ENZYMATIC HYDROLYSIS OF CALF SKIN AND PIG SKIN TROPOCOLLAGEN

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THESIS

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ABSTRACT

ENZYMATIC HYDROLYSIS OF CALF SKIN AND PIG SKIN TROPOCOLLAGEN

by Gary A. Crevasse

The objective of this investigation was to study the action of various proteolytic enzymes upon acid-soluble calf and pig skin collagen. The enzymes used were pepsin, trypsin, chymotrypsin and elastase.

The effects of these enzymes on acid-soluble calf skin collagen were evaluated using the techniques of high voltage paper electrophoresis and paper chromatography to separate the products of enzymatic digestion. All of the enzymes produced dialyzable tyrosine containing components that were largely acidic in nature. Trypsin treatment produced the largest number of tyrosine containing materials followed by pepsin and chymotrypsin and finally elastase. Other components were also produced by enzymatic action, but the number and amount varied. The dialysis tubing was shown to be contributing dialyzable material to the chromatographic pattern. The origin of this material was not pursued.

Changes in the protein subunits were monitored using disc gel electrophoresis. Chymotrypsin and elastase treatment significantly changed the banding patterns. Pepsin had less effect, while trypsin treatment had little effect. These results indicated that chymotrypsin, elastase and pepsin directly affected the intramolecular cross-links in the collagen molecule. All enzymes were able to disrupt intermolecular cross-links as shown by the absence of protein aggregates on top of the disc gels.

The proteolytic enzymes were also effective in preventing or decreasing the ability of the protein to aggregate during thermal gelation.

Elastase treatment was the most effective followed by chymotrypsin and pepsin and trypsin was the least effective.

Results of disc gel electrophoresis before and after thermal gelation showed that chymotrypsin- and elastase-treated acid-soluble calf skin collagen reduced the amount of the β -component and increased the amount of α-component. It was concluded that the β -component or its cross-link was the key factor governing polymerization of the protein. Furthermore, the disc gel patterns showed the presence of several fast moving components between the α-component and the buffer front on applying high concentrations of protein. Carbohydrate staining of the gels produced a weak positive reaction. The staining pattern was the same as the protein staining pattern except two additional small bands were observed adjacent to the buffer front. These results indicated the presence of only small amounts of carbohydrates.

Acid-soluble pig skin collagen was treated with the enzymes in the same manner as acid-soluble calf skin collagen. The results were much the same. The untreated protein showed a large proportion of the β -component to the α-component. Elastase and chymotrypsin treatment reversed this relationship and greatly inhibited fibril formation. These results strongly support the hypothesis that the β -component or its cross-link is necessary for protein polymerization. Light staining bands were also observed between the α-component and buffer front following disc electrophoresis of high concentrations of the protein.

The acid-soluble pig skin collagen preparation showed a more intense but similar carbohydrate staining pattern to calf skin collagen, which indicated a higher concentration of carbohydrate.

Lyophilization affected the aggregation properties of acid-soluble calf skin collagen. A buffer of high ionic strength was needed to initiate polymerization. The pig skin collagen polymerized in a buffer of lower ionic strength regardless of whether it was lyophilyzed or not.

ENZYMATIC HYDROLYSIS OF CALF SKIN AND PIG SKIN TROPOCOLLAGEN

By

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INTRODUCTION

Collagen research is a very broad encompassing field concerning such diverse areas as meat products, leather, adhesives and the medical discipline, including the aging process, collagen diseases, dentistry and wound healing. The biochemistry and biological significance of collagen has been vigorously pursued in the past decade in order to ascertain its physical and chemical properties. The pace has been stepped up recently, thanks to advanced technology, and increasing interest regarding human medicine.

Collagen is one of the best known fibrous proteins and is used as a model for studying this entire class of proteins. It is unique in possessing three polypeptide chains, which are helically coiled, and is commonly called tropocollagen (Schmitt, 1959). Collagen is originally laid down by the fibroblasts (Branwood, 1963). Once outside the fibroblast and into the surrounding physiological environment of connective tissue ground substance, these molecules polymerize end-to-end to form protofibrils (Schmitt, 1959). These protofibrils interact laterally to form the collagen fibers seen in the electron microscope and the larger fibers observed under the light microscope.

Collagen is an integral part of all the bones, tendons, teeth and skin, as well as a supporting element for the body organs, and encloses all tissues of the body. Therefore, any changes occurring in the total collagen picture of an animal would readily effect many vital processes.

The objective of this research was to study the action of various proteases upon acid-soluble calfskin collagen. Specifically, the enzymes trypsin, chymotrypsin, pepsin and elastase were used to probe into the nature of this collagen fraction. Their effects were monitored using the techniques of high voltage paper electrophoresis and paper chromatography to produce a "fingerprint" of the products of enzymatic digestion of collagen. Changes in the protein itself were studied using disc gel electrophoresis concomitantly with studies designed to evaluate the aggregation properties of the protein after enzymatic treatment.

Acid-soluble collagen from pig skin was also used as a substrate. The changes resulting from enzymatic treatment were studied using disc gel electrophoresis and alterations in the ability of the enzyme treated collagen fraction to aggregate.

REVIEW OF LITERATURE

General Structure and Properties of Collagen

Reviews by Bear (1952) and Harrington and von Hippel (1961) discuss at length the evolution of the current concept of the collagen molecule. Bear (1952) reviewed early structural studies utilizing X-ray defraction and electron microscope observations. Harrington and von Hippel (1961) discussed structural studies, including X-ray, electron microscope, light-scattering, and ultracentrifuge data, as well as other physical and chemical characteristics of collagen and gelatin. The amino acid composition of various collagens are thoroughly reviewed, as well as the various forms of collagen, which includes insoluble, salt- and alkali-soluble, acid-soluble and gelatin.

According to Harrington and von Hippel (1961), collagen may be recognized histologically by exhibiting swelling in acid, alkali or concentrated solutions of certain neutral salts and non-electrolytes. It is relatively inelastic and shows a higher resistance to proteolytic enzymes than most proteins. They further stated that collagen is readily degraded by collagenase, and that the fibers undergo thermal shrinkage to a fraction of their original length at a temperature characteristic for each animal species. It is converted to gelatin on heating for prolonged periods above the thermal shrinkage temperature. Collagen also exhibits some rather unique chemical and physical properties. It contains approximately one-third glycine, along with rather large amounts of

pyrrolidine-ring-containing residues of proline and hydroxyproline (Piez et al., 1960; Neuman and Logan, 1950; Woessner, 1961). Furthermore, collagen contains little to no cystyl, tryptophyl, methionyl, valyl, phenalanyl, tyrosyl or histidyl residues, and therefore, gives a minimum absorption at 280 mu (Harrington and von Hippel, 1961). Collagen also contains larger amounts of hydroxylysine than most other proteins (Tristram, 1949; Gustavson, 1956). Furthermore, significant levels of free N-terminal α-amino groups do not occur in collagen (Bowes and Moss, 1953). It displays an extensive meridian small-angle X-ray diffraction pattern and the collagen fibers are periodically-banded ($\sim$ 640A) when viewed under the electron microscope (Harrington and von Hippel, 1961). According to Harrington and von Hippel (1961), other collagens (established as collagen by their X-ray diffraction patterns) include reticulin, ichthyocol, elastodin, vitrosin, spongin, gorgonin, cornein and the secreted collagens (strands of collagen secreted by the sea cucumber and other forms of sea life).

Although the structure of collagen is still under investigation, the presently accepted model is based on the poly-L-proline model of Cowen and McGavin (1955) and the postulations of Ramachandran and Kartha (1954, 1955) and of Rich and Crick (1961), who have suggested that collagen is in the form of a coiled coil structure. Presently there is general agreement that the collagen molecule is a triple-chain coiled coil structure (Harding, 1965). It behaves as a rigid rod with a molecular weight of approximately 360,000 with dimensions of approximately 15 X 3000 A

(Boedtker and Doty, 1956; Hall, 1956; Piez et al., 1960). Collagen fibrils are the manifestation of the aggregation of collagen monomers, sometimes called tropocollagen (Schmitt, 1964).

Since the present study deals with acid-soluble native collagen, the remainder of this review will be limited to those papers dealing with the soluble collagens. The reader is referred to the reviews cited earlier (Bear, 1952; Harrington and von Hippel, 1961) along with the monographs of Gustavson (1956) and Veis (1964a) for detailed information regarding insoluble collagens and gelatin.

Soluble Collagens

Harrington and von Hippel (1961) have reviewed the early studies of soluble collagen by many foreign workers. Gallop (1955) discussed extraction of acid soluble ichthyocol. Neutral salt-soluble collagens were extracted and studied by Gross et al. (1955) and Jackson and Fessler (1955). Harkness et al. (1954) isolated a collagen fraction that was soluble in mild alkaline solutions. Collagen molecules solubilized using these techniques are essentially the same in most of their physical and chemical properties (Harrington and von Hippel, 1961). Most of the work done on soluble collagens has employed extraction with dilute citrate buffers at pH 3.5 - 4.0 and reprecipitation by dialysis against water or dilute salt solutions.

Intra- and Intermolecular Cross-links

The presence of three polypeptide chains in the tropocollagen molecule makes it necessary to distinguish between intra- and intermolecular cross-links (Harding, 1965). The latter are links between two collagen monomers, whereas, the former are links between the individual chains in a given monomer (Harding, 1965). He gives a thorough discussion of the cross-links of collagen and cites evidence that ester linkages occur and participate in intermolecular cross-linking. Blumenfeld and Gallop (1962) have shown that aspartic acid is involved in the ester-like links of collagen, which may bind the hexose molecules, as has been shown with other proteins. Evidence is also presented by Harding (1965) regarding involvement of hexoses in the intramolecular cross-links. He proposed that intermolecular cross-links involving hexoses in the presence of hydrogen bond breakers could account for the fact that insoluble collagen is rendered soluble by hydroxylamine, hydrazine or alkali. Since proteolytic enzymes will dissolve normally insoluble collagen (Rubin et al., 1965; Kühn et al., 1966), Harding (1965) suggested that the hexose may be part of the cross-link between the peptide chains or else it may be located adjacent to the cross-link on a pepsin- or trypsin-sensitive portion of the polypeptide chain.

Evidence to support the presence of the cross-links in the native collagen molecule can be found in the work of Veis and Cohen (1960) and of Veis et al. (1961). They showed that denatured collagen (gelatin) had the ability to renature. This work was verified by Rice (1960) and

Altgelt et al. (1961). Altgelt et al. (1961) also proposed that the cross-links held the chains in juxtaposition forming a nucleus for renaturation. They concluded that the cross-links had to be present in the native protein.

Veis (1964b) suggested that the tropocollagen to gelatin transformation occurs if monodispersed collagen solutions in acid are heated to 40°C. If there are no covalent bonds between chains, three randomly coiled single-stranded peptide chains result. The chains differ in composition, and probably in molecular weight. These chains are called α chains. When two chains are joined by one or more covalent linkages, it is called the β-component and when three chains are joined by two or more covalent linkages, this is referred to as the γ-component. According to Veis (1964b), only small amounts of the γ-component have been isolated from acid-soluble tropocollagen preparations.

The collagen monomer is originally laid down without intramolecular cross-links and is soluble in dilute sodium chloride and alkali buffers (Martin et al., 1963). These monomers contain little or none of the β-subunit, and no covalent intermolecular cross-links (Piez et al., 1961; Harding, 1965; Wood, 1962). Acid soluble collagen consists of α-, β- and a small amount of the γ-components (Harding, 1965). Furthermore, Piez et al. (1961, 1963) have shown that the α- and β-components can each be separated into two fractions differing in amino acid content. The α-component is made up of two α_1-chains and one α_2-chain. The β-component consists of a β_1 fraction, which is composed of one α_1- and

one α_2-chain covalently linked, and a β_2 fraction consisting of two α_1 chains.

Lathyrism

Levene and Gross (1959) studied the effect of certain simple organic compounds, such as β-aminopropionitrile and aminoacetonitrile on the connective tissue of growing animals. Administration of these compounds resulted in a weakening of the connective tissue (Levene and Gross, 1959), together with a decided increase in the extractability of collagen into neutral salt solutions (Martin and Goldhaber, 1963). The condition induced by these organic compounds is referred to as lathyrism. No differences have been detected in the physiochemical properties of lathyritic collagen and normal soluble collagen (Martin et al., 1963). Levene (1962) has shown that certain carbonyl compounds can reverse the action of lathyrogens and concluded that carbonyl groups are necessary for normal maturation of the collagen molecule, i.e., increased cross-linking. Martin et al. (1961) showed a decreased amount of the β-component in the acid extracts of lathyritic rat skin.

Recent studies by Rojkind et al. (1966), Bornstein et al. (1966a) and by Bornstein and Piez (1966) showed the presence of an aldehydic component in tropocollagen. Bornstein et al. (1966a) and Bornstein and Piez (1966) have prepared peptides from the α_1 and α_2 chains of rat skin collagen by cyanogen bromide (CNBr) cleavage and have shown that a lysyl residue in each chain is converted to the δ-semialdehyde of α-aminoadipic acid in peptide linkage. They suggested that this step is preliminary

to the formation of an intramolecular cross-link by aldol condensation. This postulation has been supported by Piez et al. (1966) using normal and lathyritic collagen. They showed that the inhibition of cross-linking was a result of blocking the lysine-to-aldehyde conversion.

In another study, Bornstein et al. (1966b) found only one cross-link per β- component. Based on the amino acid sequence and enzymatic studies, they concluded that the cross-link was located at the NH_2-terminal region of collagen. This work prompted the recent study of Kang et al. (1967), who reported the amino acid sequence of peptides from the cross-linking region of rat skin collagen. A pentadecapeptide was obtained from the α_1 chain with CNBr-cleavage and a corresponding tetradecapeptide from the α_2 chain.

Thermal Gels and Fiber Formation

Gross and Kirk (1958) showed that neutral salt solutions of collagen, at approximately pH 7, when warmed to 30-37°C, exhibited the curious property of gelling. Gross and Kirk (1958) and Bensusan and Hoyt (1958) have studied this phenomenon and have described some of the kinetics involved.

Gross et al. (1955) showed that neutral salt solutions of different ionic strength would extract various salt-soluble collagen fractions from connective tissue, but that lyophilization rendered some fractions insoluble. A gel was formed when these soluble extracts were warmed. Hydroxyproline and glycine analysis of the gel and supernatant showed that very little hydroxyproline remained in the supernatant. They further showed

that extracts at ionic strengths of 0.45, 0.7 and 1.0 M, required about 6, 3, and 0.5 hours to gel, respectively. They concluded that the source and properties of extracted collagen differ with the pH of the extraction medium and the ionic strength.

Gross and Kirk (1958) studied the effects of various chemical agents on the rate of formation of fibers from collagen solutions. They found that small concentrations of arginine would delay fiber formation, and that other amino acids, as well as urea and guanidine, would delay fiber formation in proportion to their concentration. Certain anions (SCN^-, HCO_3^- and others) would reverse the inhibitory effect of urea. Lysine and Li^+ were strong accelerators of gelation. They further showed that collagen solutions at pH 7.6 in phosphate buffers exhibited an increased opacity with increased ionic strength, but showed little difference in the precipitated collagen. Exchange of most of the phosphate for NaCl shortened the lag period and increased the rate of opacification without altering the maximum opacity.

Bensusan and Hoyt (1958) showed that increasing ionic strength inhibited the rate of fiber formation. They found that all of the protein was precipitated at the end of the reaction. Furthermore, decreasing protein concentration decreased the rate of fiber formation and increased the lag phase. The fiber formation was monitored using a wavelength of 290 mu.

Wood and Keech (1960) followed collagen precipitation tubidometrically by measuring the extinction at 400 mu. They compared the fibers

resulting from precipitation under the electron microscope. They found that fibril width decreased as the rate of precipitation increased on raising the temperature or lowering the ionic strength, but when the precipitation rate was increased by lowering the pH the fibril width increased. The precipitation curves resulting from this study, as well as from the studies of Gross and Kirk (1958) and Bensusan and Hoyt (1958), showed a lag period followed by a sigmoid growth curve. Wood and Keech (1960) concluded that most of the fibrils were formed during the lag period.

Wood (1960a) regarded fiber formation as consisting of two processes: (1) nucleation (the aggregation of collagen molecules to form nuclei), and (2) the growth of the nuclei into fibrils.

Wood (1960b) further showed that chondroitin sulfate A and C accelerated fiber formation. Chondroitin sulfate B and hyaluronic acid had no effect. Chondroitin sulfate A accelerated precipitation only when present during the lag phase. Wood (1960b), therefore, concluded that chondroitin sulfate A affects nucleation. He later postulated that the role of mucopolysaccharides in fibril formation in vivo might be one of regulating nucleation and growth, and thereby help to determine the rate of fibril formation and fibril size (Wood, 1962).

Wood (1962) using both salt-soluble and acid-soluble calf skin collagen preparations found that the acid-soluble fraction formed fibrils at a faster rate than the neutral-salt-soluble fraction. By arresting the gelation process in the lag phase and centrifuging out the aggregates that had formed, he showed that fiber formation would still occur in the

supernatant, but at a much slower rate. He concluded that both fractions of calf skin collagen are heterogeneous with respect to their ability to aggregate and form fibrils. He suggested that the fiber forming ability is a function of the subunit composition of the collagen molecule.

Bensusan and Scanu (1960) showed that iodination of tyrosine residues of native collagen increased the rate of fiber formation by decreasing the pK of the tyrosyl hydroxyl group. At pH 8.2, they stated that the diiodotyrosyl residues would be essentially all ionized, while the native tyrosyl residues would not be ionized. They concluded that the tyrosine residues must be strategically placed and that formation of ionic bonds must be a requirement for fiber formation. The role of tyrosine and its apparent importance is also supported by Hodge et al. (1960), who showed that trypsin-treated collagen showed a decreased rate of fiber formation in comparison to the control. They showed that a dialyzable tyrosine-containing-peptide was produced following enzyme treatment.

Bensusan (1960) found that lowering the dielectric constant of the medium increased the rate of fiber formation by increasing the electrostatic interaction of the molecules. Furthermore, increasing the ionic strength decreased the rate of fiber formation. The decrease was correlated with the decreased activity of the charged groups on the protein.

Effects of Proteolytic Enzymes

Nishihara and Doty (1958) demonstrated with sonication studies that soluble calf skin collagen underwent fragmentation into shorter segments while still retaining the helical structure and rigidity, which is

characteristic of the native molecule. This was confirmed by Hodge and Schmitt (1958), who further showed that sonicated collagen would no longer form end-to-end aggregates, even before the length of the molecule had been altered appreciably. Boedtker and Doty (1956) had postulated a structure for the collagen molecule based on molecular weight studies of ichthyocol. Since their results showed that the three chains produced by denaturation of collagen were of unequal weight, they postulated that the chains were arranged in a staggered fashion with a dangling chain protruding at either end, beyond the rigid three-stranded portion of the molecule. Hodge and Schmitt (1958) then postulated that these dangling peptides were necessary for native-type aggregations, which occur upon dialysis of acid solutions of collagen against 1% NaCl. They further postulated that these dangling chains are susceptible to destruction by sonication. To test this hypothesis, Hodge et al. (1960) treated intact collagen molecules with trypsin. They found that native collagen was essentially impervious to tryptic attack. However, this experiment was conducted at room temperature and some proteolysis had occurred. An acidic, tyrosine-containing peptide was isolated from the tryptic digest. The molecular length was unaffected, while the native-type aggregates were not obtained. These results suggested that trypsin attacks the end regions of the molecule. Furthermore, the aggregation properties of the treated collagen were assayed by warming neutral salt solutions of the protein to 34°C, thereby forming a thermal gel. Gel formation was severely inhibited or abolished when enzyme-treated collagen was used. This

evidence plus electron microscopy studies of the collagen strongly suggest that the proteolytic activity of trypsin is confined to the "end regions" of the molecule.

Later, Hodge and Petruska (1963) demonstrated that tropocollagen monomers in the collagen fibril overlap instead of aggregating end-to-end. This observation would imply that if peptide appendages are needed for polymerization, they are not necessarily restricted to the end regions of the tropocollagen molecule.

In studies dealing with the amount of dialyzable material released by enzymatic action, the purity of the protein preparation is of the utmost importance. Rubin et al. (1965) have described a very rigorous purification procedure for calf skin collagen. The procedure required about 8-10 weeks. The purity criteria used were optical rotation, tyrosine content, amino acid analysis, free flowing and moving-boundary electrophoresis, hexose and hexoseamine determinations. Hydroxyproline determination was also one of the more useful criteria of purification.

Following purification, Rubin et al. (1965) used the purified acid-soluble calf skin collagen as a substrate for pepsin. The digestion was carried out in dialysis tubing at 20°C for 24 hours at a pH of 3.5. The products of digestion were dialyzed for 96 hours. The dialysate was concentrated and re-dialyzed to insure that only the dialyzable material was used for amino acid analysis. The primary reason for this precaution was to eliminate the possibility that some of the collagen solution had contaminated the outside of the original dialysis sack. This procedure would avoid erroneous results from amino acid analysis due to the substrate

inadvertantly contaminating the dialysate. The dialysate was evaporated to dryness and picked up in a small volume of distilled water. HCl was added and the sample was hydrolyzed in order to prepare it for amino acid analysis. The treated protein was also analyzed for any change in amino acid content by comparing with the untreated control. The results of the amino acid analysis indicated that pepsin did, in fact, release dialyzable peptides. A total of 30-35 amino acid residues containing approximately 8% tyrosine resulted from hydrolysis of the dialyzable peptides. The higher content of tyrosine, a greater amount of acidic amino acids and absence of hydroxyproline was in contrast to that of native collagen.

An aliquot of the dialyzable material was analyzed by Rubin et al. (1965) using paper chromatography. Ninhydrin spraying revealed the presence of several compounds, and staining with amido black indicated the presence of at least four peptides, which moved slowly on the paper. Adequate resolution of these peptides was not achieved. Control experiments using dialysates from pepsin and collagen alone showed no peptidic material moving from the origin. The authors further showed that there was an increase in the proportion of the α-component to the β-component after pepsin treatment. Furthermore, the ability of the pepsin-treated tropocollagen to form a gel upon dialysis against deionized water was abolished. Finally, they discussed some of the shortcomings of previous techniques used to evaluate changes in the aggregation properties of enzyme-treated collagen. They stated that even though fibrils formed during thermal gelation are similar to native collagen, they fail to show other

physical properties, such as high tensile strength. They suggested that other physical and chemical tests need to be invoked to provide a clearer picture of the extrahelical portion of the tropocollagen molecule.

Drake et al. (1966) investigated the action of pronase, chymotrypsin, trypsin, pepsin and elastase on acid-soluble calf skin collagen and insoluble collagen. The collagen was prepared as described by Rubin et al. (1965). The enzyme to substrate ratio was 1 : 100. Digestion was carried out at 20°C for 24 hours for all enzymes except pronase. The pronase digestion period ranged from 3 to 96 hours. The amino acid composition of the dialyzable products of a 24 hour enzyme digestion (expressed as residues/mole) were as follows: pronase-128, elastase-32, chymotrypsin-50, trypsin-40 and pepsin-26. The trypsin and pepsin samples had a much larger amount of tyrosine than the other samples.

Drake et al. (1966) followed the effects of the enzymes on the protein by ultracentrifugal analysis. All enzyme-treated protein showed an increase in the α-component at the expense of the β-component. The percentage of cross-links broken, however, was not correlated with the amount of telopeptide material split off. This was shown to be quite striking in the case of trypsin-treated tropocollagen. There was a small increase in the percentage of the α-component over the control, and yet a large amount of telopeptide material was split off. On the basis of these results, the authors suggested that trypsin did not attack the cross-links.

Electron microscopy by Drake et al. (1966) showed that pronase was able to digest the tropocollagen molecule, while trypsin apparently changed

the charge profile of the precipitated collagen. Drake et al. (1966) also utilized insoluble collagen to show that all of the previously mentioned proteases had the ability to solubilize at least a portion of the normally insoluble fraction. Sequential pronase and pepsin digestion resulted in a soluble collagen solution containing a fraction that sedimented faster then the γ-component, which they called "fraction X". Therefore, fraction X was a larger aggregate than the γ-component. They then isolated fraction X and conducted an experiment that would give evidence that highly polymerized collagen with intermolecular bonds would give rise to linear polymers. A sonication study was made and the results showed that fraction X disappeared after sonication with a concomitant increase in the α-component. On the basis of this data, the authors concluded that the intermolecular cross-link in many polymer aggregates was attached $\alpha : \alpha$ or $\alpha : \beta$ rather than $\beta : \beta$, $\beta : \gamma$, or $\gamma : \gamma$.

The evidence by Drake et al. (1966) for the presence of covalently linked aggregates in soluble collagen solutions supported the previous work of Nagai et al. (1964), Veis and Anesey (1965) and Tristram et al. (1965), but Harding (1965) stated that intermolecular bonding does not occur in soluble collagen solutions.

Drake et al. (1966) further showed that pepsin will break many cross-links, but produces a smaller amount of dialyzable peptides than trypsin. They found that trypsin produced larger amounts of peptides, but broke fewer cross-links. They concluded that pepsin attacks the cross-links selectively, and that the telopeptides attacked by trypsin are not involved in the cross-links.

Finally, Drake et al. (1966) attempted to enrich fractions of tropocollagen with polymeric structures to study the ease of precipitation under selected conditions. They had only limited success with the enrichment technique, however, they concluded that precipitability was determined by chemical specificity in the molecule rather than by the presence of aggregates acting as nuclei for precipitation. This report conflicts with the earlier studies of Wood (1960a), but is similar to later conclusions reached by Wood (1964).

Bornstein et al. (1966b) studied the effects of chymotrypsin, trypsin and cyanogen bromide (CNBr) on acid soluble rat skin collagen. Results of chymotrypsin digestion show an increase in the α-component at the expense of the β-component. This change was monitored by disc gel electrophoresis and carboxymethyl cellulose column chromatography (Piez et al., 1963). Disc gel electrophoresis showed that fragments of lower molecular weight than the α-component moved between the α-component and the buffer front, as well as at the buffer front. The chymotrypsin treated sample showed only the presence of the α-component. The amino acid content of the two types of α-components (the α_1 and α_2 chain) before and after treatment showed only minor differences. This suggested that chymotrypsin was attacking the area of the cross-links, since chromatography showed a large decrease in the β-component.

The trypsin treatment used by Bornstein et al. (1966b) was limited to the neutral salt-soluble rat skin collagen. The digestion was performed in dialysis tubing against appropriate buffer. The dialyzable products of digestion were separated using phosphocellulose column chromatography

after desalting with Bio-Gel P-2. Since the amino acid analysis of CNBr-cleaved peptides from the α_1- and α_2- chains showed the presence of lysine, the authors reasoned that trypsin might be effective in the area of the cross-linkage. Trypsin was used on collagen from lathyritic rats because the cross-link located at the N-terminus area of each chain in normal collagen is believed to involve lysyl residues converted to the aldehyde form, or else condensed to form the cross-link. Trypsin would have been ineffective in this instance, therefore, lathyritic rat skin collagen was used. Two peptides were eluted from the phosphocellulose column when samples of the dialysates from trypsin-treated lathyritic collagen were applied. The amino acid composition of these peptides were identical with those of fractions from the N-terminal ends of CNBr-cleaved peptides (Bornstein and Piez, 1966).

Bornstein et al. (1966b) concluded that trypsin is apparently ineffective in breaking cross-linked components in native collagen as has been reported by Drake et al. (1966), because there are no trypsin-sensitive bonds in the cross-link at the N-terminal region of the molecule. The lysyl residues that do exist in this area occur as the aldehyde derivatives and are no longer susceptable to tryptic attack. Kühn et al. (1966) agreed with Drake et al. (1966) and Bornstein et al. (1966b) that trypsin has very little effect on the intramolecular cross-links of acid-soluble collagen.

Kühn et al. (1966) further showed that pepsin readily increased the proportion of the α-component to the β-component, which is in agreement with Rubin et al. (1965) and Drake et al. (1966). However, Kühn et al.

(1966) stated that an enzyme to substrate ratio of 1 : 100 was virtually ineffective under their experimental conditions. A ratio of 1 : 1 by weight was very effective within 2 hours, but at a ratio of 1 : 10 the reaction was incomplete after 24 hours. The authors further discussed intra- and intermolecular linkages as being of extra-helical origin, i.e., not part of the triple helix. They suggested two possible sites for the cross-links: (1) the short non-helical regions at the ends of the triple helix (Bornstein et al., 1966b) and (2) the telopeptides.

Kühn et al. (1966) further presented a current hypothesis for the origin of the telopeptides. They suggested that the three polypeptide chains are composed of six or seven subunits, which are connected by esterlike bonds. They further indicated that the telopeptides are thought to be the tails from the amino or carboxyl ends of these subunits radiating out of the triple helix.

Schmitt et al. (1964) have shown that proteolytic enzymes markedly effect the antigenic properties of collagen. They concluded that the peptides resulting from enzymatic digestion of tropocollagen by pepsin and pronase hold the key to its antigenic properties rather than its three dimensional structure. The treated protein differed very little from the native-collagen as shown by optical rotation and viscosity measurements. However, the concentration dependence of the viscosity has been shown to decrease markedly after enzymatic treatment (Rubin et al., 1965). Electron microscope studies have shown no change in the enzyme-treated protein from the native protein (Schmitt et al., 1964; Drake et al., 1966). The telopeptides released by enzymatic digestion

contained a large fraction of all the tyrosine in tropocollagen. The amino acid content of the telopeptides differs markedly from that of the remainder of the collagen molecule (Drake et al., 1966).

Since the triple helix was relatively uneffected by pronase digestion, Schmitt et al. (1964) concluded that the cross-links and antigenic sites were external to the triple-helix body. On the basis of the sonication studies by Hodge and Schmitt (1958), who showed that the end-to-end aggregation properties were affected, Schmitt et al. (1964) further postulated that some of the telopeptides are located at the end of the tropocollagen molecule. These conclusions were also supported by the work of Drake et al. (1966) and Kühn et al. (1966). A concensus of opinion seems to invision a tropocollagen molecule that has non-helical portions at the ends, and other peptides protruding from the sides, thus allowing for both side-to-side and end-to-end aggregation (Drake et al., 1966; Rubin et al., 1965; Kühn et al., 1966; Schmitt et al., 1964).

The evidence is also quite impressive for the action of pepsin on soluble and insoluble collagen. Worrall and Steven (1966), Steven (1963, 1965, 1966), Rubin et al. (1963, 1965), Drake et al. (1966), Grant and Alburn (1960) and Kühn et al. (1966) have shown that pepsin will solubilize insoluble collagen, thereby breaking intermolecular cross-links. It was further shown that the α : β ratio of acid-soluble collagen changed from an approximate 50 : 50 distribution to an increased amount of the α-component at the expense of the β-component, indicating severence of part of the intramolecular cross-links.

According to Kühn et al. (1966), there is evidence to show that insoluble collagen is very low in intramolecular cross-links. This was shown by the presence of monomeric tropocollagen following a short treatment with pepsin, whereas, the acid-soluble collagen response to this treatment is far from quantitative. This confirmed previous work done by Harkness et al. (1954), who found that neutral-salt soluble collagen (all α-component) could be converted directly into insoluble collagen. Veis and Anesey (1965) also showed that when insoluble collagen was heat denatured and fractionated, it contained a large proportion of intermolecularly cross-linked polymers along with some β - and γ-components.

Several groups of foreign workers as reported by Kühn et al. (1966) working with pepsin, ficin and trypsin have shown that the aggregation properties of collagen were greatly affected, when treated with these enzymes. The effectiveness of the enzymes was followed using either the thermal gel technique of Gross and Kirk (1958) or by dialysis of collagen solutions in citrate buffers against tap water (Hodge et al., 1960). These observations have been verified by Rubin et al. (1963) and support the conclusions reached by Hodge et al. (1960) that the action of proteolytic enzymes on collagen alters its aggregation properties.

As has been mentioned earlier, many workers have found evidence for high levels of tyrosine in enzymatic hydrolysates of collagen (Hodge et al., 1960; Steven, 1965; Rubin et al., 1965; and Drake et al., 1966). Recently, Dabbous (1966) followed the effects of a tyrosinase on soluble collagen by monitoring the fluorescent spectra of enzyme-treated and untreated collagen. He found that untreated collagen at an excitation

wavelength of 280 mu showed a characteristic fluorescence maximum at about 305 mu, which was apparently due to tyrosine residues. The tyrosinase-treated sample showed a single, but broader maximum fluorescence peak at 340 to 350 mu.

Kinetic spectrophotofluor metric studies by Dabbous (1966) showed the intermediate formation of a fluorophore with an excitation and fluorescence maxima at about 280 and 325 mu, respectively. All the evidence presented regarding tyrosine supports the earlier postulations of Hodge et al. (1960) and Bensusan and Scanu (1960) that tyrosine is a key component in the interactions of the tropocollagen molecule.

EXPERIMENTAL PROCEDURE

Protein Extraction and Purification

Extraction of calf skin

The method of Rubin et al. (1965) was used. The major steps, which with the exception of the first one were performed at approximately 4°C are described below:

(1) Holstein calf skin was washed, shaved and trimmed. It was cut into strips and ground with crushed dry ice in a commercial meat grinder. The derived wet weight was 3 kg.

(2) The ground skin was stirred and extracted for 24 hours with 9 l. of 10% NaCl. This extraction was repeated 8 times.

(3) The extracts were discarded and the residue (step 2) extracted for 24 hours with 9 l. of 0.067M Na_2HPO_4. It was filtered, and the extracts were discarded. This extraction was repeated 7 times.

(4) The residue (step 3) was extracted for 24 hours with 6 l. of a 0.15M sodium citrate buffer at a pH of 3.7 and filtered. This extraction was repeated 5 times. The final residue was discarded.

(5) Solid KCl was added to the filtrate (step 4) to bring the concentration to 0.6M KCl, and solid K_2HPO_4 was added until a pH of 5.8 was attained. The solution was allowed to stand for 48 hours, then it was centrifuged. The precipitate was referred to by Rubin et al. (1965) as fraction 2. The supernatant was

re-centrifuged after standing for another 24 hours, and the supernatant was discarded.

(6) The precipitate (step 5) was dissolved in 0.2M acetic acid overnight. The viscous solution resulting was diluted with 0.2M acetic acid to reduce the viscosity, and then was centrifuged at about 25,000 xg for 12-15 hours. The supernatant was decanted off and dialyzed against 20 l. of a 1% solution of NaCl.

(7) After several changes of the NaCl solution (step 6), a precipitate was formed. This precipitate was acid-soluble collagen (fraction 2A). The precipitate was centrifuged and stored in the frozen condition. The supernatant was made to a concentration of 15% KCl by adding solid KCl. The solution was allowed to stand for 48 hours and the precipitate formed was harvested by centrifugation and was labled fraction 2B.

Purification of Acid-Soluble Calf Skin Collagen

The method of Rubin et al. (1965) was used and is outlined below. All steps were performed at approximately 4°C.

(1) The frozen (fraction 2A) precipitate was thawed and dissolved overnight in 0.05% acetic acid. The viscous solution was made very dilute by the addition of 0.05% acetic acid to greatly reduce the viscosity, which allows removal of large protein aggregates. The solution was ultracentrifuged at approximately 25,000 xg for 12 hours at 4°C.

(2) The supernatant was decanted and filtered and dialyzed against a 1% NaCl solution. These two steps were repeated until analysis indicated 14% hydroxyproline. It was necessary to repeat the procedure four times. Complete recovery of the acid-soluble collagen was obtained after NaCl dialysis by allowing the solution to warm to approximately 20°C.

Extraction of Pig Skin

The same procedure as was used for calf skin was utilized with the following exceptions:

(1) Pig skin was obtained from the fore and hind shanks of pig carcasses that had undergone normal slaughtering procedures. The shanks were selected because of the relatively low fat content under the skin. The drained wet weight was 3.6 kg.

(2) Several changes of 1% NaCl solution were required to obtain fraction 2A at a relatively low yield (< 0.5% of the wet weight). Fraction 2B was obtained in a greater yield than fraction 2A.

Purification of Acid-Soluble Pig Skin Collagen

The purification steps were the same as those used in purifying acid-soluble calf skin collagen with the following exceptions:

(1) Fraction 2A was redissolved and reprecipitated 7 times.

(2) Fraction 2B was redissolved and centrifuged extensively for long periods of time and reprecipitated against 2% NaCl. The precipitate was redissolved in 0.05% acetic acid and centrifuged

at 25,000 xg for 24 hours. This was repeated several times, but the supernatant remained slightly cloudy. After a final dialysis against 2% NaCl the sample was frozen and stored.

Enzyme Treatment

Assay of Enzymatic Activity

Pepsin (2 x crystallized), α-chymotrypsin (3 x crystallized) and trypsin (2 x crystallized) were obtained from Worthington Biochemical Corp. and used without further purification. Elastase (2 x crystallized) in water suspension (Sigma Chemical Co.) was also used without further purification. The pepsin, trypsin and α-chymotrypsin were assayed essentially following the procedure of Anson (1939). The rate of hydrolysis of denatured hemoglobin was measured using the following procedure:

(1) A total of 25 mg of the enzyme was diluted to 50 ml in 0.001M HCl. Dilutions of 1:100, 1:50 and 1:25 were made of this stock solution.

(2) A total of 2.5 gm of hemoglobin were blended in 100 ml H_2O and filtered through glass wool. Aliquots of the filtrate were mixed in a 4:1 ratio with the appropriate enzyme reaction medium.

(3) A total of 5 ml of hemoglobin substrate were delivered into each of 6 numbered test tubes for each enzyme. The solution was placed in a water bath and allowed to equilibrate at 37°C. Tubes 1-3 were blanks. A total of 10 ml of 5% trichloroacetic acid (TCA) were delivered into each tube followed by 1 ml

of the respective enzyme dilutions. The mixtures were removed from the bath after 5 minutes and filtered.

(4) Tubes 4-6 were for the actual test. At timed intervals, 1 ml of the respective enzyme dilutions was added and the tubes allowed to incubate at 37°C for 10 minutes. A total of 10 ml of 5% TCA was then added and the mixtures were removed from the bath after 5 minutes and filtered. The filtrates must be perfectly clear.

(5) The absorbance of the filtrate at 280 mu was obtained using the appropriate blank.

Elastase was assayed for activity using a collagen substrate as follows:

(1) The enzyme and collagen solutions were dialyzed separately overnight against the reaction medium (0.05M calcium acetate pH 8.8) at 4°C.

(2) The enzyme : substrate ratio was 1:100. The substrate concentration was approximately 3.2 mg/ml.

(3) After dialysis, the enzyme was added to the dialysis tubing containing the collagen solution and the mixture was placed in fresh reaction medium at room temperature. The reaction period was 24 hours and utilized appropriate controls.

(4) The dialysate was titrated free of calcium and concentrated.

(5) Thin-layer chromatography showed that the reaction mixture differed from the controls and was taken as evidence of elastase activity.

Reaction Media

Table 1 summarizes the medium and pH, which were used with each enzyme reaction.

Table 1. Reaction media.

Enzyme	Medium	pH adjusted with	pH
α-chymotrypsin	0.1M calcium acetate	NH_4OH	7.8
Trypsin	0.05M calcium acetate	NH_4OH	7.75
Elastase	0.05M calcium acetate	NH_4OH	8.8
Pepsin	0.05% acetic acid	---	3.5

Enzymatic Digestion of Acid-Soluble Calf Skin Collagen

The general design of the experiment was very similar to that of Drake et al. (1966). The enzyme to substrate ratio was 1:100. The respective enzymes were dialyzed separately for 24 hours against their corresponding reaction media. Each enzyme was added to 10 ml of the collagen solution in dialysis tubing. The protein concentration was 7.2 mg/ml.

The reaction was allowed to proceed for 24 hours at 20°C with occasional swirling. Both enzyme and substrate controls were assayed at the same time in identical solutions. The enzyme control consisted of the same concentration of enzyme as was added to the substrate in the reaction mixture. The substrate control consisted of 10 ml of substrate minus the enzyme.

Following the 24 hour digestion period, the dialysate was exchanged for a fresh solution and dialysis was allowed to continue for another 24 hours at 4°C.

For all enzymes except pepsin, the dialysis tubing was removed and the substrate was freed from the enzyme by precipitating 3 times with 15% KCl and redissolving in 0.05% acetic acid. In order to free the substrate from pepsin, 0.02M Na_2HPO_4 was added to the 15% KCl solution (Rubin et al., 1965). Solid KCl was either added directly to the centrifuge tube in order to give a final concentration of 15%, or in some instances the protein solution was dialyzed against a 15% KCl solution. Generally, the former procedure was used.

Following 3 precipitations, the protein solution was dialyzed free of salts, lyophilized and stored over $CaCl_2$ in a dessicator. Samples could then be weighed out and dissolved in 0.05% acetic acid at 4°C.

Enzymatic Digestion of Acid-Soluble Pig Skin Collagen

Acid soluble pig skin collagen was treated in the same manner as the acid-soluble calf skin collagen, except the substrate concentration was 5.8 mg/ml. Furthermore, the protein was not lyophilized following 3 precipitations. Instead, the protein was dialyzed free of salts against distilled water and stored at 4°C. The samples were dialyzed against 0.05% acetic acid before analysis.

Dialysates from Enzymatic Digestion of Acid-Soluble Calf Skin Collagen

The dialyzable material from the enzyme-treated, acid-soluble calf skin collagen and the controls were desalted by titrating the calcium

present with oxalic acid (Drake et al., 1966). The samples were taken to dryness in a Buchler rotary evaporator following removal of the precipitated calcium oxalate. The samples were warmed to 55°C to expedite evaporation. After evaporation the samples were dissolved in 3 ml of distilled water and frozen until needed.

A similar enzymatic digestion was performed at room temperature, however, only the protein was studied. The dialysates were used as samples to establish the desalting procedure for use with the dialysates from the 20°C digestions. Furthermore these room temperature dialysates were used as samples for screening various buffer systems for the electrophoresis studies as well as for evaluating the separations obtained from various chromatography solvent systems.

Dialysates from Enzymatic Digestion of Acid-Soluble Pig Skin Collagen

These dialysates were not analyzed.

Dialysis Tubing

The method of Drake et al. (1966) was used and consisted of boiling the tubing for 2 hours in a 10% Na_2CO_3 solution. The tubing was rinsed with distilled water and numerous final rinses of 0.05% acetic acid. The tubing was stored in 0.05% acetic acid at 4°C.

Hydroxyproline Analysis

All protein samples were run in duplicate at two different protein concentrations. The method of Woessner (1961) was used as described below:

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Hydroxyproline Analysis

All protein samples were run in duplicate at two different protein concentrations. The method of Woessner (1961) was used as described below:

(1) A total of 25 mg of dried L-hydroxyproline was dissolved in 250 ml of 0.001N HCl. This was the stock solution. Standards were prepared daily by diluting the stock with water to obtain concentrations of 1-5 ug/2 ml.

(2) A buffer solution containing 50 gm of citric acid (monohydrate), 12 ml of glacial acetic acid, 120 gm sodium acetate (trihydrate) and 34 gm sodium hydroxide were made to a final volume of 1 l. in distilled water. The pH was adjusted to 6.0 and the buffer was stored under toluene in the refrigerator.

(3) A 0.05M solution of chloramine T (K & K Labs) was prepared daily by dissolving 1.41 gm in 20 ml of water. A total of 30 ml methyl cellosolve and 50 ml of buffer was added.

(4) A 3.15M solution of perchloric acid was prepared using 70% perchloric acid and distilled water.

(5) A 20% solution of p-dimethylaminobenzaldehyde in methyl cellosolve was prepared shortly before use.

(6) The collagen samples in solution were added to small digestion vials and concentrated HCl was added to give a final concentration of 6N. The samples were sealed in the vials and hydrolyzed overnight in an autoclave at a temperature of 121°C at a pressure of 15 psi. The tube was then opened and the contents rinsed into a volumetric flask. Several drops of 0.02% methyl red indicator were added, followed by the theoretical amount of 2.5N NaOH required for neutralization. Final

adjustments were made with dilute acid or base until the indicator turned slightly yellow.

(7) A 2 ml sample containing approximately 1-5 ug of hydroxyproline was delivered into test tubes for analysis. Standard solutions were also prepared containing 0-5 ug hydroxyproline/2 ml. Hydroxyproline oxidation was then initiated by adding 1 ml chloramine T solution to each tube in a predetermined sequence. The tube contents were mixed and allowed to stand for 20 minutes at room temperature. The chloramine T was then destroyed by adding 1 ml perchloric acid solution to the tubes in the same order as before. The contents were mixed and allowed to stand for 5 minutes. Finally, 1 ml of the p-dimethylaminobenzaldehyde solution was added, and the mixture was shaken until no schlieren could be seen. The tubes were then placed in a 60°C water bath for 20 minutes, then cooled in tap water for 5 minutes. The absorbancy of the solutions was determined spectrophotometrically at 557 mu. The hydroxyproline values were determined directly from a standard curve.

Protein Concentration

Nitrogen Content

The nitrogen content was determined on duplicate samples by the micro-Kjeldahl procedure as outlined by A.O.A.C. (1960). A value of 17.6% nitrogen was used to calculate the concentration of acid-soluble calf skin collagen in solution (Rubin et al., 1965). The nitrogen

content of acid-soluble pig skin was determined by lyophilyzing an aliquot of the solution and drying over $CaCl_2$ for 2 days in a dessicator. Triplicate samples of the dried protein were used to determine the percent nitrogen. From the determinations, it was possible to calculate the protein concentration of acid-soluble pig skin in solution from the total nitrogen as determined by the micro-Kjeldahl procedure (A.O.A.C., 1960).

Absorbancy

The protein concentration was also compared between samples using absorbance at 230 mu (Piez et al., 1962).

Amino Acid Analysis

Two protein samples were analyzed. They were an aliquot of untreated acid-soluble calf skin collagen and an aliquot of acid-soluble calf skin collagen that had been treated with pepsin at room temperature (25°C). The concentrations were 3.16 mg/ml for the untreated collagen and 2.25 mg/ml for the enzyme-treated collagen. The aliquots were placed in digestion vials and enough concentrated HCl was added to bring each solution to 6N. The samples were then frozen and sealed under vacuum. They were hydrolyzed for 20 hours at 105°C. The samples were then diluted to 5 ml and a 0.2 ml aliquot of each was taken for analysis.

A partial amino acid analysis was performed using a Beckman/Spinco, model 120, automatic amino acid analyzer. The procedure described by Moore et al. (1958) was used. This procedure does not permit direct determination of hydroxyproline, and hydroxylysine elutes at approximately

the same time as tryptophan. Consequently, the results of the amino acid analysis do not include hydroxylysine and hydroxyproline.

Spectrophotofluorimetric Analysis

Acid-soluble calf and pig skin collagen were analyzed for tryptophan using an Aminco-Bowman spectrophotofluorimeter (Dabbous, 1966). The solutions were compared with standard solutions of L-tyrosine and L-tryptophan. The presence of tryptophan in the protein solutions would indicate an impurity.

The standards were dissolved in dilute NaOH, and the protein was dissolved in 0.05% acetic acid. The relative fluorescence was compared between enzyme-treated samples of the same species with the protein concentrations adjusted to approximately the same absorbance at 230 mu or else by dilution to a known concentration.

Disc Electrophoresis of Collagen

Both acid-soluble calf skin and acid-soluble pig skin collagen were analyzed using the procedure of Nagai et al. (1964) modified as outlined below:

(1) The protein was desalted by dialysis against cold 0.05% acetic acid at pH 3.5. Following dialysis, the protein was heat denatured by warming the acid solutions to 40-45°C for 20 minutes. The lyophilized protein was dissolved in 0.05% acetic acid by stirring overnight at 4°C. Protein concentration was determined by weighing out lyophilized samples or by using absorbancies at 230 mu.

(2) Cyanogum (E-C Apparatus Corp.) was used for making the gel instead of acrylamide and N,N'-methylenebisacrylamide.

(3) No sample gel was used. Photopolymerization with riboflavin was also omitted.

(4) The upper gel was a 3.1% gel composed of 2 volumes of 12.5% cyanogum, 1 volume of upper gel buffer and 1 volume of distilled water. This solution was diluted with an equal volume of ammonium persulfate solution.

(5) The lower gel was a 7.5% gel as described by Nagai et al. (1964), except for the use of a higher concentration of ammonium persulfate.

(6) A solution of 0.4% ammonium persulfate in water was used instead of 0.15% to facilitate polymerization.

(7) The current per tube was generally 3 to 4 millamperes.

(8) The density of the denatured protein solution was increased by adding sucrose. This solution was then applied on the upper gel. The sample was applied with a 100 ul pipet, which was immersed in the buffer solution overlaying the gel. The increased density of the protein solution permitted layering the solution directly on the gel by displacment of the less dense buffer.

Staining Procedures

Protein Staining

The protein staining technique was similar to that of Smithies (1955). Two grams of Amido Black 10 B dye were dissolved in a mixture of 250 ml water, 250 ml methanol and 50 ml acetic acid. The gels were submerged in this solution for approximately 20 minutes and were destained by soaking overnight in a 7% acetic acid solution.

Test for Carbohydrates

Testing for carbohydrates was accomplished using the techniques of Keyser (1964). The reagents required were:

(1) ethanol (both absolute and 95% by volume).

(2) methanol-water-acetic acid (8:10:1 by volume).

(3) periodic acid reagent (3 gm of H_5IO_6 and 1.66 gm of sodium acetate (3 H_2O) dissolved in 500 ml of water, stored in the refrigerator and diluted 6:4 with 95% ethanol before use).

(4) thiosulfate - metabisulfite reagent (5 gm of potassium metabisulfite and 30 gm of sodium thiosulfate dissolved in 1 l. of water and diluted 1:1 with ethanol before use).

(5) fuchsin-sulfite reagent (8 gm of potassium metabisulfite dissolved in 1 l. of water and exactly 10.5 ml concentrated HCl was added. Then 4 gm of finely powdered basic fuchsin was added and the mixture was stirred for 2 hours at room temperature. After standing for 2 more hours, the solution was treated with a small amount of decolorizing charcoal, filtered within 15 minutes and stored in the refrigerator).

(6) ethanol-sulfite wash (5 gm of potassium metabisulfite dissolved in 1 l. of distilled water; 1 l. of 95% ethanol and 9 ml of concentrated HCl were then added).

After electrophoresis, the gels were removed from the tubes and immersed for 10 minutes in 95% ethanol, and then for 10 minutes in methanol-water-acetic acid (8:10:1). The periodic acid-Schiff procedure was then applied as shown in table 2. All treatments were carried out in shallow dishes with shaking, especially those steps that involved ethanol solutions.

Table 2. Carbohydrate testing procedure.

Step	Treatment	Time (min.)
1	Periodic acid oxidation	12
2	Thiosulfate-metabisulfite reduction	5
3	Rinsing (water)	3
4	Fuchsin-sulfite staining	20
5	Ethanol-sulfite washing	
	two washes	5
	then repeated washes	30
	then final wash	overnight
6	0.1N HCl	15-30

All treatments were carried out in shallow dishes with shaking, especially those steps that involved ethanol solutions.

High-Voltage Paper Electrophoresis

The apparatus used was similar to that used by Katz et al., 1959. The tank held approximately 35 gallons of the coolant (oleium spirits) and 5 gallons of formic-acetic acid buffer at pH 2.0 (Efron, 1960). A 5,000 volt, d.c. power source (Savant Instruments, Inc.) was used to supply the necessary current. The coolant was cooled with running tap water passing through the stainless steel coils at the top of the tank. Generally, the temperatures of the coolant at the beginning of a run was 16°C but had increased to approximately 26°C at the conclusion.

A roll of Whatman 3 MM chromatographic paper was cut into different lengths for the electrophoretic run. A length of 72 cm and a width of 45 cm were the most common dimensions used. The paper was divided longitudinally as well as latitudinally with pencil lines. This facilitated the application of four samples on one sheet (72 x 45 cm). Each sample was placed 2 cm toward the cathode side of center and 5 cm from the anode edge of the paper. All samples were located 2 cm from the outer edge of the paper and were run in duplicate on the same paper.

The samples were applied in 1 cm lengths with a 100 ul pipet and were dried after each application with a laboratory hot air blower (Precision Scientific Co.). Approximately 20 applications, performed by reversing the direction after each application, were required to deliver 200 ul to the area. Following application of the sample and drying, one half of the paper was dipped into the buffer and blotted. Blotting entailed placing the wetted paper between two dry sheets of heavy filter

paper and pressing on the upper blotter to insure contact with the wet paper. This was repeated for the other half. In both instances, care was taken to avoid wetting the area containing the sample. After blotting, the paper was hung on an electrophoresis rack. The sample area was sprayed with buffer and the rack was lowered into the tank. Most of the electrophoresis was performed using high voltages (Gross, 1955). Voltages of 70 volts/cm were commonly used and produced currents of approximately 5 or 6 ma/cm. The normal run lasted for 25 minutes, after which the paper was removed from the tank and dried.

Paper Chromatography

N-butanol/acetic acid/pyridine/H_2O, (15:3:10:12) was used as the solvent system (Randerath, 1964a). Ascending chromatography was carried out in a large glass cylinder at room temperature (25°C). After the paper was dried following electrophoresis, it was cut along the center lines resulting in four rectangles of 36 x 22.5 cm. Each rectangle was rolled along the longitudinal axis into a cylinder and the free ends of the paper were stapled together. Care was taken to prevent the ends from touching each other. The cylinders were then placed in the chromatography tank with the point of application down and the edge of the paper was immersed in the solvent to a depth of about 1 cm. Chromatography required approximately 6 hours.

Color Reactions

The dried chromatograms were selectively stained for tyrosine, ninhydrin positive areas and peptides using the procedures described by Easley (1965).

Ninhydrin Staining

Buffered ninhydrin was prepared by adding 1 ml of pyridine and 1 ml of glacial acetic acid to 98 ml of a 0.3% ninhydrin solution in acetone. The paper was dipped through this solution and dried. It was then placed in an oven at 70-80°C for color development.

Tyrosine Staining

This procedure was used in combination with the ninhydrin method. The paper was first treated with ninhydrin and developed. The spots were marked with a pencil and then the paper was treated for tyrosine. Tyrosine staining required the following two solutions: Solution A, which contained 0.1% α-nitroso-β-napthol in acetone, and Solution B, which contained 10 ml of concentrated HNO_3 plus 90 ml of acetone, freshly prepared. The paper was dipped through solution A and dried, then through solution B. The paper was dried and then warmed carefully by moving back and forth over a hot plate. Tyrosine spots were identified by a rose colored appearance.

Chlorination

This procedure was used to detect areas that would not react with ninhydrin. Peptides could be located, since the intensity of the stain

was proportional to the number of peptide linkages, more specifically, this procedure reacts with N-H groups. Chlorination entailed the preparation of the following two solutions: solution A, 1% v/v tertiary butyl hypochlorite in cyclohexane, and solution B with 1% soluble starch, 1% KI in H_2O (boiling water was added to the starch suspended in a few ml. of water then the KI was added).

The paper was dipped through solution A and allowed to aereate in a stream of cold air under the hood for 1 hour. Then it was sprayed with the freshly prepared solution B, while the solution was still hot. The positive areas appeared dark blue on a blue background. Both the front and back were sprayed. This technique does not allow any other staining procedure to be used on the paper.

Gelation and Fibril Formation

Long Term Experiments

The procedure for producing gelation was a modification of the fibril and gel formation procedures of Gross and Kirk (1958) and Kühn et al. (1966). Basically, the procedure involved adding equal volumes of the protein in 0.05% acetic acid and phosphate buffers of various ionic strengths (pH 7.4) at room temperature. The absorbancies at 230 mu were recorded and the samples were allowed to remain at 35°C for 12 hours. The solutions were then filtered and the absorbancies of the filtrates at 230 mu were again recorded. The final reading indicates the amount of protein in solution.

Short Term Experiments

Continuous monitoring of the gelation process was conducted using a Beckman DU-2 monochronomotor with a Gilford automatic cuvette positioner and optical density converter (Gilford Instrument Co.) connected to a Sargent recorder, Model LSR (E. H. Sargent and Co.). The gelation was initiated by diluting 1.5 ml of protein solution in a cuvette with 1.5 ml of phosphate buffer.

Fractions of acid-soluble calf skin collagen were diluted with a NaH_2PO_4-Na_2HPO_4-NaCl buffer at an ionic strength of 1.2M (0.45M NaCl) and pH of 7.4, until the ionic strength was approximately 0.6M. The acid-soluble pig skin collagen fractions were diluted with KH_2PO_4-Na_2HPO_4 buffer (pH 7.4) until the ionic strength was 0.1M. The temperature of gelation was approximately 33°C. Four samples were run at once, each being monitored for 30 seconds at 1.5 minute intervals. The wavelength setting was 230 mu for all samples including all the enzyme-treated proteins. The protein solutions were diluted with 0.05% acetic acid to adjust their respective absorbancies to approximately the same value when measured at 230 mu.

Monitoring continued until the resulting curves showed gelation was complete or non-existent. Generally, the absorbancy range on the recorder was 0.0-3.0 O.D.

Disc Electrophoresis

Samples of the initial gelling mixtures and of the filtrates following gelation were taken for evaluation on disc gel electrophoresis. The

initial samples were dialyzed free of salts against 0.05% acetic acid. They were then examined by disc gel electrophoresis as previously described. The filtrates were dialyzed free of salts and concentrated by pervaporation at room temperature and subsequently analyzed using disc gel electrophoresis.

Column Chromatography

Dialysates of enzymatic digests of acid-soluble calf skin collagen, digested at room temperature, were concentrated and applied to a column of P-2 Bio-Gel (Bio-Rad Corp.) for desalting purposes. The column bed was 26 x 3 cm and had a void volume for Blue Dextran of 69 ml. The column was equilibrated over night with distilled water.

Samples of 2-5 ml were applied to the column and the effluent was monitored with an Isco dual beam detector (Isco Corp.) at both 280 and 254 mu at a flow rate of 180 ml/hr. The effluent was also monitored with 1M oxalic acid to detect the presence of calcium. When the sample had been previously titrated with excess oxalic acid, the effluent was monitored with a solution of 1M calcium acetate. Fractions were collected from the column that were salt free and others that contained most of the salts present in the samples. The salt free fractions were taken to dryness with a Rinco rotary evaporator (Rinco Instruments Co., Inc.) and then picked up in 3 ml of water. An aliquot of these samples were chromatogrammed using thin layer chromatography for a rapid analysis of the degree of separation achieved by the column.

The resolution obtained using this technique was poor, and therefore, it was abandoned and replaced by the method of direct titration of the calcium present with oxalic acid (Drake et al., 1966).

Thin-Layer Chromatography

The technique described by Randerath (1964b) was used for preparing the Silica Gel G plates. The same solvent was used as with the paper chromatography. The plates were dried and sprayed with 0.5% ninhydrin-ethanol solution.

RESULTS AND DISCUSSION

Acid-Soluble Calf Skin Collagen

Protein Purification

The criteria used to determine protein purity were hydroxyproline content, spectrophotofluorimetric assay and a partial amino acid analysis.

Acid-soluble calf skin collagen (fraction 2A) was redissolved and precipitated several times after extraction. The apparent hydroxyproline percentage was measured colorimetrically. The percent hydroxyproline increased from about 12% in the first precipitation to approximately 14.3% in the final purified product. This value agrees very well with values obtained by Woessner (1961), for gelatin from calf skin and Rubin et al. (1965), for acid-soluble calf skin collagen.

Fluorimetric analysis of fraction 2A indicated that the protein preparation was free from tryptophan. The fact that collagen is free from this amino acid and that most other proteins contain significant amounts of tryptophan would establish the purity of the collagen preparation. Tryptophan is a strong fluorophore and would be readily detected if excited at 280 mu in the spectrophotofluorometer. Figure 1 shows the fluorescence spectra of L-tryptophan (0.5 ug/ml), L-tyrosine (0.5 ug/ml), acid-soluble calf skin collagen and redissolved, lyophilyzed acid-soluble calf skin collagen when excited at 280 mu.

The protein samples were dissolved in 0.05% acetic acid and the tyrosine and tryptophan standards were dissolved in dilute NaOH. L-tyrosine

Figure 1. Fluorescence spectra of tyrosine, tryptophan, lyophilyzed and non-lyophilyzed acid-soluble calf skin collagen. Tyrosine value is uncorrected for scattering effects. Excitation wavelength-280 mu. Code: R.I. = Relative Intensity; mu = emission wavelength; tyr. = tyrosine (0.5 ug/ml); try. = tryptophan (0.5 ug/ml); C. = non-lyophilyzed collagen and l.c. = lyophilyzed collagen.

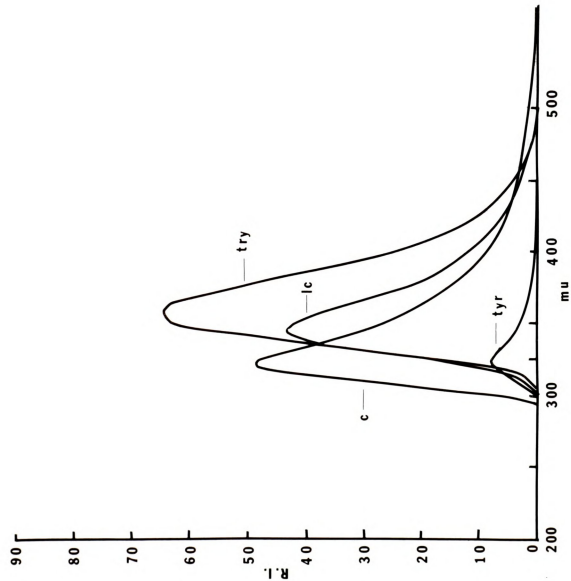


Figure 1.

has a fluorescence maximum at approximately 303 mu in neutral solution when excited at 275 mu. The shift of the tyrosine peak (figure 1) to approximately 325 mu is probably due to the interference peak from light scattering, since the excitation wavelength (280 mu) and the known emission wavelength (303 mu) are so close together. The effects of light scattering (interference peaks) were obtained with the Aminco-Bowman instrument at an excitation wavelength of greater than 300 mu. Tryptophan had an emission maximum at approximately 355 mu, when excited at 280 mu. In neutral solutions, tryptophan has an emission maximum at 348 mu upon excitation at 287 mu (Udenfriend, 1962).

The fluorescence maximum for acid-soluble calf skin collagen can readily be seen to approximate that of the standard L-tyrosine. Dabbous (1966) observed a fluorescence maximum at 305 mu for acid-soluble calf skin collagen. In a preliminary experiment, equal concentrations of tryptophan and tyrosine were mixed and the fluorescence spectrum was determined at an excitation wavelength of 280 mu. One peak was observed at an emission wavelength of 355 mu. This would indicate that in the presence of tryptophan, fluorescence of tyrosine was completely masked. This is in agreement with the results of Teale and Weber (1959), who showed that tryptophan and tyrosine-containing proteins exhibited only tryptophan fluorescence with wide differences in both absolute quantum yield and wavelength of maximum fluorescence emission. A shift of this maximum to shorter wavelengths was accompanied by a comparable shift of the absorption maximum to longer wavelengths.

The shift in the fluorescence maximum of lyophilyzed acid-soluble calf skin collagen as compared to the non-lyophilyzed protein was unexpected. The reason for this is unknown, but perhaps lyophilyzation affected the fluorescing residues by slightly altering the configuration of the protein.

These fluorescent studies give additional support as to the purity of the collagen preparation, because no fluorescence was observed in the tryptophan range and the fluorescence maximum of the protein corresponded to that of pure tyrosine.

Table 3 shows a partial amino acid composition of untreated calf skin tropocollagen (fraction 2A). This analysis was made on a 20 hour hydrolysate and is not corrected for any amino acid decomposition, which might have occurred during hydrolysis. It does not include values for hydroxyproline and hydroxylysine. The value for aspartic acid is higher than that reported by Rubin et al. (1965), as are several other amino acids, but to a lesser degree. Better agreement probably would have been obtained had several analyses been performed and corrections applied.

A value of 17.6% nitrogen was used in conjunction with micro Kjeldahl nitrogen determinations to calculate the concentration of protein in solution (Rubin et al., 1965).

Table 3. Partial amino acid composition of acid-soluble calf skin collagen and of pepsin-treated acid-soluble calf skin collagen. No corrections are included for any amino-acid decomposition which might have occurred during hydrolysis. Values for hydroxyproline and hydroxylysine are not included, and, as they are present, the values shown will be high.

Amino acids[a]	Mole percent amino acid	
	Untreated	Treated
Lysine	3.49	2.65
Histidine	0.66	0.50
Arginine	4.34	5.66
Aspartic acid	6.57	5.84
Threonine	2.46	1.77
Serine	3.63	4.07
Glutamic acid	8.33	8.31
Proline	12.55	13.62
Glycine	36.83	35.02
Alanine	12.43	13.97
Valine	2.69	2.83
Methionine	0.35	0.35
Isoleucine	1.41	1.24
Leucine	2.93	2.83
Tyrosine	0.47	0.09
Phenylalanine	1.29	1.24

[a]Analysis performed by J. R. Brunner, Food Science Department, Michigan State University.

Enzymatic Treatment of Acid-Soluble Calf Skin Collagen

Dialysates

Plate 1 shows a tracing of the combined 2-dimensional chromatograms of the controls. The composite consists of two chromatograms, one stained with ninhydrin and one with a chlorination stain. Chlorination staining is indicated only if it differed from the ninhydrin staining. Spots numbered 1 and 2 were present in several enzyme controls. The remaining spots resulted when all enzyme and substrate controls were separated by the 2-dimensional techniques. However, these spots were found to be derived from the dialysis tubing after numerous experiments. This was shown by dialyzing distilled water against distilled water and concentrating the dialysate. The resultant chromatogram gave a total of 15 spots, which matched the enzyme and substrate controls in every case.

The deionized water used to make up all the reaction media was tested by evaporating 500 ml to dryness. A small portion of water was added to the flask, and the liquid subjected to the 2-dimensional separation. No positive test for ninhydrin or chlorination was observed. These observations, if made on the collagen substrate alone, might lead one to suspect the presence of non-protein nitrogen in the soluble collagen preparation. Steven and Tristram (1962) observed the presence of 12 chromatographic components when analyzing the dialysates of soluble collagen. These preparations were dialyzed at three different pH values and the dialysates were concentrated and chromatographed. Each mixture gave approximately the same number of ninhydrin positive spots. These

Plate 1. Two dimensional electrophoresis and chromatography of the dialyzable material from the control samples used during enzymatic digestion of acid-soluble calf skin collagen. Half-filled circles indicate positive chlorination staining areas which gave no positive ninhydrin test. Dotted lines indicate weakly staining areas. Black circles show ninhydrin positive areas. Spots labeled 1 and 2 were present in some enzyme controls. The "O" designates the point of application of the sample.

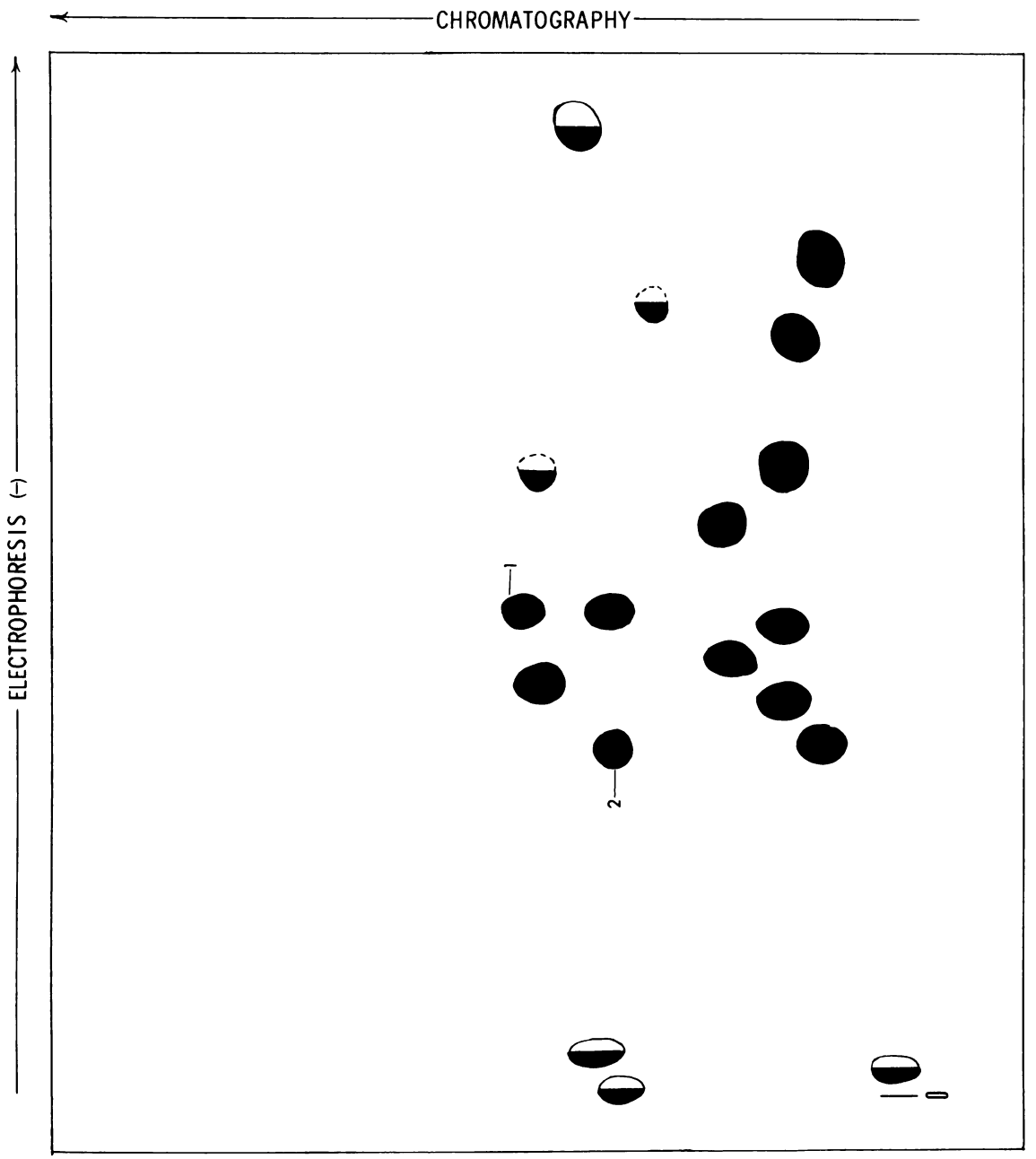


Plate 1

workers methodically checked the water, as well as the desalting resin bed, and in both instances found no evidence of ninhydrin positive material. They, therefore, concluded that the chromatographic spots obtained from dialysis of the collagen solutions were evidence for non-protein nitrogen. They did not analyze the dialysis tubing alone.

The presence of dialyzable nitrogenous material in the dialysis tubing seems to involve one of two alternatives. First, the nitrogenous material could be present after manufacture and survive the tubing treatment prior to use, or second, molds or yeasts growing on the dialysis tubing at the acid pH in the refrigerator might produce dialyzable materials. There was no evidence to indicate the presence of molds or yeasts in the tubing in the refrigerator. The origin of the nitrogenous material remains obscure.

Plates 2, 3, 4 and 5 show tracings of the composite chromatograms and the composites minus the controls. The latter shows the dialyzable materials due to enzymatic activity. Comparison of the electrophoretically slower moving components, i.e., those spots directly above the origin (0), reveals that the leading spot resulting from trypsin, elastase and chymotrypsin digestion has a similar R_f value. The average R_f values from duplicate samples of each enzymatic digest were 0.719, 0.731 and 0.726 for trypsin, elastase and chymotrypsin digests, respectively. However, only the spot from trypsin digestion was positive to tyrosine staining. Chymotrypsin digests, when analyzed by electrophoresis in only one dimension, indicated a positive tyrosine staining area close to the origin.

Plate 2. Two dimensional electrophoresis and chromatography of the dialyzable materials from pepsin-digested acid-soluble calf skin collagen. Digestion occurred at 20°C for 24 hours. Black spots indicate material in the control; half-filled circles indicate positive staining with the chlorination stain where different from ninhydrin positive areas. Outlined circles indicate ninhydrin positive areas. The letter T indicates a positive tyrosine test. The letter O represents the point of sample application. The composite chromatogram represents two chromatograms, one stained for tyrosine and ninhydrin positive areas and one stained with the chlorination procedure.

2-a. A composite chromatogram of the dialyzable materials following pepsin digestion of acid-soluble calf skin collagen.

2-b. Resultant chromatogram after subtracting the control spots.

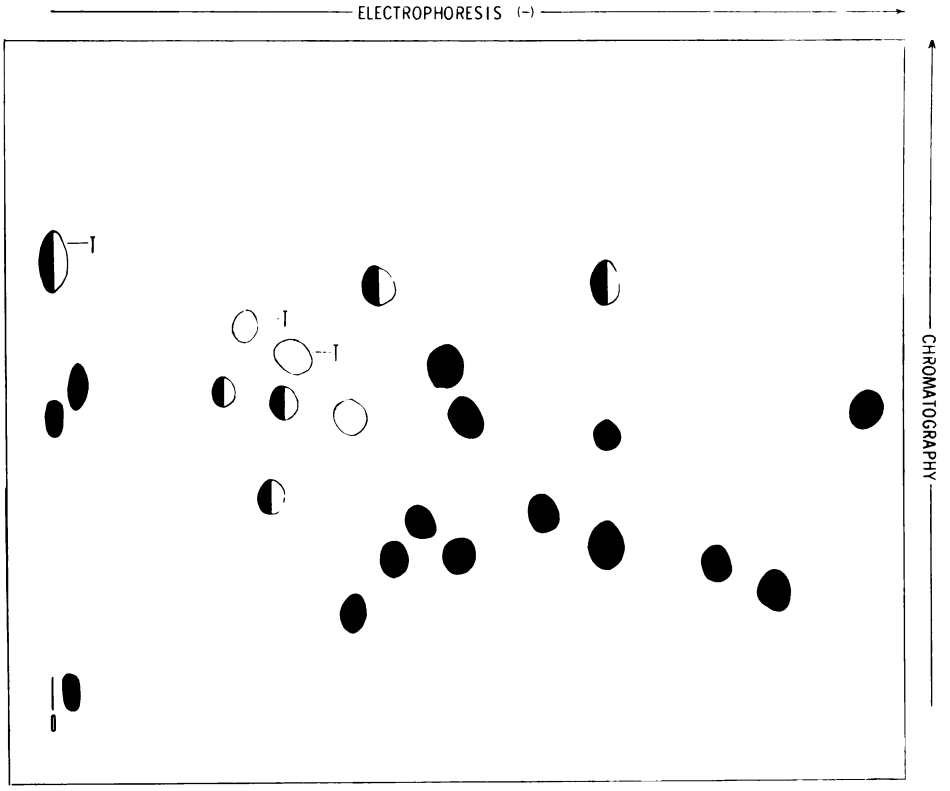


Plate 2-a

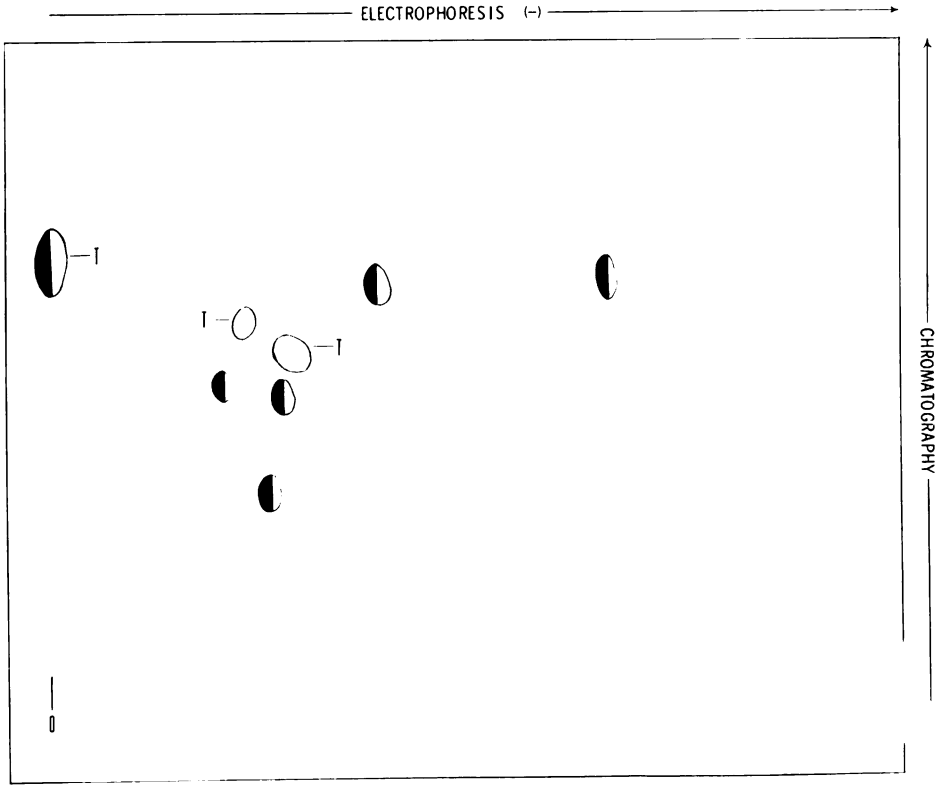


Plate 2-b

Plate 2.

Plate 3. Two dimensional electrophoresis and chromatography of the dialyzalbe materials from trypsin-digested acid-soluble calf skin collagen. Digestion occurred at 20°C for 24 hours. Black spots indicate material in the control; half-filled circles indicate positive staining with the chlorination stain where different from ninhydrin positive areas. Outlined circles indicate ninhydrin positive areas. The letter T indicates a positive tyrosine test. The letter O represents the point of sample application. The composite chromatogram represents two chromatograms, one stained for tyrosine and ninhydrin positive areas and one stained with the chlorination procedure.

3-a. A composite chromatogram following trypsin treatment.

3-b. Resulting chromatogram after subtraction of the control spots.

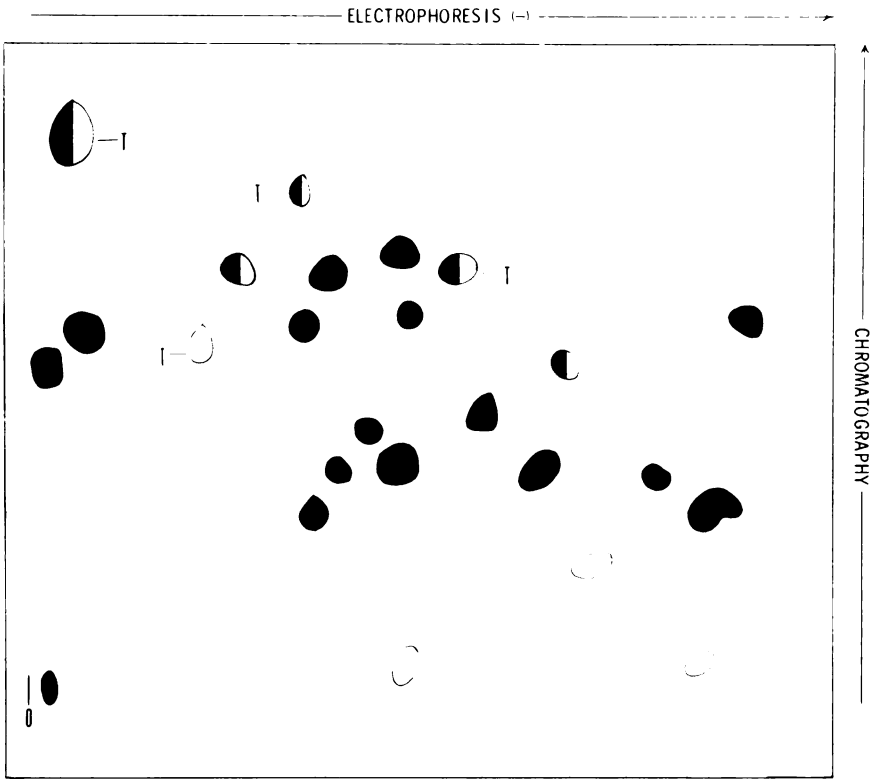


Plate 3-a

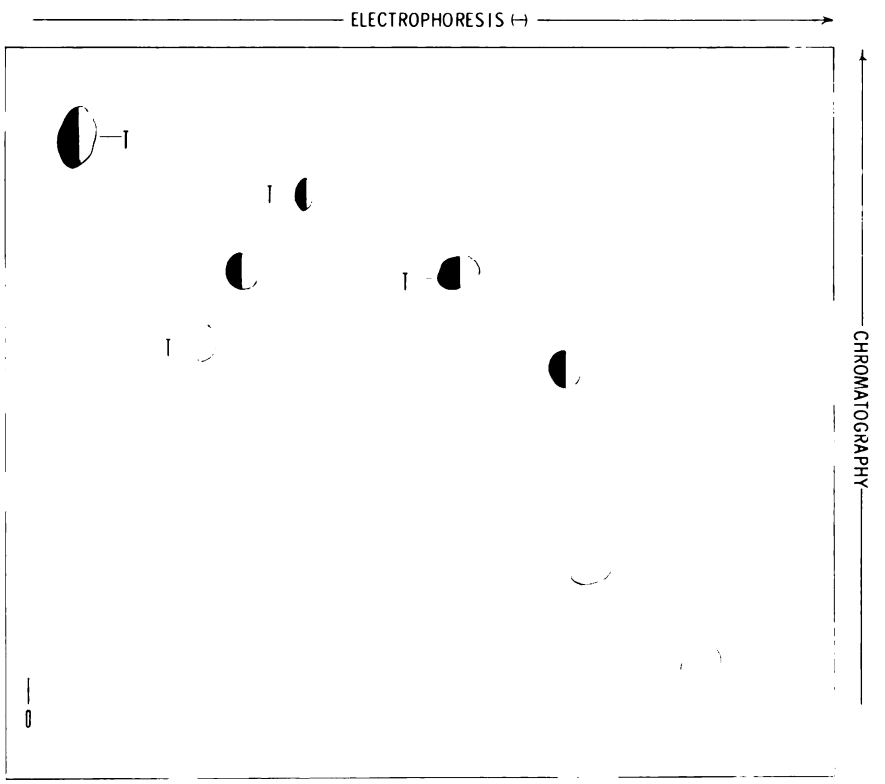


Plate 3-b

Plate 3.

Plate 4. Two dimensional electrophoresis and chromatography of the dialyzable materials from chymotrypsin-digested acid-soluble calf skin collagen. Digestion occurred at 20°C for 24 hours. Black spots indicate material in the control; half-filled circles indicate positive staining with the chlorination stain where different from ninhydrin positive areas. Outlined circles indicate ninhydrin positive areas. The letter T indicates a positive tyrosine test. The letter O represents the point of sample application. The composite chromatogram represents two chromatograms, one stained for tyrosine and ninhydrin positive areas and one stained with the chlorination procedure.

4-a. A composite chromatogram following chymotrypsin treatment.

4-b. Resulting chromatogram after subtraction of the control spots.

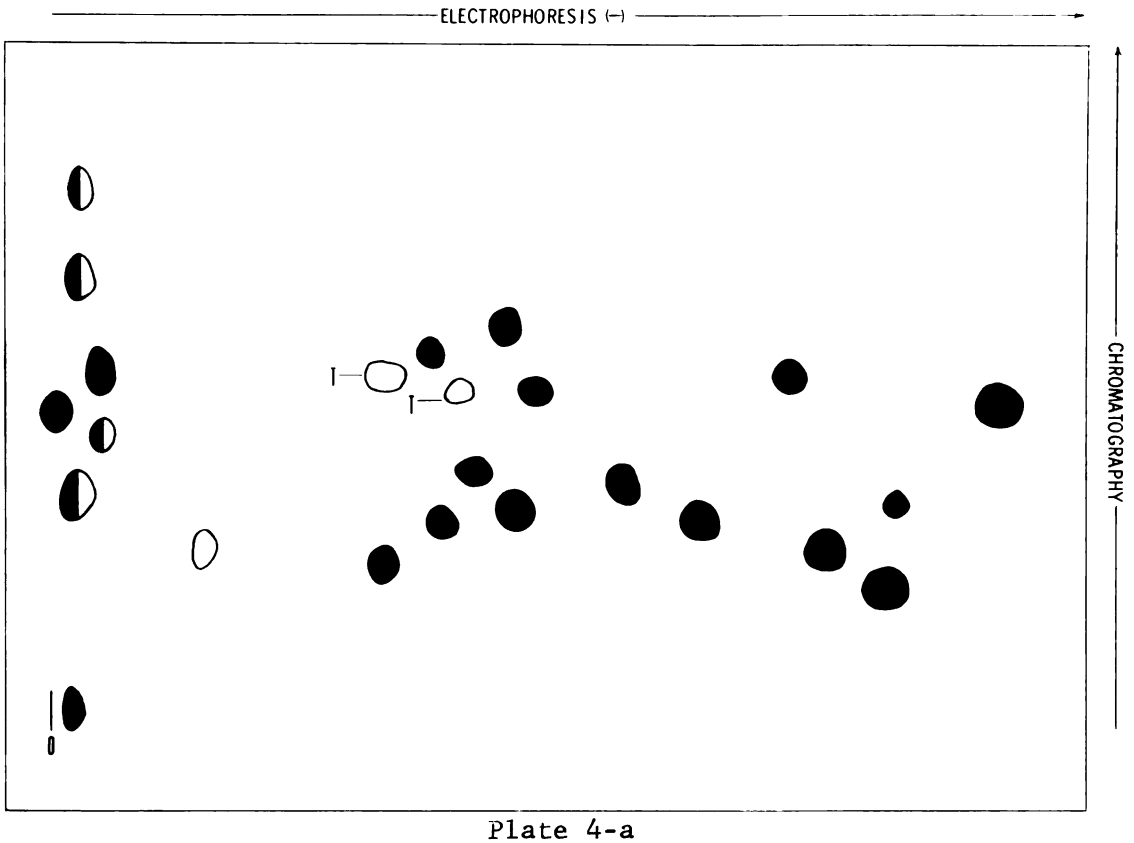


Plate 4-a

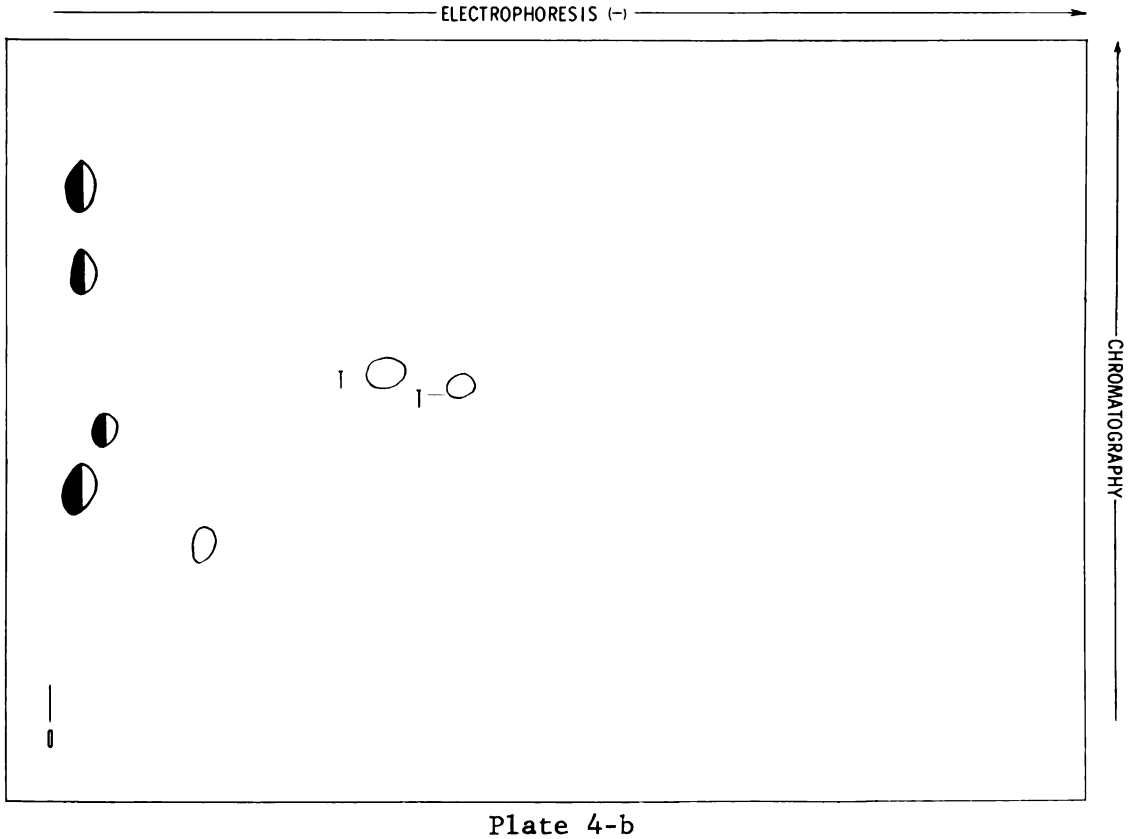


Plate 4-b

Plate 4.

Plate 5. Two dimensional electrophoresis and chromatography of the dialyzable materials from elastase-digested acid-soluble calf skin collagen. Digestion occurred at 20°C for 24 hours. Black spots indicate material in the control; half-filled circles indicate positive staining with the chlorination stain where different from ninhydrin positive areas. Outlined circles indicate ninhydrin positive areas. The letter T indicates a positive tyrosine test. The letter O represents the pointof sample application. The composite chromatogram represents two chromatograms, one stained for tyrosine and ninhydrin positive areas and one stained with the chlorination procedure.

5-a. A composite chromatogram following elastase treatment.

5-b. Resulting chromatogram after subtraction of the control spots.

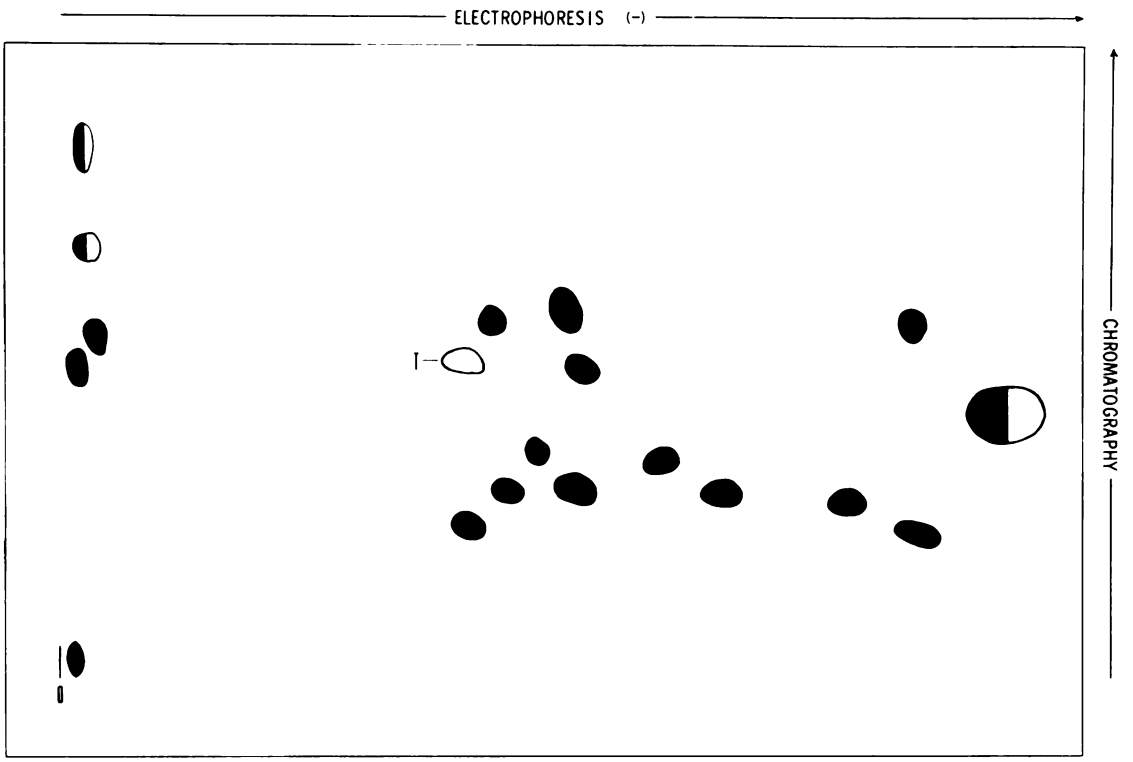


Plate 5-a

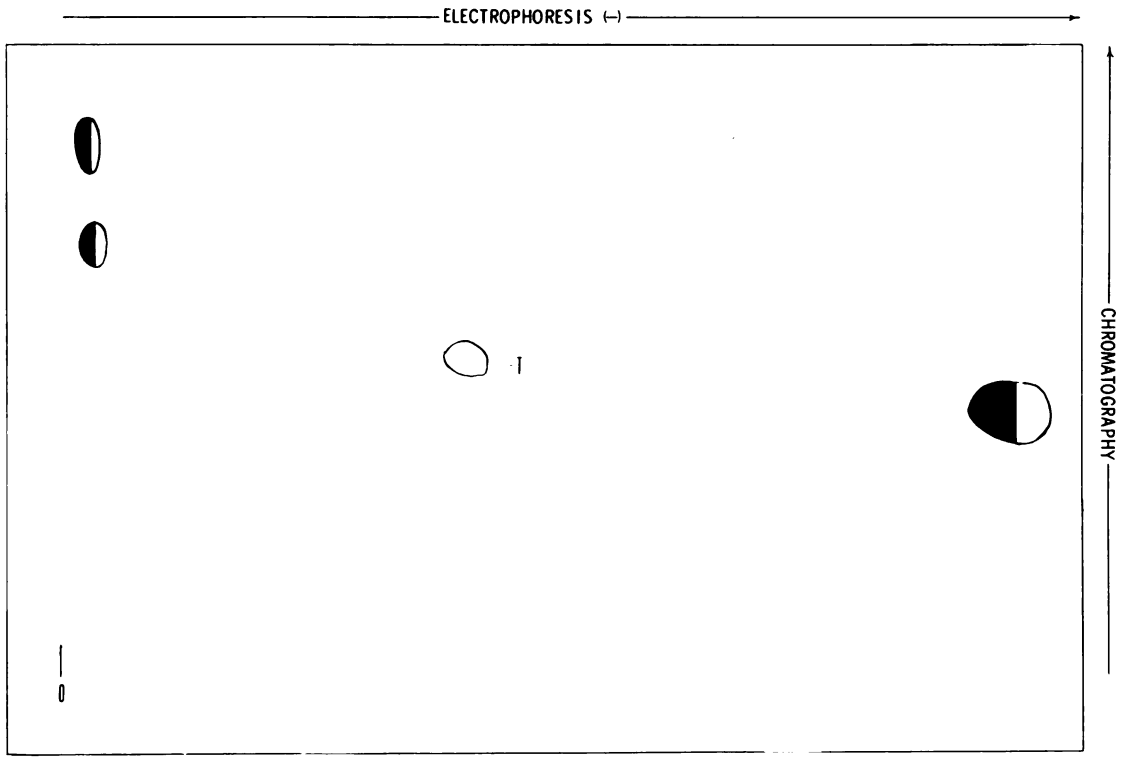


Plate 5-b

Plate 5.

This area had a different electrophoretic mobility compared to the similar spot of the trypsin digests run on the same paper in one dimension. Two-dimensional analysis of the chymotrypsin digests failed to show a positive tyrosine test in the area above the origin.

The second leading spot in the elastase and chymotrypsin digests (above the origin) and the spot above the origin in the pepsin digest have R_f values that are quite comparable. The average R_f values were 0.600, 0.623 and 0.675 for the elastase, chymotrypsin and pepsin digests, respectively. Only the spot from the pepsin digest gave a positive tyrosine test, and therefore, is probably distinctly different from the other spots. The proximity of the two spots described above in the various digests, plus the apparent lack of electrophoretic mobility at pH 2.0, indicates that these compounds are acidic in nature and may differ by only a few amino acid residues.

The remaining spots above the origin in the chymotrypsin digest have no counterparts in the other digests, and consequently, are unique to chymotrypsin.

The tyrosine staining areas in the various digests, which were electrophoretically mobile, differed both in mobility and R_f values between digests, as well as in the number of positive areas discernible. However, the spot labeled 1 on the control chromatogram (plate 1) gave a tyrosine positive test in the elastase digest, but did not give positive tests in numerous control samples. The tyrosine positive areas in the chymotrypsin digests have similar R_f values to that of the spot for the elastase digest. The R_f

values were 0.462 for the spot in the elastase digest and 0.495 and 0.473 for the two spots in the chymotrypsin digest. Since the spots are so close together, it is difficult to say that they are different. They may, however, have subtle differences, which would account for the variation in their R_f values and electrophoretic mobilities.

A comparison of the elastase and chymotrypsin digests reveals that they differ very little. The chymotrypsin digest has an extra positive tyrosine area and two other spots directly over the origin. The elastase digest differs in that it has a large chlorination positive area at the leading edge of the electrophoretic front. Other than the similarities previously discussed, the pepsin and trypsin digests showed no similar tyrosine staining spots. The remaining spots appear to have no counterparts, when all chromatograms are compared.

The repeatability of the electrophoretic runs was very good when duplicate samples were analyzed on the same paper. However, day-to-day repeatability was variable with respect to the relative mobility of a given spot. This was probably due to variations in the temperature during the run and the degree of wetness of the paper, as well as to fluctuations in current flow.

Control samples for all enzymes and substrates were run at the specific pH of the reaction media. All the controls were very similar, and indicated that the pH of the reaction media had no effect on the collagen substrate.

Enzyme-treated Acid-soluble Calf Skin Collagen

Amino acid analysis

Table 3 shows the amino acid composition of a 20-hour hydrolysate of acid-soluble calf skin collagen following pepsin-treatment at room temperature for 24 hours. On comparing the values for the pepsin-treated and the untreated sample, a loss of amino acids was apparent. Values that are the same or lower than the untreated values indicate that at least some of the particular amino acid was released due to enzymatic action. Values that are higher than the untreated ones indicate that the amino acid in question was either absent or only present in small amounts in the dialyzable products following enzymatic treatment. These conclusions are, of course, based on the assumption that the enzyme did, in fact, release dialyzable materials from the soluble collagen. This was confirmed by the 2-dimensional chromatography previously discussed. If material was removed from the original protein, then the remaining unaffected amino acids would show an increase in their relative proportion.

Spectrophotofluorimetric Analysis

Fluorimetric measurements were made to follow the changes in the fluorescing residues. Figure 2 shows the results of this experiment. The fluorescence spectra are uncorrected for light scattering effects. All samples were redissolved from the lyophilyzed state in 0.05% acetic acid for analysis. The untreated soluble collagen plus all treated samples showed a fluorescence maximum at 248 mu on excitation at 280 mu. Compared to the unlyophilyzed, untreated soluble collagen, which showed a fluorescence maximum at 325 mu (figure 1), there was a shift to the right of 23 mu.

Figure 2. Fluorescence spectra of enzyme-treated acid-soluble calf skin collagen. Excitation wavelength = 280 mu. Code: R.I. = Relative Intensity; mu = emission wavelength; 1 = pepsin treated sample; 2 = untreated collagen; 3 = chymotrypsin treated sample; 4 = trypsin treated sample; 5= elastase treated sample; and 6 = solvent blank. All samples had been lyophilized and redissolved in 0.05% acetic acid for analysis at approximately identical concentrations.

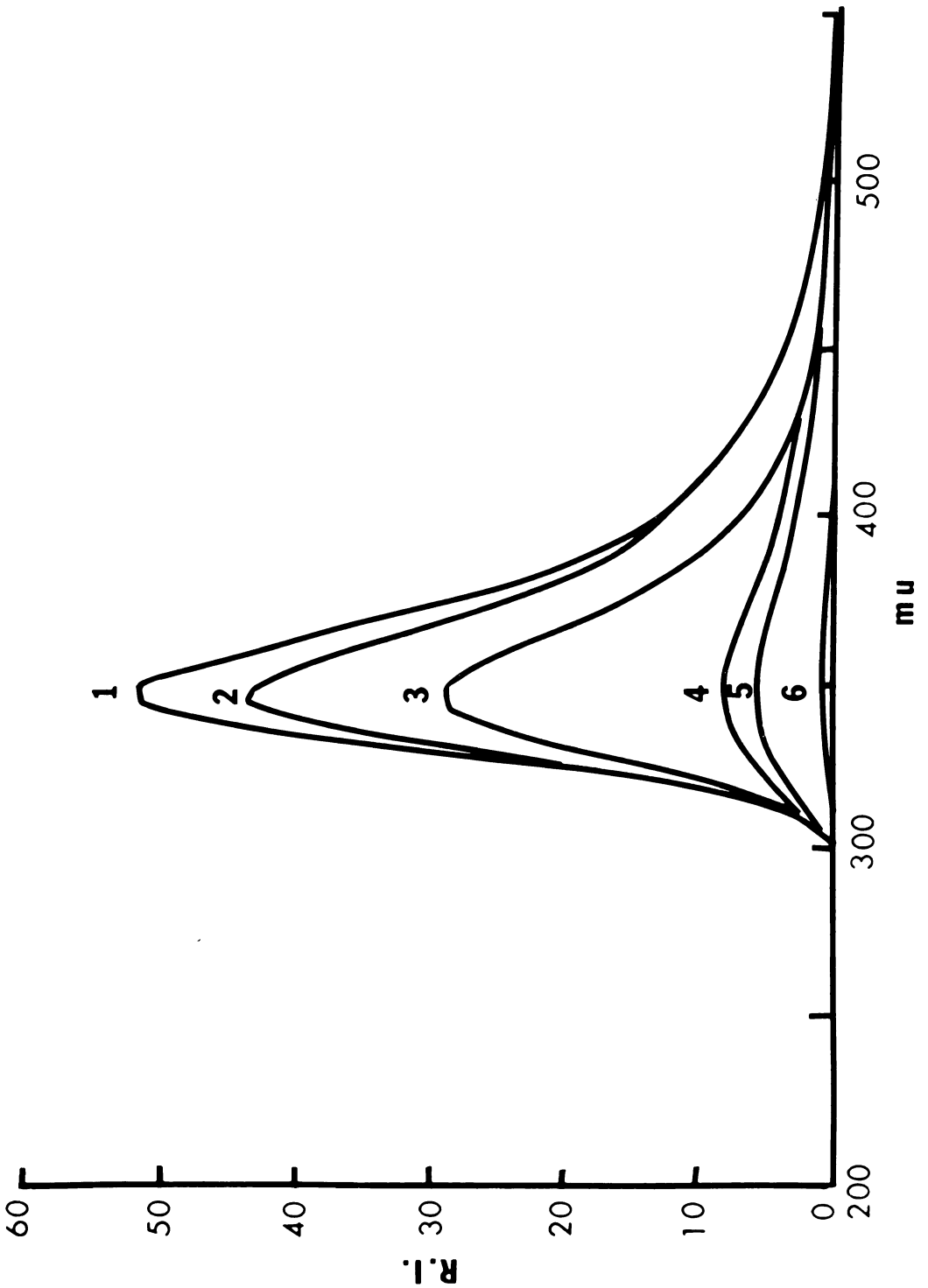


Figure 2.

The protein concentration was determined by weighing samples of the lyophilyzed protein and diluting to approximately the same concentration. The absorbance at 230 mu was recorded and used in conjunction with relative intensity to calculate the theoretical relative intensity. Assuming a linear relationship existed between fluorescence and concentration, the ratio of relative intensity to optical density at 230 mu for the untreated soluble collagen was calculated as follows:

$$R.I./O.D. = 44/.883 = 49.8$$

This value was then multiplied by the optical density obtained for each enzyme-treated protein solution, giving the theoretical relative intensity. The theoretical value was then compared with the actual relative intensity in order to clarify the recorded data. Table 4 shows the results for the values from figure 2 after being computed as described above. These values are uncorrected, i.e., the relative intensity of the blank was not subtracted, since its contribution was negligible.

The results of elastase- and trypsin-treatment of acid-soluble calf skin collagen are readily apparent. These enzymes produced a marked drop in the relative intensity of the protein, amounting to a decrease of approximately 88% for the elastase-treated sample, 82% for the trypsin treatment and 39% for the chymotrypsin-treated sample. On using more sensitive instrument conditions, the pepsin-treated sample yielded lower relative intensities than the control. However, under the conditions used in this experiment the pepsin-treated sample showed no apparent difference.

Table 4. Computed theoretical relative intensities of enzyme-treated acid-soluble calf skin collagen and the actual relative intensities obtained on the spectrophotofluorometer. Values are not corrected for the contribution of the solvent blank.

Treatment	Optical density at 230 mu	Relative intensity		
		Theoretical	Actual	% of theoretical
Pepsin	1.044	52.0	52.0	100
Trypsin	0.896	44.6	8.2	18.4
Elastase	0.966	48.1	6.0	12.3
Chymotrypsin	0.948	47.2	29.0	61.4
Untreated	0.883	44.0	44.0	100

Generally, these data are supported by the results of the chromatography study reported earlier. All of the dialysates, except the elastase dialysate, showed the presence of several tyrosine containing components. Therefore, a reduction in the intensity of the fluorescence of the respective protein solutions would be expected. The elastase-treated sample showed very little tyrosine in the dialysate, and yet this enzyme had the greatest effect on the fluorescing properties of the protein.

These results show that the loss of tyrosine does lower the fluorescence intensity of the protein. Furthermore, it is indicated, especially by elastase treatment, that the enzymes may affect the fluorescing components by cleavage of key residues, but not necessarily tyrosine. This could result in configurational changes that surpress or quench the fluorescence. Another consideration could be that tyrosine residues,

are in fact cleaved, but are present in the dialysate in some form not detectable by the procedure utilized.

Disc Gel Electrophoresis

Acid-soluble calf skin collagen digested at room temperature with the four proteolytic enzymes produced the patterns shown in plate 6-a. The protein samples were heat denatured at 40-45°C before electrophoresis. The banding pattern is indicated. The α-component is the first dark staining band behind the buffer front, followed by the β-component and then a small amount of the γ-component. Penetration of the 7 1/2% lower gel by the γ-component is usually not observed. The penetration shown in plate 6-a was probably due to the fact that the gel was not 7 1/2% at the interface, but was diluted in layering with water.

The gel patterns for the various enzyme treatments show that all of the protein applied entered the gel. However, the control sample showed protein remaining on top of the 3 1/2% upper gel. This indicates that all enzymes must have had an effect in de-polymerizing the larger protein aggregates. A close inspection of the gel patterns reveals a decrease in the amount of the γ-component in the pepsin- elastase- and chymotrypsin-treated protein and a decided increase in the α-component in the chymotrypsin-treated protein. The elastase-treated sample also shows an increased amount of α-component as well as an increase in the amount of the β-component. The trypsin-treated sample, except for the lack of large aggregates on the upper gel, shows little to no change from the control. The pepsin-treated sample exhibited about equal proportions of

Plate 6. Disc gel patterns of enzyme-treated, acid-soluble calf skin collagen. The protein was treated with the enzymes at room temperature for 24 hours. Code: BSC = untreated protein; BPR_x = pepsin-treated protein; BTR_x = trypsin treated protein; BER_x = elastase-treated protein and BCR_x = chymotrypsin-treated protein. The banding patterns are coded: a = α-component; b = β-component; c = γ-component and f = the buffer front.

6-a. The protein samples were heat denatured at 40-45°C for 20 minutes prior to electrophoresis.

6-b. The protein samples were not heat denatured prior to electrophoresis.

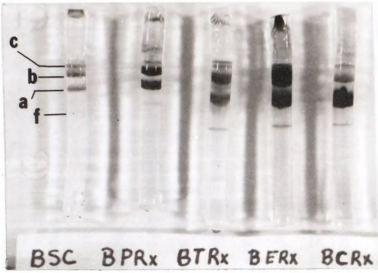


Plate 6-a

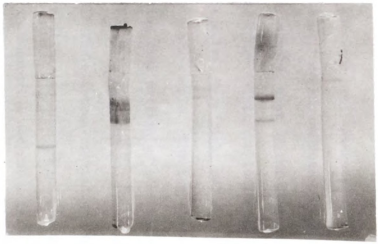


Plate 6-b

Plate 6.

the α- and β-components. The concentration of the enzyme-treated protein and the untreated sample was different as shown by the widths of the various staining components.

These same protein samples were run under identical conditions, but were not heat denatured. This study was conducted in an effort to show that the enzymes would produce definite changes in the protein and that such changes would be apparent without previous heat denaturation. Plate 6-b shows the results of such an experiment, and though not apparent, except in the case of the pepsin-treated sample, most of the protein sample applied to the upper gel remained there. This was indicated by the dark staining area at the top of the gels. Very light α-, β - and γ-bands were present in the untreated protein sample. The trypsin-treated sample also showed very light bands in the same pattern as the control. The pepsin-treated sample showed definite staining of α- and β-components similar to the same sample in plate 6-a. The elastase-treated sample showed a dark staining α-component and a light staining β-component. The chymotrypsin-treated sample showed that material had moved into the lower gel, but this tube apparently received very little current as was evidenced by the buffer front, which had moved very little. Nevertheless, it was apparent that the enzymatic effects could be monitored without heat denaturation.

During the tenure of these experiments, the gel tubes received 5 ma of current for approximately 1 hour. The tubes warmed up rapidly and were very warm to the touch at the conclusion of the experiment. The

experiment was repeated using a current of 1-2 ma/tube for approximately 3 hours. The tubes were not warm to the touch at the conclusion of the run. Staining of the gels revealed no differences between the control and enzyme-treated samples. It was therefore concluded that at the higher current, some heat denaturation of the protein must have occurred, thereby giving the results in plate 6-b. Nevertheless, the enzyme-treated samples were definitely stronger staining than the control. However, the heat denaturation occurring in the tubes seemed to complicate the interpretation of enzymatic treatment. Therefore, all future samples used for disc electrophoresis were heat denatured prior to electrophoresis.

A similar experiment was performed using the protein from a 20°C digestion for 24 hours. These results are shown in plate 7. Plate 7-a shows the result of heat denaturation of the enzyme-treated tropocollagen at 40-45°C for 20 minutes. The control gel pattern is labeled, showing that the various components are present and moved as previously described. However, several other lightly staining bands appeared between the α-component and the buffer front in all samples. These light staining bands will be discussed later.

The control sample in plate 7-a shows that large aggregates on the upper gel did not penetrate, even after heat denaturation. Two other control samples (not shown) revealed similar patterns. All of the enzyme-treated samples showed that no protein remained on the upper gel. The lower gel of the various enzyme-treated protein samples shows an increase in the amount of the α-component in the pepsin- elastase- and chymotrypsin-

Plate 7. Disc gel patterns of acid-soluble calf skin collagen digested at 20°C for 24 hours. Code: BSC = untreated protein; BPRx = pepsin-treated protein; BTRx = trypsin-treated protein; BERx = elastase-treated protein and BCRx = chymotrypsin-treated protein. The banding patterns are coded: a = α-component; b = β-component; c = γ-component and f = the buffer front.

7-a. The protein was heat denatured prior to electrophoresis.

7-b. The protein was not heat denatured prior to electrophoresis.

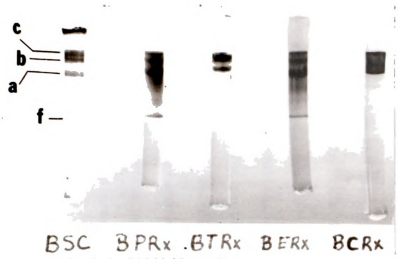


Plate 7-a

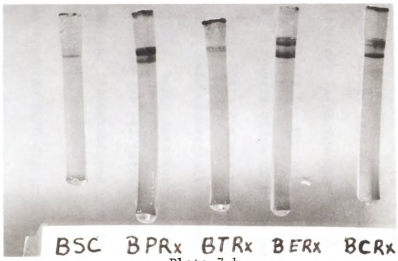


Plate 7-b

Plate 7.

treated samples. Very little of the β-component remained in the elastase- and chymotrypsin-treated samples.

Similar results were obtained by Bornstein et al. (1966b) for chymotrypsin-treated, acid-soluble rat skin collagen. In the present study, the trypsin-treated sample was similar to that of the control, except that no protein remained on the upper gel. Comparison of Plates 6-a and 7-a shows that the gel patterns are similar, except that the elastase-treated protein in Plate 6-a shows a relatively large amount of the β-component. The gel in plate 6-a was apparently overloaded with protein, as is obvious by the extra width of the α- and β-components. The protein concentration in Plate 7-a was much less and may account for the discrepancy. The protein concentrations were different between experiments for all samples, being less for the 20°C digestion. The protein concentrations used for the gel patterns in Plate 7-a were approximately the same, but the concentrations used in the gel patterns shown in Plate 6-a varied.

The enzyme-treated protein resulting from the 20°C digestion was treated as previously described, but was not heat denatured. The gel patterns are shown in plate 7-b. Note the presence of protein staining on top of the upper gel in every case. Inspection of the lower gel reveals a very light staining α-component in the control sample. The pepsin-treated and elastase-treated samples show strong staining α- and β-components. The elastase- and chymotrypsin-treated samples show the presence of both α- and β-components, with the α-component being about

twice the size of the β-component. The trypsin-treated sample shows some staining at the top of the lower gel. The bands in the lower gel are apparently due to a small amount of the α- and β-component which have not fully entered the gel. As the results following electrophoresis of the native protein were similar to those previously discussed, heat denaturation was utilized on all subsequent samples.

The results from the heat denatured samples following digestion at 20°C for 24 hours showed that the elastase- and chymotrypsin-treated protein gave very similar patterns. Both of these treatments resulted in an increased amount of the α-component at the expense of the β-component. The effect on the γ-component was not readily discernible due to the small amount present. Drake et al. (1966) showed that pepsin-, elastase- and chymotrypsin-treated acid-soluble calf skin collagen resulted in an increased amount of the α-component at the expense of the β-component, when analyzed on the analytical ultracentrifuge. They further showed no change in the amount of the γ-component. However, it was present in such small amounts that subtle changes probably would not have been observed. Rubin et al. (1965) using column chromatography and ultracentrifuge data also showed that pepsin treatment of acid-soluble calf skin collagen resulted in an increase in the α-component at the expense of the β-component. This was also confirmed by Kühn et al. (1966).

In the present study, trypsin treatment of the acid-soluble collagen showed very little difference on disc gel electrophoresis. Drake et al.

(1966) showed a slight increase in the amount of the α-component following trypsin treatment in comparison to an untreated sample.

The disc gel results of the present investigation confirm the previously mentioned work. The results demonstrate that elastase and chymotrypsin, and probably pepsin, cleave intramolecular cross-links as shown by the increase in the α-component relative to the β-component. Trypsin treatment apparently has very little effect on the intramolecular cross-links. All the enzymes seem to readily reduce protein aggregation as shown by the absence of protein staining on the upper gel. This observation would indicate that all the enzymes were effective in disrupting intermolecular cross-links.

Thermal Gelation

Figure 3 shows the results of short time studies on the ability of the enzyme-treated protein to polymerize on warming to 33-35°C in a phosphate-NaCl buffer at an ionic strength of 0.6M and a pH of 7.4. Preliminary experiments at various protein concentrations showed that the lag phase of the curves were concentration dependent. These results confirmed those of Gross and Kirk (1958) who also showed the dependence on the initial concentration. The apparent similarities of the pepsin- and trypsin-treated protein curves can be explained on this basis. The trypsin-treated protein was less concentrated than the pepsin-treated sample by 0.13 optical density units (measured at 230 mu). Subsequent experiments at similar concentrations showed that the trypsin-treated protein had a slightly increased lag phase over the untreated sample. Hodge et al.

Figure 3. Thermal gelation curves of lyophilyzed, enzyme-treated, acid-soluble calf skin collagen. Thermal gelation occurred at 33°C using a 0.6M phosphate-NaCl buffer at a pH of 7.4. The optical density (O.D.) was monitored at 230 mu. Code: sc = untreated protein; p = pepsin-treated protein; t = trypsin treated; e = elastase treated and c = chymotrypsin treated.

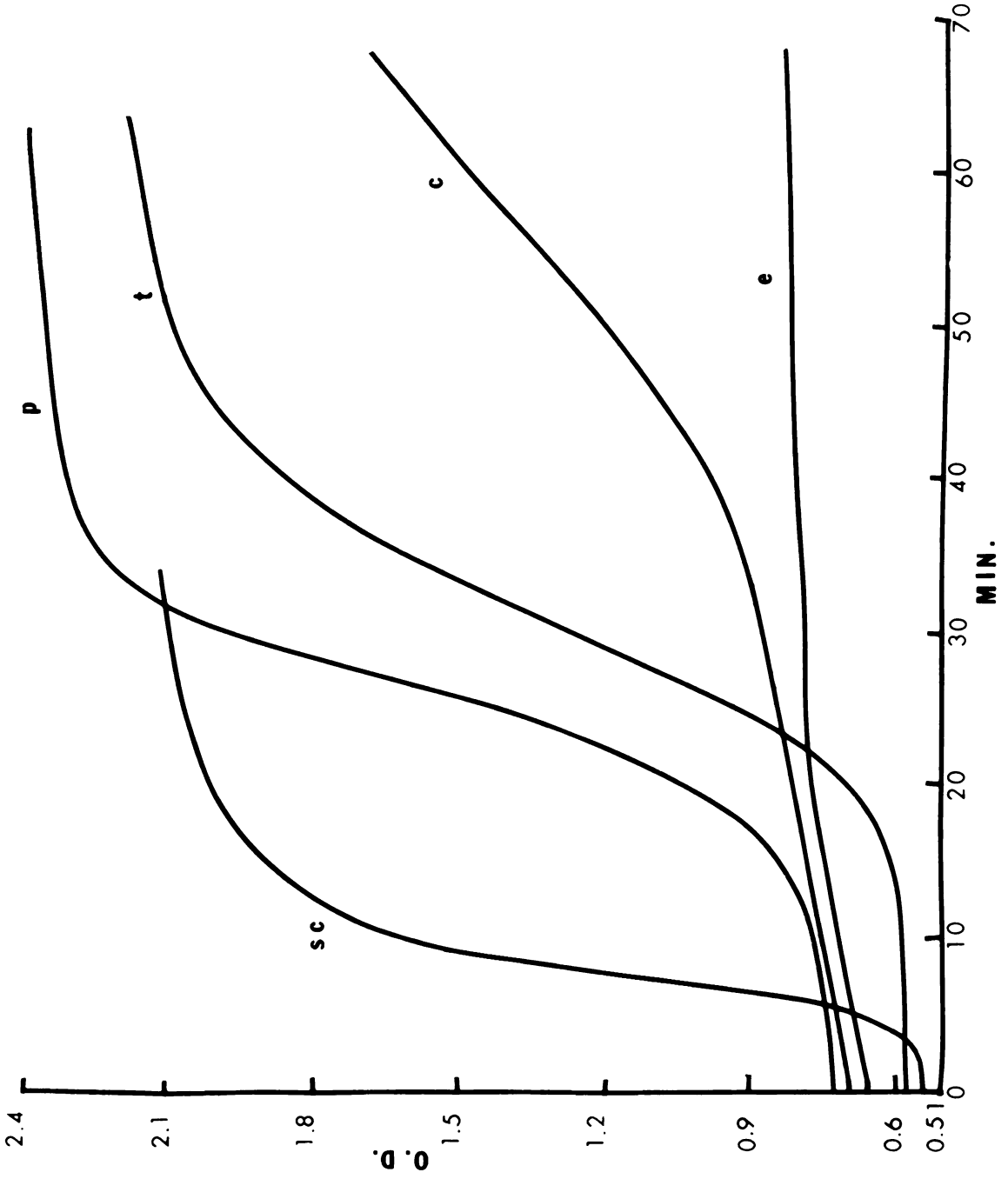


Figure 3.

(1960) also showed that trypsin-treated collagen exhibited a longer lag phase than an untreated control. The untreated protein curve (figure 3) was also less concentrated than the enzyme-treated samples, because higher concentrations resulted in an instantaneous polymerization of the protein in the cuvette and an incomplete curve.

The curves in figure 3 show an increase in the lag phase for the curves following pepsin- or trypsin treatment of the protein. Chymotrypsin treatment resulted in a curve with a very gradual slope. The elastase-treated protein never formed a gel or fibrous precipitate during the duration of these short term experiments. On the other hand the untreated sample readily formed a slightly opaque gel, as indicated by the sharp slope of the curve.

The physical appearance of the enzyme-treated samples at the conclusion of the experiment indicated that the precipitate was more fibrous than gel-like. The trypsin- and pepsin-treated samples were composed of fibers in a gel-like matrix. However, the pepsin-treated samples were more fibrous than the trypsin-treated ones. Both samples appeared homogeneous as opposed to the chymotrypsin-treated sample, which contained a small amount of fibrous precipitate that eventually floated to the top of the solution. The elastase-treated sample remained clear with no gel or fiber formation being observed.

Listed in order of increasing ability to prevent aggregation, the enzymes would be arranged as follows: trypsin < pepsin < chymotrypsin < elastase. Elastase was the most effective in preventing aggregation.

The disc gel electrophoretic data previously discussed, as well as the results of other workers (Drake et al., 1966; Rubin et al., 1965; and Kühn et al., 1966) if arrayed in increasing order of the effectiveness of the enzymes to cleave intramolecular bonds, would give a similar sequence. The effectiveness of the enzymes would be as follows: trypsin < pepsin < chymotrypsin and elastase. Chymotrypsin and elastase had approximately equal effects.

The results of long term thermal gelation experiments (5-24 hours duration) in tables 5 and 6 give a somewhat different view. The percentages of the original protein remaining in solution are reasonably close, except for the value for the chymotrypsin-treated sample. The discrepancy in the two values for chymotrypsin prompted other experiments with this protein sample. The results showed that after 24 hours the chymotrypsin-treated protein had values similar to those of the elastase-treated sample shown in tables 5 and 6. Both the elastase- and chymotrypsin-treated samples seemed to require more time for precipitation of small amounts of protein.

Trypsin treatment seemed to be more effective in keeping the protein from aggregating than pepsin treatment. If the enzymes were again arrayed in order of increasing ability to prevent polymerization, they would appear as follows: pepsin < trypsin < chymotrypsin < elastase. Elastase was consistently the most effective.

The results of disc gel electrophoresis of heat denatured, enzyme-treated collagen and the short term thermal gelation experiments indicated that the polymerization of the protein depends to a large degree on the

Table 5. Long term thermal gelation experiment of 5 hours duration. The percent protein remaining in solution was determined by the changes in absorbance at 230 mu before and after thermal precipitation.

Collagen treatment	Absorbance (O.D.)		% protein remaining in solution
	Before gelation	After gelation	
Untreated	0.660	0.317	48.0
Pepsin	0.425	0.317	74.6
Trypsin	0.356	0.308	86.5
Chymotrypsin	0.581	0.388	66.8
Elastase	0.638	0.597	93.6

Table 6. Long term thermal gelation experiment of 24 hoursduration. The percent protein remaining in solution was determined by the changes in absorbance at 230 mu before and after thermal precipitation.

Collagen treatment	Absorbance (O.D.)		% protein remaining in solution
	Before gelation	After gelation	
Pepsin	0.720	0.514	71.4
Trypsin	0.775	0.690	89.0
Chymotrypsin	0.720	0.568	78.9
Elastase	0.660	0.642	97.3

integrity of the intramolecular cross-link(s) of the β -component or even the intact β -component itself. The results of the long term thermal gelation studies indicated that the enzymatic effects might be more complicated than the mere cleavage of the covalent cross-link(s) of the β -component.

In light of the previous results, it was postulated that the β -component is the major factor necessary for polymerization of collagen. If this is true, a greater proportion of the α-component should be present in the supernatant of the samples, which failed to gel or form extensive fiber networks, following long term thermal gelation.

Plate 8 shows the disc gel patterns of enzyme-treated collagen both before and after long term thermal gelation. The samples that fitted the above hypothesis included the chymotrypsin- and elastase-treated samples. Plate 8-b shows that both of these samples exhibited substantial amounts of the α-component (especially the chymotrypsin-treated sample) and smaller amounts of the β -component, as well as some lightly stained bands between the α-component and the buffer front. The control sample shows traces of these two components and numerous smaller bands. The pepsin- and trypsin-treated samples showed the presence of a small amount of the α-component and traces of the β -component, as well as numerous smaller bands located between the α-component and the buffer front. The trypsin-treated sample also showed staining in the buffer front, indicating the presence of some fast moving components.

Results of disc gel electrophoresis of the elastase- and chymotrypsin-treated protein and those from the filtrates of the two enzyme-treated

Plate 8. Disc gel patterns of enzyme-treated, acid-soluble calf skin collagen and a control before and after thermal gelation. All samples were heat denatured at 40-45°C for 20 minutes prior to electrophoresis. Enzyme digestion conducted at 20°C for 24 hours. Gel code: BSC = untreated acid-soluble calf skin collagen; BPRx = pepsin treated; BTRx = trypsin treated; BERx = elastase treated and BCRx = chymotrypsin treated.
Gel pattern code: a = α-component; b = β-component; c = γ-component and f = buffer front.

8-a. Before thermal gelation.

8-b. After thermal gelation at 35°C for 12 hours.

Plate 8-a

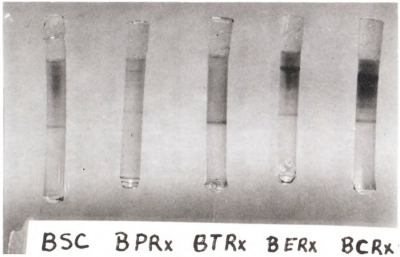


Plate 8-b

Plate 8.

samples support the hypothesis that the cross-link in the β -component is an important factor in the polymerization of the protein. However, other considerations may be proposed, for example, the enzymes may be cleaving other peptides at strategic loci concomitantly with the cleavage of the cross-link of the β -component. This could lead to the same results. Furthermore, the β -component itself may be necessary to fulfill certain steric requirements, as yet unknown. Finally, the α-component observed after gelation in the elastase- and chymotrypsin-treated samples may not all be derived from the β -component. The enzymes may also cleave certain residues on the α-chains in the intact collagen molecule, and thereby prevent this component from participating in polymerization.

The light staining bands observed between the α-component and the buffer front were readily observed when large amounts of the protein were applied on disc gel electrophoresis. Unfortunately, the majority of these bands did not photograph. Plate 9 shows a magnified photograph of the lower gel. The light bands were not visible, but a schematic drawing (column B) accompanies each gel to indicate the location of these light staining bands. Columns A and B show the photograph and drawings, respectively, of the gel patterns for the enzyme-treated protein before gelation. Columns D and E show the photograph and drawings, respectively, of the gel patterns of the enzyme-treated protein after gelation.

Though the bands were apparent in every electrophoretic run and seemed to exhibit similar patterns between treatments, emphasis on their significance and actual similarities will need to await more detailed study.

Plate 9. Magnified gel patterns and schematic drawings of enzyme-treated, acid-soluble calf skin collagen. Drawings show the approximate location of fast-moving, weakly staining bands between the α-component and the buffer front before and after thermal gelation. The banding patterns following carbohydrate staining are also shown schematically. Columns A, B and C may be directly compared within and between treatments. Columns D and E may be similarly compared. Code: BSC = untreated acid-soluble calf skin collagen; BPRx = pepsin treated; BTRx = trypsin treated; BERx = elastase treated and BCRx = chymotrypsin treated. Staining intensity is indicated by width of the drawn bands, i.e., the wider the band, the more intense the staining.

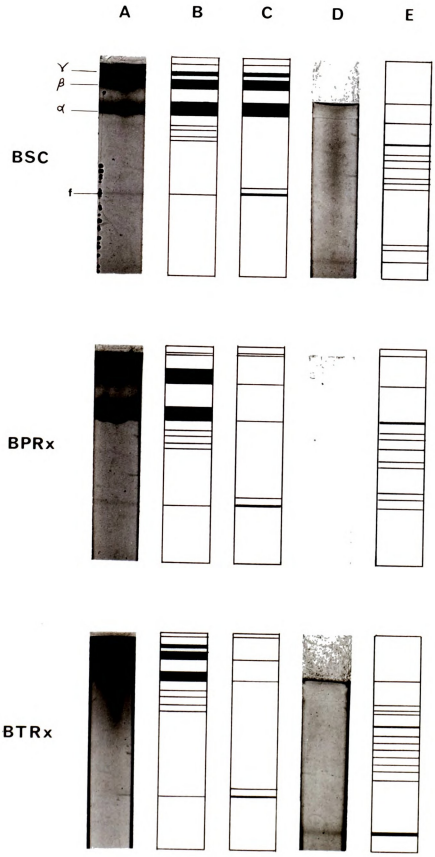
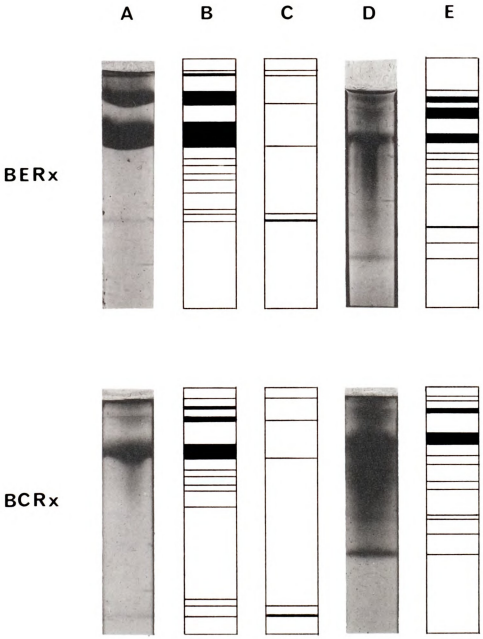


Plate 9.



A - Disc gel stained for protein before thermal gelation of the acid-soluble collagen.

B - Drawing of A showing strong and weak staining bands.

C - Drawing of carbohydrate staining patterns.

D - Disc gel stained for protein after thermal gelation of the acid-soluble collagen.

E - Drawing of D showing strong and weak staining bands.

Plate 9. (continued)

They are shown to point out their existence and are commented on in passing. The most significant difference noted was that the trypsin-treated protein after gelation exhibited a larger number of lightly staining bands than any other sample. Similar experiments showed that trypsin treatment resulted in a darker staining buffer front than any of the other samples. The presence of numerous bands in the trypsin-treated sample support the data previously reported in tables 5 and 6, which show that the trypsin-treated sample had a relatively larger amount of protein in solution than the other enzyme-treated samples, with the exception of those treated with elastase. However, the elastase-treated sample did not display as an extensive banding pattern as did the trypsin-treated sample, but it did have a much higher concentration of the α-component. The chymotrypsin-treated protein also had a higher concentration of the α-component than the trypsin-treated sample. Furthermore, the photograph of banding patterns of the chymotrypsin-treated protein (plate 9, column D) clearly shows the presence of small bands between the α-component and the buffer front.

The acrylamide gels, which contained the enzyme-treated protein following electrophoresis, were sliced longitudinally with a scalpel. One-half of the gel was stained with the protein stain and the other half was stained for carbohydrates. The light red staining components resulting from the carbohydrate staining procedurewere too faint to photograph, but a schematic drawing in column C of plate 9 shows the position of the carbohydrate positive areas relative to the protein stained areas of the

photograph in column A. The carbohydrate staining resulted in very weak bands that matched the α-, β - and γ-components in most cases. However, all samples showed two other positive bands, one at the buffer front and one just behind the buffer front. These results indicated that the protein contained only small amounts of carbohydrates, and that the enzymes had no obvious effect on the staining of the carbohydrates in the protein as compared to the control.

In all the disc gel patterns discussed, the enzyme blanks were analyzed for their contribution. No banding pattern was visible in any of the enzyme controls.

Acid-Soluble Pig Skin Collagen

Protein Purification

The yield following extraction of acid-soluble pig skin collagen (fraction 2A) was much less than that of acid-soluble calf skin collagen. Furthermore, the treatment of the skin prior to extraction was markedly different. The normal slaughtering procedure for pigs involves scalding for easy removal of the hair. The heat treatment may affect the solubility or alter some of the properties of pig skin collagen. The pigs used were approximately 4-6 months of age, which could account for the lower yield of acid-soluble collagen from the skin as compared to the yield from the one month-old calf.

The extracted acid-soluble pig skin collagen was redissolved and re-precipitated numerous times to effect purification. The purity criteria

used were hydroxyproline analysis and spectrophotofluorimetry. A portion of the solubilized protein was lyophilized and dried over $CaCl_2$ for 2 days. The nitrogen content of triplicate samples was 17.79, 17.77 and 17.74%, resulting in an average of 17.76%. This value was used to calculate the protein concentration of solutions of pig skin collagen from micro Kjeldahl analysis. The final hydroxyproline content obtained, based on the protein content, was 13.2%. The hydroxyproline recovery was 98 percent on adding it to protein solutions.

Figure 4 shows that the untreated acid-soluble pig skin collagen had a maximum fluorescence peak at approximately 325 mu, which was identical to the tyrosine and acid-soluble calf skin collagen as shown in figure 1. However, analysis of the pig skin collagen sample utilized a wider light path (slit width) for excitation and emission, and consequently shows the presence of a shoulder between 375 and 420 mu. Acid-soluble calf skin, when similarly analyzed, exhibited a fluorescence spectrum that was nearly identical. The difference was a somewhat more gentle slope to the shoulder on the calf skin collagen peak. Furthermore, at approximately the same concentration, the acid-soluble calf skin collagen exhibited a relative intensity that was 44% more than the acid-soluble pig skin collagen.

The acid-soluble pig skin collagen, when analyzed with the same slit width arrangement as the acid-soluble calf skin collagen (figure 1), produced a very weak fluorescence spectrum. The sensitivity of the instrument was increased to the maximum, but resulted in a wavy, unsteady

Figure 4. Fluorescence spectra of enzyme-treated acid-soluble pig skin collagen. Excitation wavelength = 280 mu. Code: R.I. = Relative Intensity; mu = emission wavelength; 1 = trypsin treated sample; 2 = chymotrypsin treated sample; 3 = elastase treated sample; 4 = pepsin treated sample; 5 = untreated sample and 6 = solvent blank. These samples were not lyophilyzed prior to analysis.

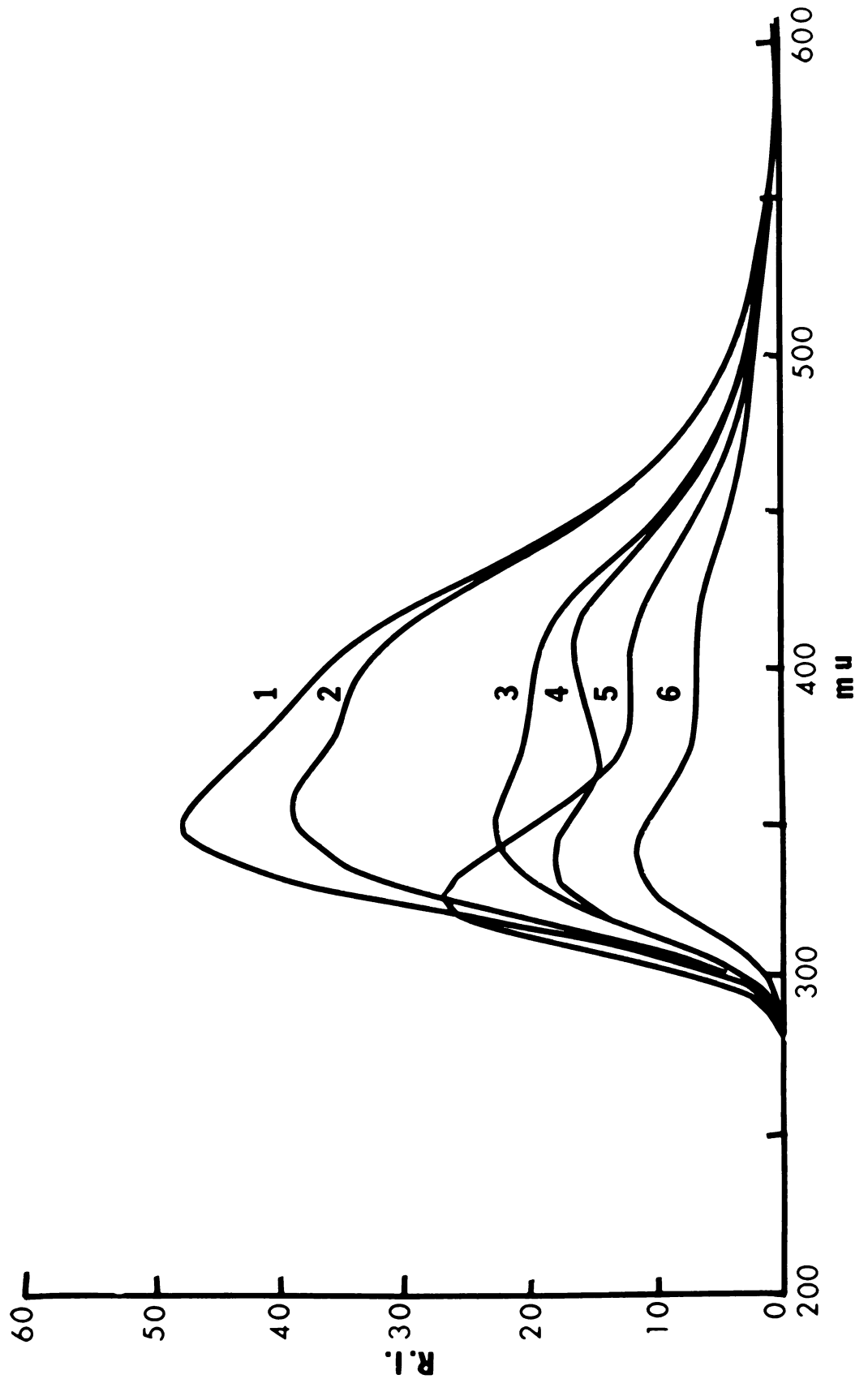


Figure 4.

tracing. Therefore, the greater slit width was used to avoid the unsteady response and produced the spectrum shown in figure 4.

Results suggest that at similar protein concentrations, the acid-soluble calf skin collagen produced higher fluorescent energy yields than the pig skin collagen. Furthermore, results indicate that the fluorescence spectrum of the untreated acid-soluble pig skin collagen (figure 4) did not contain tryptophan.

Enzyme Treated Acid-Soluble Pig Skin Collagen

Spectrophotofluorimetric Analysis

Fluorimetric measurements were made to observe any loss in fluorescing residues, which would be shown by a decrease in relative intensity, or any change in the fluorescence spectrum associated with the action of the enzymes upon the acid-soluble collagen. Figure 4 shows the result of such an experiment. The excitation wavelength was 280 mu, and the spectra are uncorrected for light scattering. The samples had not been lyophilyzed prior to analysis, but were dissolved in 0.05% acetic acid. Examination of figure 4 reveals that the fluorescence maxima of the chymotrypsin-, trypsin- and elastase-treated protein samples shifted towards a longer wavelength of approximately 350 mu. The pepsin-treated sample shifted to 337 mu, while the untreated protein has a maximum at approximately 327 mu.

Furthermore, the relative intensity of fluorescence of the chymotrypsin- and trypsin-treated samples are much greater than the untreated control. All of the enzyme-treated samples show changes in the shape of

the fluorescence spectrum with respect to the untreated sample. Table 7 shows these results on the basis of the actual intensities versus the calculated theoretical intensities as previously described. Table 8 shows the results, expressed on the basis of protein concentration, as determined by micro Kjeldahl of duplicate samples. A ratio of the relative intensity to protein concentration (mg/ml) was found for the untreated protein as follows:

$$\text{R.I./mg./ml.} = \frac{15.2}{.975} = 15.6$$

This value was thenmultiplied by the protein concentration obtained for each enzyme-treated protein, resulting in the theoretical relative intensity. A linear relationship of protein concentration to relative fluorescence was assumed.

The discrepancy between the absorbance at 230 mu and the protein concentration of the elastase-treated sample was noted. Consequently, a dilution series was made on the untreated protein, measuring the absorbance at 230 mu on known concentrations of the protein. The absorbancies given in table 7 were plotted on the curve to obtain the protein concentration. These data and the ratio of optical density to concentration are shown in tables 7 and 8 and are compared in table 9.

It is evident from table 9 that the apparent linearity shown by the ratio of optical density to protein concentration (in the range between 0.814 and 1.23 optical density units) is not followed by the pepsin- or trypsin-treated protein. Most of the values shown for the untreated,

Table 7. Computed theoretical relative intensities of enzyme-treated, acid-soluble pig skin collagen and the actual relative intensities obtained on the spectrophotofluorometer. The contribution by the solvent blank has been subtracted.

Protein treatment	Absorbance (O.D.) at 230 mu	Relative intensity		% of theoretical
		Theoretical	Actual	
Untreated	1.231	15.2	15.2	100.0
Pepsin	0.894	11.0	6.0	54.5
Trypsin	0.909	11.2	36.0	321.0
Chymotrypsin	0.988	12.2	27.0	225.0
Elastase	0.814	10.0	11.0	110.0

Table 8. Theoretical relative intensities computed on the basis of protein concentration (mg/ml) of enzyme-treated, acid-soluble pig skin collagen and the actual relative intensities observed on the spectrophotofluorometer. The contribution by the solvent blank has been subtracted.

Protein treatment	Protein concentration (mg/ml)	Relative intensity		% of theoretical
		Theoretical	Actual	
Untreated	0.975	15.2	15.2	100.0
Pepsin	0.598	9.3	6.0	64.5
Trypsin	0.598	9.3	36.0	387.1
Chymotrypsin	0.776	12.1	27.0	223.1
Elastase	0.634	9.9	11.0	111.1

Table 9. Comparison of protein concentrations obtained from a standard dilution curve (absorbancy at 230 mu versus protein concentration) and concentrations determined by the micro Kjeldahl procedure of enzyme-treated samples used in fluorescence study.

	Protein concentration (mg/ml)			O.D.
Protein treatment	Micro Kjeldahl	Dilution curve	Absorbance (O.D.) at 230 mu	Kjeldahl determined concentration
Untreated	0.975	1.01	1.231	1.26
Pepsin	0.598	0.73	0.894	1.50
Trypsin	0.598	0.75	0.909	1.52
Chymotrypsin	0.776	0.80	0.988	1.28
Elastase	0.634	0.66	0.814	1.28

chymotrypsin- and elastase-treated protein appear to be within the range of experimental error. The optical density to Kjeldahl-determined concentration ratios are in good agreement, except for the trypsin- and pepsin-treated protein. These data along with the results of the fluorimetric analysis (figure 4) indicate that pepsin and trypsin have marked, but different effects on the protein.

The decreased relative intensity shown by the pepsin-treated sample indicates a loss of fluorescing residues. The increased absorbance at 230 mu (table 9) suggests that the enzyme has other specific effects. The same is true in the case of the trypsin-treated sample, except that this enzyme must allow other residues, which were masked prior to treatment, to fluoresce. This would result in an increased relative intensity in comparison to the control. While the evidence presented in table 9 is by no means conclusive, it indicates that trypsin and pepsin release different components from the protein.

The fluorescence spectrum of the chymotrypsin-treated protein resembles that of the trypsin-treated sample. The same type of reasoning as was explained above for the trypsin-treated protein must be invoked to explain the increased fluorescence. It is possible that contamination may be responsible, although there was no evidence of microbial contamination.

Thermal Gelation and Disc Gel Electrophoresis

Plate 10-a shows the disc gel patterns of enzyme-treated, acid-soluble pig skin collagen. The untreated sample showed a greater proportion of the β-component than the α-component. Large aggregates were stained on top of the gel as in the case of acid-soluble calf skin collagen. Most of the enzyme-treated samples showed no staining at the top of the upper gel. The chymotrypsin-treated protein showed a trace of staining at the top of the upper gel. All of the treated samples show a definite change in the α- to β -component ratio, when compared with the untreated sample. The elastase- and chymotrypsin-treated samples are markedly reduced in the amount of β -component and show increased amounts of the α-component. The pepsin-treated sample showed a more even distribution of the α- and β -components, but favored the α-component. The trypsin-treated sample exhibited approximately an even distribution of the two components.

These patterns were obtained prior to thermal gelation of the protein. Plate 10-b shows the gel patterns of the filtrates following thermal gelation and filtration. These patterns are similar to those obtained

Plate 10. Disc gel patterns of enzyme-treated, acid-soluble pig skin collagen and a control, before and after thermal gelation. All samples were heat denatured at 40-45°C for 20 minutes prior to electrophoresis. Enzyme digestion conducted at 20°C for 24 hours. Gel code: PSC = untreated acid-soluble pig skin collagen; PPRx = pepsin treated; PTRx - trypsin treated; PERx - elastase treated and PCRx = chymotrypsin treated. Gel pattern code: a = α-component; b = β-component; c = γ-component and f = buffer front.

10-a. Before thermal gelation.

10-b. After thermal gelation at 35°C for 12 hours.

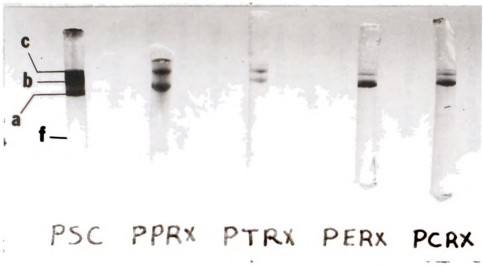


Plate 10-a

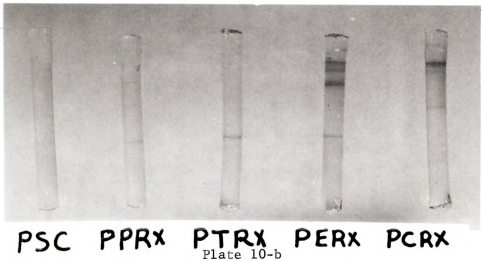


Plate 10-b

Plate 10.

with acid-soluble calf skin collagen. Both the elastase- and chymotrypsin-treated samples show large amounts of the α-component, whereas, the other samples have only weak staining areas. Observations again seem to imply that the -component and/or cross-link is of importance in the polymerizing process.

Table 10 shows the results of absorbancy measurements made before and after long term thermal gelation (24 hours at 35°C). All of the thermal gelation experiments with acid-soluble pig skin collagen were initiated in 0.1M phosphate buffer at pH 7.4. None of the samples had been lyophilyzed. The values shown in table 10 are averages of duplicate samples. These data show that the untreated and trypsin-treated samples have approximately the same amount of protein remaining in solution. The pepsin-treated sample has less protein in solution than the untreated sample. The reason for this is not clear, however, the gel patterns for the untreated, trypsin- and pepsin-treated samples show similarities. The inability of the electrophoretic procedure to produce gel patterns with 40-50% of the protein in solution is also puzzling. The chymotrypsin- and elastase-treated samples support the solubility data given in table 10.

Light staining bands were also noted moving between the α-component and the buffer front -- both before and after thermal gelation. Magnified photographs of the gel patterns before and after thermal gelation are shown in plate 11. Schematic drawings are used to show the location of these light bands since they were difficult to photograph. The photograph of the pepsin-treated protein gel pattern shows a good example of

Table 10. Absorbancy measurements before and after long term thermal gelation of enzyme-treated, acid-soluble pig skin collagen. The duration of the gelation period was 24 hours. The absorbancies were measured at 230 mu.

Collagen treatment	Absorbancy (O.D.)		% protein remaining in solution
	Before gelation	After gelation	
Untreated	0.422	0.211	50
Pepsin	0.560	0.224	40
Trypsin	0.506	0.237	47
Chymotrypsin	0.486	0.481	99
Elastase	0.382	0.311	81

the small bands and their location. Column D shows the photographs of the gel patterns following 24 hour thermal gelation. Column E contains schematic diagrams showing the location of all visible bands. The presence of these bands, although not too extensive may explain, to some degree, the high values obtained in table 10 for the percent of protein remaining in solution.

The disc gels before gelation were treated the same as the acid-soluble calf skin collagen and were stained for carbohydrates. The resulting gel patterns were too faint to appear on a photograph, but are schematically shown in column C of plate 11. The carbohydrate patterns showed that the acid-soluble pig skin collagen stained more intensely than the acid-soluble calf skin collagen. This indicated the presence of a higher concentration of carbohydrate. The banding patterns were similar to those of the acid-soluble calf skin collagen samples, and enzymatic digestion did not appear to have any effect on the carbohydrate content.

Plate 11. Magnified gel patterns and schematic drawings of enzyme-treated, acid-soluble pig skin collagen. Drawings show the approximate location of fast-moving, weak staining bands between the α-component and the buffer front before and after thermal gelation. The banding patterns following carbohydrate staining are also shown schematically.
Columns A, B and C may be directly compared within and between treatments. Columns D and E may be similarly compared.
Code: PSC = untreated acid-soluble pig skin collagen; PPRx = pepsin treated; PTRx = trypsin treated; PERx = elastase treated and PCRx = chymotrypsin treated. The staining intensity is indicated by the width of the drawn bands, i.e., the wider the band, the more intense the staining.

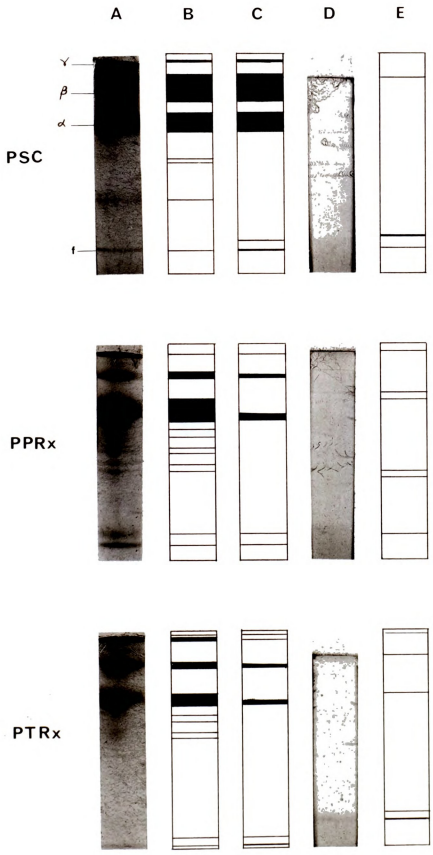
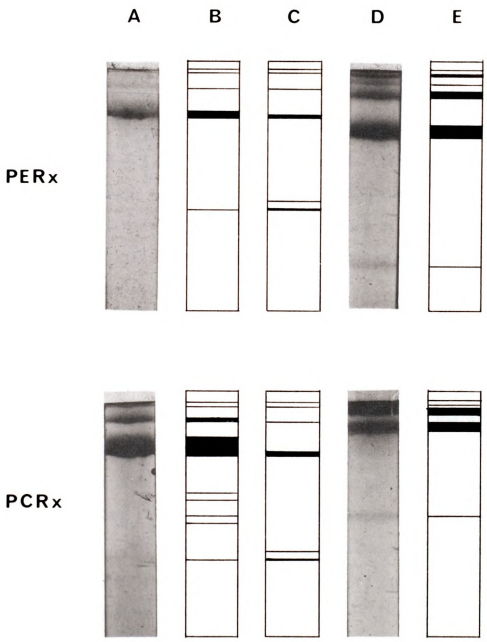


Plate 11.



A - Disc gel stained for protein before thermal gelation of the acid-soluble collagen.

B - Drawing of A showing strong and weak staining bands.

C - Drawing of carbohydrate staining patterns.

D - Disc gel stained for protein after thermal gelation of the acid-soluble collagen.

E - Drawing of D showing strong and weak staining bands.

Plate 11. (continued)

Short term thermal gelation experiments were performed with the enzyme-treated acid-soluble pig skin collagen. The protein had not been lyophilyzed prior to gelation. The gelation was initiated in 0.1M phosphate buffer at pH 7.4 and at approximately 33°C. The use of buffers of higher ionic strength resulted in an almost instantaneous polymerization of the untreated protein. Therefore, the gelation experiments were carried out in 0.1M buffers to facilitate monitoring of the untreated protein polymerization.

The gelation curves of the enzyme-treated and untreated protein were run in duplicate and were identical. These curves are shown in figure 5. The protein concentration of the pepsin-treated protein and the untreated protein were almost the same. The trypsin-treated sample had a slightly lower concentration, while the elastase- and chymotrypsin-treated samples were still lower. The pepsin-treated sample and the control sample had very similar maximum values, however, the lag period of the pepsin-treated sample was much longer. The untreated sample had a very short lag period. The trypsin-treated sample also had a rather short lag phase and had a decidedly lower maximum value.

The ratios of the maximum value to the initial value for the untreated, pepsin-treated and trypsin-treated protein were 4.7, 4.5 and 4.5, respectively. This indicates that the initial protein concentration effects the final opacity, as would be expected. The gelation curves of the elastase- and chymotrypsin-treated samples varied but little from the original concentration, indicating no polymerization. Similar experiments using

Figure 5. Thermal gelation curves of enzyme-treated, acid-soluble pig skin collagen. Thermal gelation occurred at 33°C using an 0.1M phosphate buffer at a pH of 7.4. Code: sc = untreated protein; p = pepsin treated protein; t = trypsin treated; e = elastase treated; and c = chymotrypsin treated.

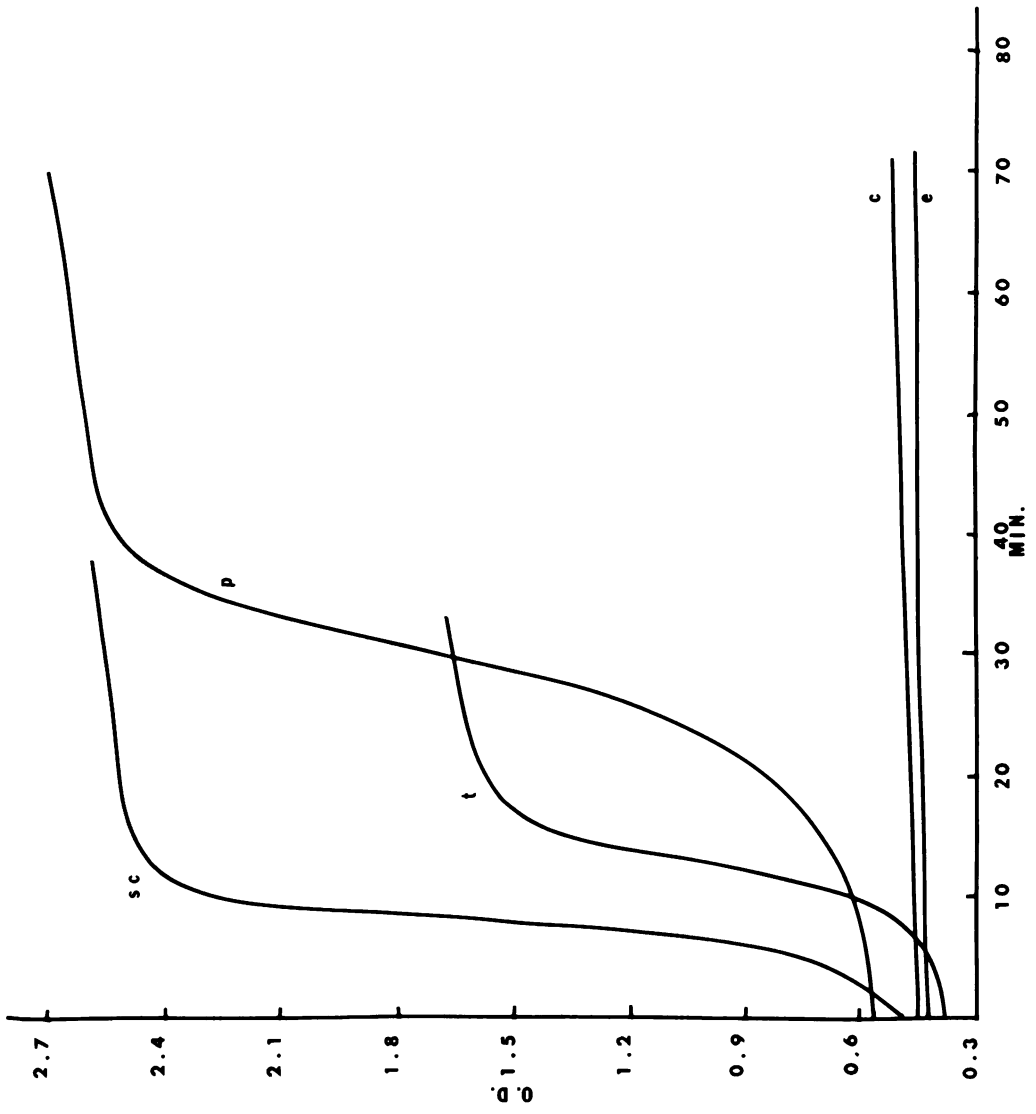


Figure 5.

higher concentrations of these two samples produced identical results. Elastase and chymotrypsin obviously had the greatest effect of any of the enzymes on polymerization of acid-soluble pig skin collagen. It will be recalled that a similar conclusion was reached in the acid-soluble calf skin study. However, there was some fiber formation in the chymotrypsin-treated calf skin collagen sample after a very long lag period in 0.6M buffer.

Several gelation experiments were conducted to establish the effect of lyophilyzation on the gelation process. Acid-soluble calf skin collagen and acid-soluble pig skin collagen after lyophilization produced the gelation curves shown in figure 6 using 0.1M phosphate buffer. The pig skin collagen polymerized rapidly, but the calf skin collagen did not. Other experiments using lyophilyzed and non-lyophilyzed calf skin collagen gave similar results. The lyophilyzed sample of acid-soluble calf skin collagen would not polymerize in 0.1M phosphate buffer at pH 7.4 during the duration of the experiment (1-2 hours), using both enzyme-treated and untreated samples. Tristram et al. (1965) stated that lyophilyzation or freezing of precipitated collagen at -20°C would reduce the solubility. Thus, the changes occurring during the freezing process must alter the gelation properties of the acid-soluble calf skin collagen samples and require the use of a buffer of higher ionic strength. The reason that the freeze-drying process does not alter the gelation properties of the acid-soluble pig skin collagen is not known.

The results following enzyme treatment of acid-soluble pig skin collagen are generally very similar to those obtained using acid-soluble

Figure 6. Thermal gelation curves of lyophilyzed acid-soluble calf skin collagen (1b) and lyophilyzed acid-soluble pig skin collagen (1p). Gelation was initiated at 33°C using an 0.1M phosphate buffer at a pH of 7.4.

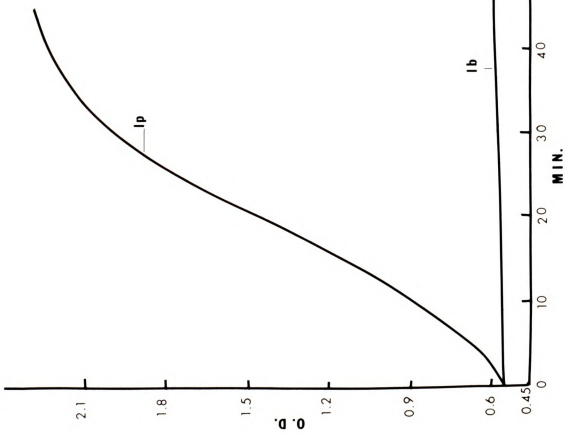


Figure 6.

calf skin collagen. Some specific differences that were found could be clarified by further study into the fluorescent properties from both protein sources, as well as to establish the nature of the protein remaining in solution following thermal gelation. The significance of the light-staining, fast moving bands observed in the disc gel studies would be of interest.

SUMMARY AND CONCLUSIONS

Dialyzable materials were separated by 2-dimensional electrophoresis and chromatography to determine the action of pepsin, trypsin, chymotrypsin and elastase on acid-soluble calf skin collagen. Differential staining showed a total of 4 tyrosine components following trypsin attack, 3 tyrosine positive areas in the pepsin and chymotrypsin digests and only 1 tyrosine containing component in the elastase digest. The major components were acidic in nature.

The dialysis tubing was shown to contribute dialyzable material to the chromatographic pattern. The exact origin of the material was not determined.

Disc gel electrophoresis of enzyme-treated acid-soluble calf skin collagen showed that elastase and chymotrypsin readily cleaved intramolecular cross-links. Pepsin had a similar effect but to a lesser degree, while trypsin had no discernable effect on the intramolecular cross-links. However, all the enzymes appeared to disrupt intermolecular cross-links.

Long and short term thermal gelation experiments indicated that all enzymes affected the ability of the protein to polymerize. The effectiveness of the enzymes in preventing protein aggregation was as follows: trypsin < pepsin < chymotrypsin < elastase.

It is postulated that the β-component or its cross-link is the key factor governing protein polymerization. This hypothesis is supported by the fact that disc gel electrophoresis of the solutions of elastase- and chymotrypsin-treated collagen, which did not polymerize, contained

largely the α-component. Thus, it was concluded that the β-component had been converted to the α-component by elastase and chymotrypsin, thus destroying the ability of the protein to polymerize.

While the fiber formation observed in these experiments may not be similar to that of fibrinogenesis in vivo, it does afford a tool for observing enzymatic effects on acid-soluble collagen.

Disc gel electrophoresis patterns of acid-soluble calf skin collagen also showed the presence of 6-8 lightly staining bands, moving between the α-component and the buffer front. On staining the gels for carbohydrates, only small amounts were shown to be present and stained in the familiar α-, β - and γ-component pattern with two additional bands at the solvent front.

Acid-soluble pig skin collagen was treated with the same proteolytic enzymes. The disc gel electrophoresis banding patterns of the enzyme-treated collagen were very similar to those described for acid-soluble calf skin collagen. The untreated pig skin collagen showed a much higher proportion of the β-component in comparison to the α-component than the acid-soluble calf skin collagen.

Chymotrypsin and elastase treatment greatly reduced the α- to β-component ratio in favor of the α-component. Pepsin treatment had a similar effect but to a lesser degree. Treatment with trypsin also appeared to reduce the amount of the β-component, however, a significant increase in the α-component was not observed. It was evident from these results that all the enzymes cleaved at least some of the intramolecular

cross-links. Furthermore, these enzymes also showed the ability to reduce intermolecular cross-links.

Thermal gelation studies showed that the effectiveness of the enzymes to prevent protein polymerization was the same as was observed with the acid-soluble calf skin collagen. Chymotrypsin and elastase were the most effective.

The large proportion of the β-component in the untreated samples, which was drastically reduced by elastase and chymotrypsin digestion, and the inability of the treated protein to polymerize supports the theory that the β-component or its cross-link is the key to protein polymerization.

Disc gel electrophoresis patterns of acid-soluble pig skin collagen also showed the presence of fast moving, lightly staining components between the α-component and the buffer front. Carbohydrate staining produced more intense bands than those observed with acid-soluble calf skin collagen, which indicated the presence of a higher concentration of carbohydrates.

Lyophilyzation decreased the ability of the acid-soluble calf skin collagen to polymerize. However, polymerization of the lyophilyzed protein could be achieved by using a buffer of high molarity. Acid-soluble pig skin collagen was not affected by lyophilyzation, being readily polymerized in a 0.1M phosphate buffer.

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