen units. In this respect it is probably significant that Pronase, which effects a greater reduction in component IV than trypsin also produces a greater reduction in viscosity.

It is considered that, of the spectrum of aggregates of increasing strength of cross-linking comprising component IV only the more weakly interlinked aggregates are depolymerised by trypsin. This then results in the formation of monomers which on thermal denaturation give rise to only α-units, the constituent β-units having been broken down to α-units by the action of the enzyme. The increased degradation brought about by Pronase, would in addition to this, depolymerise more strongly cross-linked aggregates. Some of these aggregates, in addition to having stronger intermolecular links, might also contain monomers with stronger and more extensive intramolecular links. On thermal denaturation these monomers (γ -units) would not dissociate and would thus account for the appearance of additional component III.

The presence of hexosamine in the end-chains as suggested from results in Table X, and discussed later, might be of significance since Orekhovitch et al. (1960)

have suggested that the bond linking two α-components as a β-component is an ester link. Pectinesterase however does not appear to convert the β-component into α-component (fig. XI(f)) and also sisal enzyme releases the same amount of hexosamine as Pronase, but does not convert β to α, suggesting that hexosamine is not involved in the link.

Neither hyaluronidase nor sisal enzyme have any significant effect upon the sub-unit composition of the collagen. In view of previous results, the lack of activity of hyaluronidase is to be expected. Since sisal enzyme does not cause any alteration in the fractionation pattern, although it does bring about slight proteolysis it seems that proteolysis by sisal enzyme must be limited to the extreme termini of the end-chains at a point external to the site of the bond linking α-components together into a β-component.

As all the enzymes which convert the β-component to α-component (Pronase, trypsin and pepsin) also release tyrosine from collagen it would seem possible that tyrosine might be involved in the cross-link.

From the results discussed so far it is apparent that Pronase has a comparable effect to trypsin, but it would seem that the effect of Pronase is more extensive.

Hodge et al. (1960) reported that treatment of collagen with trypsin prevented the thermal gelation of the collagen solutions, i.e. inhibited the formation of native fibres as a result of removal of end-chains. However, Kühn et al. (1961) have reported that trypsin-treated collagen is capable of forming native-type fibres, and similar results have been reported by Itoi (1961) for pepsin-treated collagen. The results reported in Table XII show that collagen treated with either trypsin or Pronase is still capable of thermal gelation, which would appear to be in accordance with the results of Kühn et al. and Itoi. However, it was not possible to determine whether or not the fibres formed had the native spacing of 700Å, but it is possible that some native-type fibres are present, since a part of the IVth component remains unaltered by treatment with these enzymes, and this would presumably be capable of producing native fibres. Another possibility is that, even if the unchanged material is not capable of producing native fibres, it would act as a nucleus onto which tropocollagen monomers, after removal of end-chains, would bind by lateral interaction and in this way produce a gel.

The results discussed so far consider the action of enzymes only upon soluble collagen, and many workers, e.g.

Mandl (1961) have suggested that native insoluble collagen is a much more suitable substrate.

Preliminary experiments involving the use of the congo-red derivative as substrate reveal that the enzymes have some action upon insoluble collagen. The results shown in Table VII reveal that the action of Pronase upon insoluble collagen results in the solubilisation of a certain amount of nitrogen, and since hydroxyproline is also solubilised the nitrogen must be of collagenous origin. The results reported in section IX.1.(b) show that the effect of Pronase on insoluble collagen is more extensive than is suggested by the results shown in Table VII, since lowering of the pH results in a much greater amount of the collagen being solubilised. Nishihara (1960) has shown that trypsin also has this effect and it is due to the enzyme cleaving the end-chains which link the tropo-collagen monomers in end-to-end aggregates. Since Pronase has been shown to have the same action as trypsin in that it hydrolyses in the end-chain region the same mechanism is active. Enzymic cleavage of the end-chains converts the linear polymers into monomeric units which are still extensively crosslinked, by lateral ionic links involving the ionic side chains of the polar amino acids, and thus remain insoluble. Bensusan *et al.* (1960, 1962) have shown

the involvement of the phenolic hydroxyl group of tyrosine and the ionic side-chains of basic amino acids in the formation of crosslinks necessary for fibre formation. In the present discussion the contribution of tyrosine can probably be ignored since this amino acid appears to be present only in the end-chain regions and would consequently be removed by enzyme action (although the amino acid analyses reported in Table XVI reveal that some of the tyrosine does in fact remain). Suppression of the ionisation of these groups by lowering the pH results in the weakening of these lateral links and consequent solubilisation of the tropocollagen monomers.

Similar experiments using sisal enzyme (IX(ii)) reveal that this enzyme has very little activity on insoluble collagen, which is to be expected since digestions using soluble collagen as substrate reveal that interlinking end-chains are not hydrolysed to any extent.

The fact that Pronase and some other enzymes, e.g. trypsin, solubilise collagen at neutral pH's is of interest since it was considered for a long time that collagenolysis by enzymes other than collagenase did not occur unless the collagen was swollen, e.g. by exposure to acid pH's. Sherry, Troll and Rosenblum (1954) had suggested a two-stage mechanism for the solubilisation of collagen by pepsin,

the first stage consisting of swelling caused by exposure to low pH's, and the results of Grassmann et al.(1937) are in accord with this, since inhibition of swelling, by addition of salt, prevented digestion of collagen by trypsin. Various claims have been made that enzymes would attack collagen at neutral pH's, e.g. Grant and Alburn (1960), but these digestions were carried out in the presence of calcium chloride, which is a lyotropic agent having a similar swelling effect to that caused by acids.

Table XIV shows the N-terminal amino acids found in insoluble collagen. Bowes and Moss (1951) detected no N-terminal amino acids in ox-hide insoluble collagen, but 10 species of N-terminal residue are found here. (See also Steven, Tristram and Tyson (1961)). All of these amino acids were also found as N-terminal residues in soluble collagen (Table VI), but the three additional N-terminal residues detected in trace amounts in soluble collagen were not detected in insoluble collagen. This however does not exclude the possibility that they are present - but in amounts too small to be detected. The significance of these N-terminal residues will be discussed later in relation to the non-protein nitrogen component of insoluble collagen.

The results of N-terminal analyses of Pronase-collagen

digests (Table XIV) shows a large increase in the number of N-terminal residues, as a result of proteolysis, but no new species of N-terminal amino acid is produced. However, the apparent reduction in chain length suggested by these results is misleading (as was also the case with digests of soluble collagen) since further results (fig. XIV) reveal the presence of considerable amounts of free amino acids.

Table XV shows that insoluble collagen is extensively solubilised by crude Pronase, and fractions I and III, at an acid pH. Solubilisation to this extent at neutral pH's does not occur, for reasons already discussed. As in the case of soluble collagen digests approximately 6% of the total nitrogen is reduced to a dialysable form, but the percentage of the total hydroxyproline which is dialysable is once again extremely low, indicating proteolysis in the end-chain regions only. The dialysable fraction after enzyme treatment also contains hexose and hexosamine (cf. soluble collagen). The non-protein nitrogen of the control contains no hexosamine, unlike the non-protein nitrogen of soluble collagen. This is probably due to a certain amount of the hexosamine present in collagenous material being non-collagenous in origin, as suggested by Kühn, Kühn and Hannig (1961). This non-

collagenous hexosamine would appear to be completely removed by the exhaustive extraction procedure used to prepare insoluble collagen, while this is not the case with soluble collagen, where some of the hexosamine is retained, possibly being co-precipitated with the collagen during the purification. Additional hexose is found in the non-protein nitrogen fraction after treatment with Pronase, which does not occur in the case of digests with soluble collagen. However in view of the variations in hexose content reported in this experiment this may not be of significance.

Tabulated in Table XVI are the results of the amino acid analysis of the dialysable material, i.e. the amino acid composition of the material liberated from the end-chains. The content of tyrosine is high, in agreement with previous results, and large amounts of glycine and alanine are also present. Hydroxyproline appears to be absent, but Table XV reveals that small amounts are present and are detectable by the more sensitive colorimetric analysis method. Proline, leucine, aspartic acid and glutamic acid are present in considerable amount, in agreement with the results of Rubin et al.(1963). The values of serine, threonine, (cf. soluble collagen digests) lysine, histidine and methionine (and proline) are much

higher than the values reported by Rubin et al. (1963), and this would appear to be a further indication that digestion with Pronase results in more extensive degradation of end-chains than does pepsin.

The presence of both hexose and hexosamine in the soluble non-dialysable fraction in these experiments (and also in the case of soluble collagen digests) would suggest that these carbohydrate components are an integral part of the triple-helix "body" of the collagen molecule. In view of the results of Kühn, Kühn and Hannig (1961) who found that treatment with trypsin effected almost complete removal of hexosamine, the presence of this component after Pronase treatment is difficult to account for. In the case of soluble collagen it can be seen that removal of tyrosine is not complete, suggesting that the reaction may not have gone to completion, and hence all the hexosamine might not be removed. However, it is possible that a portion of the end-chain containing hexosamine which is degraded by trypsin is not attacked by Pronase.

Chromatography of the dialysable fraction after hydrolysis (figs. XII and XIII) reveal the presence of glucose, galactose and possibly fucose. Since no hexose component was detected chromatographically prior to hydrolysis of the dialysable fraction, it would seem that the hexose is present

in a bound form in the end-chain and is not just fortuitous material, and as the reagents used to detect the sugars on chromatograms are dependent upon free reducing groups, it is suggestive that the reducing group of the hexose component is involved in some linkage. The hexose could be linked either to an amino acid, or could be present as a non-reducing oligo-saccharide which is then bound to protein by an ester link. The latter suggestion would seem more probable in view of the findings of Konno and Altman (1968) who reported the isolation of a polysaccharide-glycine complex from muscle collagen, the polysaccharide being linked to a C-terminal amino acid, presumably of the end-chain.

As would be expected the hexoses which are not dialysable also do not appear on chromatograms prior to hydrolysis of the non-dialysable fraction. It is again possible that the hexoses are present as a non-reducing polysaccharide, but Hörmann (1960) has suggested that in mature collagen the hexoses are linked to protein via ester links and through links involving the reducing group. It would appear therefore that the carbohydrate component in the end-chains are linked differently to those in the helical portion of the molecule. Gustavson (1956) stresses the importance of using native collagen, which

has not been swelled in preparation, as substrate for enzymic digestions, and Mandl (1961) states that pre-treatment of collagen with acid increases its susceptibility to attack by trypsin. It was of interest therefore to examine the effect of Pronase upon skin which had not previously been treated with acid. Results (Table XVII) reveal that Pronase has a similar effect upon this material to its effect on insoluble collagen, indicating that the prolonged acid treatment required to produce insoluble collagen does not increase its susceptibility to proteolysis by Pronase.

In the experiments reported in XIII some dialysable and acetone soluble peptides from collagen-Pronase fraction I digests are examined. Table XIX shows that the dialysable material contains large amounts of free amino acids and also peptides, which contain large amounts of imino acids. This is not consistent with the results of other digestions where the dialysable fraction contains very little hydroxyproline. However, since the soluble material was reduced in volume at 40°C prior to dialysis it is probable that the collagen was denatured, allowing complete proteolysis, and hence the reduction of the helical portion of the molecule to peptides of dialysable size.

It is of interest to note that in this experiment

acidification of the collagen solution resulted in almost complete precipitation of the collagen nitrogen. In the enzyme treated collagen, however, even taking into account the dialysable nitrogen produced by proteolysis, the amount of the total nitrogen precipitated by acidification is significantly less than in the control. This is probably a manifestation of the alteration of aggregation properties suggested by Hodge et al. (1960) to be the result of end-chain hydrolysis, and which was not detected here by the thermal gelation tests (Table XII).

Examination of acetone soluble material also reveals significant amounts of free amino acids (Table XIX) and several peptides (figs. XVI and XVII). In this case no hydroxyproline was found in the peptides examined, confirming that the hydroxyproline found in the dialysable material described above is a result of enzymic attack on denatured collagen.

As may be observed from the results reported, the various Pronase fractions appear to have similar effects upon collagen, i.e. they reduce the viscosity of solutions of collagen and effect solubilisation of insoluble collagen, as a result of hydrolysis of the end-chains. The degree of activity varies for the different fractions, with fraction I being the most active. Table I shows that this

fraction is also quantitatively the most predominant. It has also been shown that a large proportion (almost 50%) of the total nitrogen of the commercial enzyme can be removed as non-protein-nitrogen by dialysis of a solution of the enzyme. Nomoto and Narahashi (1959(d)) reported that the enzyme is extremely unstable during dialysis, and it is probable that the non-protein-nitrogen found here are the result of autodigestion. In view of this it would seem likely, since all the fractions have a similar effect upon collagen, that the different fractions are products of autodigestive processes which still retain enzymic activity, with fraction I being the undegraded enzyme, or the least degraded fraction.

Fig. III shows that removal of the non-protein-nitrogen by dialysis greatly decreases the activity of the enzyme, but since re-addition of the dialysable material to the enzyme does not restore activity it is apparent that loss of activity on dialysis is not due to removal of a cofactor. Since large decreases in activity do not occur when the enzyme is not dialysed, it appears that the non-protein-nitrogen products of autodigestion act as inhibitors of autolysis. According to Nomoto and Narahashi (1959(d)) loss of activity during dialysis can be prevented by addition of calcium ions to the enzyme solution, which

would suggest that these ions inhibit enzyme activity, thereby preventing autodigestion. Calcium however does not prevent digestion of collagen by Pronase, since certain digestions here have been carried out in the presence of calcium.

Of the enzymes examined in this work, only Pronase and its fractions have any significant collagenolytic activity. The effect of Pronase on collagen is such that it is possible that the enzyme could be utilised to pretreat collagenous material prior to its conversion to gelatine. Nomoto and Narahashi (1959(d)) suggest that Pronase will hydrolyse almost all non-fibrous proteins, in which case it will effect a removal of non-collagenous impurities. In addition it has been found here that treatment of collagen with Pronase, without producing major changes in the collagen structure, enhances the ease with which the collagen can be converted to gelatine on warming. Since the enzyme is reported to be secreted by Streptomyces griseus, extraction and purification of the enzyme for pretreatment should not be necessary, and it might be possible to pretreat the collagen merely by treatment with a culture of the organism.

The results reported in Table XX (see also Steven, Tristram and Tyson, 1961) reveal that a non-protein-nitrogen

(N.P.N.) fraction can be removed from insoluble collagen by dialysis or acetone precipitation. This N.P.N. accounts for less than 1% of the total collagen nitrogen, compared with the 2% of the total nitrogen which can be removed from soluble collagen (Steven and Tristram, 1962).

Chromatography of the N.P.N. material (fig. XVI(i)(ii)) shows that it contains 10 free amino acids, which differ slightly from those found in soluble collagen N.P.N. in that tyrosine is absent, but traces of lysine are present. In addition to free amino acids the N.P.N. also contains peptide material. Chromatographic examination of N.P.N. after treatment with FDNB (fig. XIX) reveals the same 10 free amino acids together with trace amounts of a further three (tyrosine, proline and hydroxyproline) frequently occurring.

The peptide material on hydrolysis is found to contain 9 amino acids, differing from the N.P.N. peptide material of soluble collagen only in that phenylalanine is absent. Chromatography of the DNP-peptides (fig. XX(i)(ii)) reveals that there are 3 DNP-peptides, two of which are soluble in ethyl acetate. This constitutes another difference from the peptides in soluble collagen N.P.N., where of the 3 DNP-peptides found, only one was soluble in ethyl acetate.

In addition to amino acids and peptides the N.P.N. examined here contains a significant amount of a hexose

component, and possibly a trace amount of hexosamine (14, iv). Hexose is also found in the N.P.N. of calf-skin insoluble collagen (Table XV(i)) and both hexose and hexosamine in the N.P.N. of soluble collagen (Table X).

N-terminal analysis of ox-skin insoluble collagen reveals the presence of 10 apparent N-terminal residues (Bowes and Moss (1951); Bowes, Elliot and Moss (1957), found no N-terminal amino acids in ox-skin collagen) and the same N-terminal residues were found in calf-skin insoluble collagen (Table XIV) and calf-skin procollagen (Table VI; Steven and Tristram, 1962). The amino acids found as N-terminal residues in insoluble collagen are the same as the free amino acids found in the N.P.N. material, as is also the case with soluble collagen, and Steven and Tristram (1962) have suggested that the free amino acids of the N.P.N. account for the apparent N-terminal residues found in soluble collagen. It is probable that this also applies in the case of insoluble collagen.

Since the total free α-amino groups of N.P.N. and residual collagen, after removal of N.P.N., much exceed the apparent N-terminal residues of untreated insoluble collagen, and in addition removal of N.P.N. does not produce a corresponding decrease in the apparent N-terminal residues of collagen (cf. Tables XXIII and XXI), it would seem that

some of the free amino acids which can be removed as N.P.N. are not available for reaction with FDNB prior to removal. Steven and Tristram (1962) reported similar findings with respect to soluble collagen, and also found that the N.P.N. could only be removed from soluble collagen at extremes of pH, and as a result of these findings have concluded that the N.P.N. is physically bound to collagen by ionic links. It is found here, however, (Table XX) that N.P.N. can be removed from insoluble collagen at neutral pH's and thus there is insufficient evidence to state conclusively whether or not the N.P.N. is in fact physically bound to collagen or is present merely as fortuitous material. However, considering the large number of extractions required to produce insoluble collagen it is likely that fortuitous material would be removed from the collagen, making the former possibility more probable. In this respect it is of interest to consider the differences between the N.P.N. of soluble and insoluble collagens, consisting of a difference in the peptides, and the absence of hexosamine from the N.P.N. of insoluble collagen. It is possible therefore that the N.P.N. component of soluble collagen which contains hexosamine is fortuitous material, which is removed by the exhaustive extraction required to produce insoluble collagen, but is

retained with the soluble collagen as a result of co-precipitation.

Although the origin of the N.P.N. fraction is uncertain and may not be of significance as regards the integrity of collagen, its presence is important in work where it is required to examine small amounts of nitrogenous material released by enzymic hydrolysis. In this respect it is essential to perform adequate controls to ensure that the N.P.N. produced is in fact the result of enzymic hydrolysis.

S U M M A R Y

1. The literature on collagen has been reviewed, with especial emphasis on structural aspects of collagen and the collagenolytic effects of enzymes.

2. The proteolytic enzyme of Streptomyces griseus (Pronase) has been examined and shown to consist of four fractions, each having proteolytic activity.

3. The collagenolytic effect of the above enzyme and its fractions, and to a lesser extent of other enzymes has been examined by a variety of methods, both physical and chemical, using both soluble and insoluble collagen as substrates.

4. Pronase has been shown to have a collagenolytic effect on collagen in both the soluble and insoluble states, and the products of collagenolysis have been examined.

5. The action of Pronase has been shown to result in peptide bond cleavage only in the terminal regions of the collagen molecule, resulting in the liberation of small peptides and free amino acids, while leaving the body of the molecule intact. Fractionation of Pronase-treated collagen after denaturation showed

that the three constituent chains of the collagen molecule had been separated, indicating that the enzyme is also affecting lateral cross linkages.

6. An extract of sisal has been shown to have a proteolytic effect using gelatine as substrate, but a very slight collagenolytic effect, which is limited to collagen in solution.

7. Insoluble collagen has been shown to contain a small quantity of non-protein-nitrogen material which can be removed by physical methods, and which consists of free amino acids, small peptides and a carbohydrate component.

B I B L I O G R A P H Y

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