A STUDY OF HUMAN COLLAGEN UTILIZING BACTERIAL COLLAGENASE DIGESTION AND HIGH PRESSURE LIQUID CHROMATOGRAPHY

by

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ABSTRACT

With a view to facilitating the study of human genetic connective tissue diseases we have developed a technique of analyzing the primary structure of collagen.

One mg samples of purified human type I, II, and III collagens and α1(I) and α2 chains were digested with clostridiopeptidase A and the released peptides analyzed by ion exchange high pressure liquid chromatography. Specific fingerprints were produced for each type of collagen. The specificity of these fingerprints appeared to be related to the primary structure of these collagens.

Type I collagen fingerprint patterns were obtained when this technique was applied to 5 mg samples of human dermis, tendon and bone and type II collagen fingerprint patterns were obtained from 5 mg samples of human costal cartilage.

It is suggested that this technique may be suitable for the study of the primary structure of collagen in small tissue samples obtained from patients with genetic connective tissue diseases.

Le collagène chez l'homme: une étude basée sur la digestion à la collagénase bactérienne et la chromatographie à haute pression.

par

William G. Cole

RESUME

Dans le cadre d'un projet d'étude des maladies génétiques du tissu conjonctif chez l'homme, nous avons mis au point une technique d'analyse de la structure primaire du collagène.

Nous avons soumis des échantillons d'un mg des collagènes humains de type I, II et III ainsi que des chaînes $\alpha 1(I)$ et $\alpha 2$ à une digestion par la clostridiopeptidase A. Les petits peptides ainsi obtenus ont été analysés par chromatographie en phase liquide à haute pression. Les profils d'élution obtenus forment des cartes peptidiques spécifiques qui sont liées à la structure primaire de chaque type de collagène étudié.

Les analyses par cette technique d'échantillons de 5 mg de derme, de tendon et d'os humain ont produit des profils semblables à celui du collagène de type I tandis que des profils similaires à celui du collagène de type II ont été obtenus par analyse de cartilage costal humain.

Nous proposons l'emploi de cette technique pour l'étude de la structure primaire du collagène dans les petits échantillons de tissu que l'on peut prélever à des patients atteints de maladies génétiques du tissu conjonctif.

ORIGINAL CONTRIBUTIONS

1. A method of analyzing the primary structure of small amounts of collagen using clostridiopeptidase A digestion, separation of the released peptides by high pressure ion exchange liquid chromatography and their reaction with ninhydrin was developed.

2. Specific fingerprints were obtained when this technique was applied to one mg samples of human type I, type II and type III collagens and $\alpha 1(I)$ and $\alpha 2$ chains.

3. When this technique was applied to 5 mg samples of human tendon, bone and dermis fingerprints almost identical to the purified human type I collagen fingerprint were obtained. There was a high yield of peptides.

4. When it was applied to 5 mg samples of costal cartilage fingerprints almost identical to the purified type II collagen fingerprint were obtained. A good peptide yield was obtained.

5. Based on these observations, it is suggested that this may be a valuable technique for the study of small tissue samples from patients with suspected primary structure anomalies of collagen.

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CHAPTER I

INTRODUCTION

Collagen is a major connective tissue protein (Gallop and Paz, 1975). It is abundant in tendons, ligaments, fascia, joint capsules, bone and cartilage. It also forms an important part of the framework of organs such as the liver and lung and of basement membranes (Bornstein, 1974).

Using the electron microscope tissue collagen has been shown to consist of fibrils with a highly organized structure characterized by repeating axial periods measuring approximately 640 Å (Schmitt et al., 1955). The basic unit of the fibril is the collagen molecule referred to by Gross (1956) as tropocollagen and measures approximately 2,800 Å in length and 15 Å in width. To produce the 640 Å periodicity it was proposed in the quarter stagger hypothesis that the polarized molecules were arranged in parallel rows staggered by one quarter of their length in respect to their neighbours (Gross, 1956).

This concept has been extended by Hodge and Petruska (1963) who showed that the molecule measured 4.4 times the axial period. They suggest that there is a microfibril intermediate of five molecules (depicted in fig. 1) in which the molecules are staggered in such a manner that there is a region where the five molecules overlap (measuring 0.4 of the axial period) and a gap region in which only four molecules overlap (measuring 0.6 of the axial period). The studies of Smith (1965, 1968), Veis et al. (1967), Hulmes et al. (1973) and Bruns and Gross (1974) support this concept.

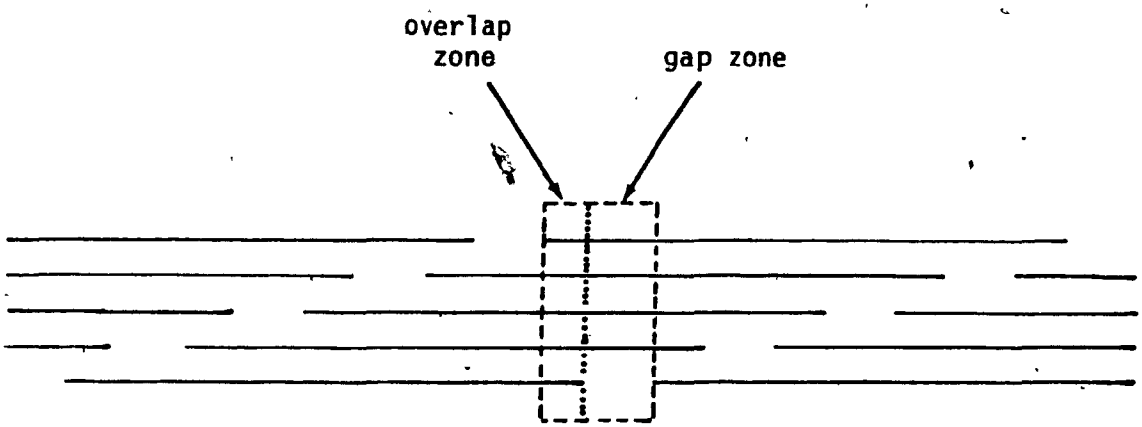

FIGURE 1. Schematic representation of the packing of collagen molecules in staggered array in a native type fibril.

The collagen molecule consists of a triple helical rod formed by the wrapping of three polypeptide chain around each other, as shown in fig. 2 (Yee et al. 1974). Except for short terminal regions referred to as telopeptides each polypeptide chain is coiled in the form of a polyproline type II helix (Ramachadran and Kartha, 1954, 1956; Rich and Crick, 1955). Each polypeptide chain is formed by the linear arrangement of amino acids linked by peptide bonds (Bornstein and Piez, 1965). This arrangement is referred to as the primary structure of collagen (Gross, 1974).

A close relationship exists between the primary structure and biomechanical properties of collagen (Levene and Gross, 1959); Pinnell et al., 1972). In man the closeness of this relationship is well illustrated in the Ehlers-Danlos syndrome. McKusick and Martin (1975) have defined seven forms of this syndrome in which there are varying degrees of connective tissue laxity. Abnormalities in the biochemical structure of collagen have been detected in the four recessive forms and defects in the primary structure of collagen are also suspected in the three dominant forms. Two other diseases, osteogenesis imperfecta and Marfan's syndrome demonstrate connective tissue laxity and are also suspected of having primary structure anomalies (Penttinen et al., 1975; McKusick, 1972).

In view of this relationship between structure and function and because of the limited information available concerning human collagen abnormalities this project was directed towards the study of the primary structure of human collagen.

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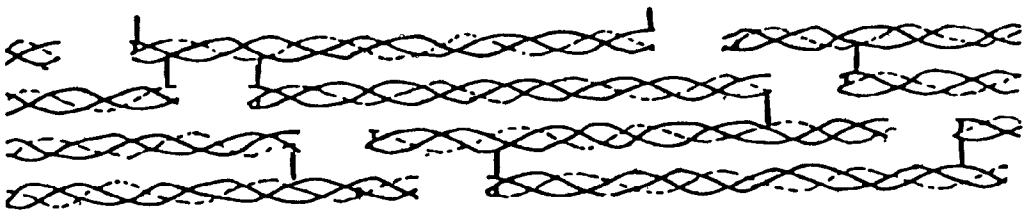

FIGURE 2. Schematic representation of triple helical tropocollagen molecules in a collagen fibre.

CHAPTER II

PREVIOUS INVESTIGATIONS:
NORMAL PRIMARY STRUCTURE OF COLLAGEN

2.1. TYPES OF COLLAGEN

Up to 1969 only one type of collagen was known to exist. This type of collagen was shown by Piez et al. (1961, 1963) to contain two distinct chains referred to as α1 and α2 in accord with their order of elution when chromatographed on CM-cellulose. The α1 chain has been renamed α1(I) to distinguish it from the other more recently described α1 chains. Each type I collagen molecule contains two α1(I) and one α2 chain designated as $[\alpha 1(I)]_2\alpha 2$. This form of collagen is found in many tissues in particular adult dermis, tendon, bone and dentin (Miller, 1973; Epstein, 1974).

In 1969 Miller and Matukas extracted a previously unrecognized form of collagen from lathyritic chick cartilage which consisted of α1 chains with a distinct amino acid and cyanogen bromide peptide composition. This new form of collagen referred to as type II collagen has the chain composition $[\alpha 1(II)]_3$. Their results were confirmed soon after by Trelstad et al. (1970) and Miller (1971). In addition to articular and costal cartilages this type of collagen is found in the intervertebral disc (Eyre and Muir, 1974; Herbert et al., 1975).

In 1971 Miller et al. showed that two of the cyanogen bromide peptides obtained from the insoluble residue of human foetal dermis could not be attributed to the α1(I), α1(II) or α2 chains. Subsequently, Chung and Miller (1974) and Epstein (1974) isolated the type III collagen which

produced these peptides, from human vessels, leiomyoma and foetal dermis. It has the chain composition $[\alpha 1(III)]_3$ and is also found in the intestine and synovium (Eyre and Muir, 1975; Pope et al, 1975).

Also in 1971 a fourth type of collagen was identified in basement membranes by Kefalides (1971). This form of collagen contains α1 chains described as $[\alpha 1(IV)]_3$. It is found in the basement membranes of many tissues including the glomerulus, Descemet's membrane and the aorta (Kefalides, 1972; Trelstad, 1974).

There may be other types of collagen as suggested by a recent report of an apparently new type of collagen consisting of two similar and one dissimilar chain in human foetal membranes and human leiomyoma (Hollister et al., 1976).

These four types of collagen have similar helical structures and physical dimensions but they have significant primary structure differences as outlined below.

Type I collagen

Before 1965, the primary structure of collagen was analyzed following cleavage of the molecule with mild acid or alkaline hydrolysis, clostridiopeptidase A digestion and sequential digestion with other enzymes (Schroeder et al., 1953; Michaels et al., 1958; Franzblau et al., 1964). It was shown that glycine accounted for one third of the amino acids and because of the position of glycine in isolated tripeptides such as Gly-Pro-Pro, Gly-Pro-Hyp and Gly-Pro-Ala it was concluded that glycine occupied each third position in the molecule. However the order of these triplets was not determined nor was it clear whether the molecule was composed of several subunits linked by non peptide bonds as was suggested by Blumenfeld and Gallop (1962).

In 1965 Nordwig and Dick and Bornstein and Piez described the cleavage of collagen with cyanogen bromide. Bornstein and Piez (1965) applied this technique to chromatographically purified α1(I) and α2 chains and clearly showed that the collagen molecule was composed of polypeptide chains consisting of a linear arrangement of amino acids linked by peptide bonds and it did not contain large repeating subunits joined by ester like bonds. This technique was also a major advance in the amino acid sequence studies as the peptides produced by cleavage of the collagen chains at their methionine bonds were easier to study than the parent chain (Bornstein and Piez, 1965). Another advantage of this technique was the ability to determine the order of these peptides in the parent chain using chemical overlapping techniques, electron microscopic studies and pulse labelling data (Piez et al., 1969; Igarashi et al., 1970; Vuust et al., 1970).

Recent studies of the primary structure of cyanogen bromide peptides from known regions of the parent chains using clostridiopeptidase A, carboxypeptidase, trypsin, chymotrypsin and automated sequential Edman degradation have provided important data concerning the amino acid sequences of various chains (Kang et al., 1967; Kang and Gross, 1970; Becker et al., 1975; Clark et al., 1975). This data is however incomplete but the entire α1(I) chain sequence has been compiled from rat and calf α1(I) sequences (Hulmes et al., 1973).

α1(I) chains

Human α1(I) chains have a typical collagen amino acid composition (Table I) of approximately one third glycine and a high content of proline and hydroxyproline (Bornstein and Piez, 1966). Since only limited information is available concerning the order of these amino acids in human α1(I) chains the complete sequence assembled from calf and rat data reported by Hulmes et al. (1973) will be described.

This data shows that the α1(I) chain contains 1052 amino acids with pyroglutamic acid at the amino terminal end and tyrosine at the carboxyterminal end. As previously suggested by Bornstein and Piez (1965) all the amino acids are linked by peptide bonds and there are no large repeating subunits.

The amino terminal end of 16 residues and the carboxyterminal end of 25 residues are the non helical telepeptides. In contrast to the helical region these peptides contain less than one third glycine, less hydroxylysine and no hydroxy proline. In these regions the lysyl and hydroxylysyl residues play an important role in intra- and intermolecular cross linking (Eyre and Glimcher, 1973; Mechanic, 1974).

The large helical region commences at the seventeenth residue with the typical Gly-X-Y triplets which continue for 337 triplets to residue 1027. These triplets consist of :

TABLE I

Amino acid analyses of human collagens *

Amino acid	Skin(1) α1(I)	Skin(1) α2	Articular cartilage 1(II)	Aorta(3) α1(III)	Glomerular basement membrane (4) α1(IV)
3-Hydroxyproline	1	1	2	-	11
4-Hydroxyproline	91	82	93	125	130
Aspartic acid	43	47	42	42	51
Threonine	17	19	20	13	23
Serine	37	35	27	39	37
Glutamic acid	77	68	90	71	84
Proline	135	120	121	107	61
Glycine	333	337	333	350	310
Alanine	115	105	100	96	33
Cysteine	-	-	-	2	8
Valine	21	33	18	14	29
Methionine	5	5	9	8	10
Isoleucine	7	15	9	13	30
Leucine	20	30	26	22	54
Tyrosine	2	5	1	3	6
Phenylalanine	12	12	13	8	27
Hydroxylysine	4	8	14	5	45
Lysine	30	22	23	30	10
Histidine	3	10	2	6	11
Arginine	50	51	51	46	33

* Values expressed in residues/1000 amino acid residues.
References : (1) Bornstein & Piez (1966); (2) Miller & Lunde (1973); (3) Chung & Miller (1974); (4) Kefalides (1974).

115 Gly-Pro-Y where Y is hydroxyproline in 38 instances and alanine in 31.

77 Gly-X-Hyp where X is any amino acid other than proline

145 Gly-X-Y where X and Y are any amino acids other than proline and hydroxyproline.

Further details regarding the composition of these triplets are given by Gallop and Paz (1975).

Apart from one 3-hydroxyproline residue all the hydroxyproline residues are in the 4-hydroxyproline form (Hulmes et al., 1973). Hydroxyproline is however not incorporated into the polypeptide chain during translation but arises by the hydroxylation of certain prolyl residues which occupy the Y triplet position (Stetten and Schoenheimer, 1944; Miller and Udenfriend, 1970). There are 115 such sites but the completeness of the hydroxylation varies in different parts of the molecule. The overall completeness of prolyl hydroxylation was estimated by Gallop and Paz (1975) to be 80 %.

Hydroxylysine is also not incorporated into the polypeptide chain during translation but arises from the hydroxylation of certain lysyl residues in the Y triplet position (Grant and Prockop, 1972). Lysyl residues are hydroxylated to varying extents and significant differences exist in the hydroxylysine content of $\alpha 1(I)$ chains from different tissues and in the same tissue at different ages (Spiro, 1972; Kivirikko et al. 1973).

This variation is demonstrated by comparing the 4-5 hydroxylysine residues in dermal α1(I) chains to the 15 residues in bone α1(I) chains (Stoltz et al., 1973). Some of the additional hydroxylysine residues in bone α1(I) chains are located in the telopeptides (Eyre and Glimcher, 1973).

Two to three of the hydroxylysine residues are linked to glucosyl-galactose or galactose residues (Morgan et al., 1970). Pinnell et al. (1971) showed that the ratio of glucosylgalactosyl-hydroxylysine to galactosylhydroxylysine to be 2.06 in human dermis as opposed to 0.47 in human bone collagen. In addition, they showed that in these tissues approximately one third of the hydroxylysine residues were glycosylated.

α2 chains

When compared to the α1(I) chain human α2 chains are noted to contain more leucine, isoleucine, histidine and hydroxylysine but similar amounts of hydroxyproline (Bornstein and Piez, 1966).

The completeness of prolyl and lysyl hydroxylation are also quite variable in different parts of the α2 chain (Gallop and Paz, 1975). Tissue variations in the degree of lysyl hydroxylation also exist as shown by 9 residues in dermis and 14 residues in bone (Stoltz et al., 1973). Hexoses in the form of galactosyl or glucosylgalactosyl residues are linked to two of these hydroxylysines (Aguilar et al., 1973). Although the bone α2 contains more hydroxylysine residues there is no increase in the degree of glycosylation (Gallop and Paz, 1975). This also applies to the α1(I) chains in bone.

The telopeptides contain less than one third glycine, low levels of hydroxylysine and no hydroxyproline but despite these similarities the α1(I) and α2 telopeptides have different amino acid sequences (Fietzek and Kuhn, 1973).

There is only limited α2 amino acid sequence information available. Comparison of the sequenced regions to corresponding regions of the α1(I) chain reveals many interchain substitutions but the overall charge patterns which appear to be important for the stability of the fibrils are essentially the same (Gallop and Paz, 1975).

Type II collagen

As a consequence of the insolubility of human type II collagen the amino acid composition given in Table I has been derived from the composition of the type II cyanogen bromide peptides (Miller and Lunde, 1973). It contains the typical collagen content of glycine and imino acids.

Compared to the α1(I) chain it is noted that type II collagen contains approximately 4-5 times as many hydroxylysine residues and 9 times as many hexoses which are mainly in the glucosylgalactosyl form (Miller, 1973). In addition, type II collagen also contains more glutamyl residues.

Only limited sequence data is available for this type of collagen.

Type III collagen

This form of collagen is isolated from tissues as a trimer (γ) as a result of the disulphide bonds established between cysteine residues in the helical region of this molecule. These bonds are readily reduced with β-mercaptoethanol to yield α1(III) chains (Epstein, 1974).

As shown in Table I human type III collagen differs from α1(I) chains in having two cysteine residues, more hydroxyproline, glycine and histidine but it has similar amounts of hydroxylysine (Chung and Miller, 1974). The type III cyanogen bromide peptides were analyzed by Chung and Miller (1974) and their results showed that 7 of the 9 peptides contained more hydroxyproline than proline and three contained more than one third glycine. The two cysteine residues were located near the carboxy terminal end of the chain.

Only limited data concerning the amino acid sequence in these peptides is available.

Type IV collagen

In comparison with the other collagens type IV collagen contains high levels of 4-hydroxyproline, 3-hydroxyproline, hydroxylysine, cysteine, leucine, isoleucine and phenylalanine and low levels of alanine and arginine (Kefalides, 1974). In addition it characteristically contains 40 hexoses mainly glucosylgalactosyl residues but also small amounts of mannose and hexosamine linked to hydroxylysine residues.

There is also very limited amino acid sequence information available for this type of collagen.

2.2. Biosynthesis of collagen

The primary structure of collagen described in section 2.1 is the result of a complex series of biosynthetic steps which are illustrated in Fig. 3. Collagen is not only unique because of its triple helical structure and amino acid composition but also because of the large number of post translational modifications required before the final product is obtained (Gross, 1974).

The normal translational and post translational events in the synthesis of collagen will be described because anomalies of these events (discussed in Chapter III) can produce profound abnormalities in the primary structure and function of collagen.

Translation

Collagen is synthetized by connective tissue cells such as fibroblasts, chondrocytes and osteoblasts and by epithelial cells (Gross, 1974). The translational process appears to take place on membrane bound ribosomes in a similar manner to other proteins (Kivirikko, 1974). The polypeptide chains are formed by the stepwise addition of amino acids at the rate of 209 residues per minute and the chain is completed in 6 minutes (Vuust and Piez, 1970). Hydroxyproline and hydroxylysine residues are not incorporated into the polypeptide chain but are formed at a later stage by hydroxylation of selected prolyl and lysyl residues (Stetten and Schoenheimer, 1944; Stetten, 1949; Miller, 1971). The completed chains are longer than the tropocollagen chains as they contain additional amino and carboxy extensions (registration peptides) the structure and function of which will be described later.

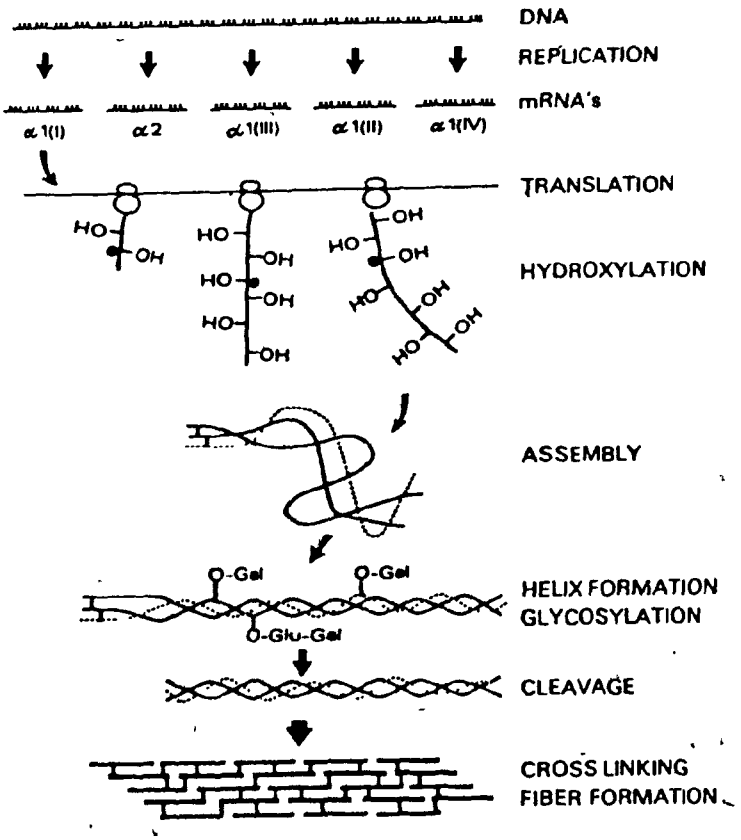

FIGURE 3. Biosynthesis of collagen.

If the prolyl and lysyl hydroxylation steps are blocked unmodified translated chains called protocollagen are obtained (Berg and Prockop, 1973).

It is not clear whether procollagen messenger RNA (mRNA) is monocystronic or polycistronic in nature. The physical characteristics of isolated procollagen mRNA would suggest a monocistronic form (Larazides and Lukens, 1971; Harwood et al, 1974a; Boedtker et al, 1974; Pawlowski et al, 1975). Vuust and Piez (1970) concluded from their kinetic studies that the pro α chains are synthesized simultaneously in keeping with a monocistronic form of procollagen mRNA. An alternative view proposed by Church et al (1971) and by Park et al. (1975) is that procollagen mRNA is polycistronic. They propose that the three chains comprising a procollagen molecule are synthesized in series so that a long chain is formed with each α chain joined by non collagenous segments.

Post-translational modifications

a) Protocollagen prolyl hydroxylation

This step is mediated by the enzyme protocollagen prolyl hydroxylase (Grant and Prockop, 1972). The enzyme has been purified from new born rat skin and chick embryo extracts (Halme et al. 1970; Rhoads and Udenfriend, 1970). It is an acidic protein which probably exists within the cell as an inactive precursor (Kuttan et al., 1975). It has also been shown to be a tetramer consisting of two dissimilar subunits of 64,000 and 60,000 daltons which are probably stabilized by disulphide bonds in the active state (Popenoe et al., 1969; Berg and Prockop, 1973).

This enzyme has been localized to the rough endoplasmic reticulum where it is concentrated on the cisternal side of the membrane (Harwood et al., 1974b; Olsen et al., 1973; Al-Adnani et al., 1974; Cutroneo et al., 1974). This is close to the site where the polypeptide chains are synthesized and it appears that prolyl hydroxylation occurs while the chains are still attached to the ribosomes (Vuust and Piez, 1972; Rosenbloom et al., 1976).

This enzyme hydroxylates certain prolyl residues in the $(Gly\text{-}X\text{-}Pro)_n$ triplets where n is greater than two (Miller and Udenfriend, 1970; Kivirikko et al., 1969; McGee et al., 1971). The enzyme has a stronger affinity for the unhydroxylated protocollagen and is only able to hydroxylate prolyl residues while the chain is in a random form (Kivirikko et al., 1972,1973; Rosenbloom et al., 1976).

This enzyme requires molecular oxygen, ferrous ions, α ketoglutarate and a reducing agent such as ascorbic acid (Fujimoto and Tamiya, 1962; Kuttan et al., 1975; Uitto et al., 1975). There is a stoichiometric coupling of oxidative decarboxylation of α ketoglutarate (converted to succinate) with hydroxylation of peptidyl proline to 4-hydroxyproline (Rhoads and Udenfriend, 1970).

It is uncertain what factors control the degree of prolyl hydroxylation. In view of the poor affinity of the enzyme for triple helical procollagen it is possible that the rate of helix formation is one controlling factor (Schoefield et al., 1974; Uitto and Prockop, 1974).

b) Protocollagen lysyl hydroxylation

This step is mediated by the enzyme protocollagen lysyl hydroxylase (Grant and Prockop, 1972). This enzyme has a molecular weight of approximately 350,000 daltons and is located on the membrane of the rough endoplasmic reticulum (Popenoe and Aronson, 1972; Harwood et al., 1974b).

It has many features in common with protocollagen prolyl hydroxylase They are both situated on the rough endoplasmic reticulum, they have the same cofactor requirements and both appear to act on nascent chains (Uitto and Prockop, 1974; Rosenbloom et al., 1976). However each enzyme is specific for its own substrate (Grant and Prockop, 1972).

This enzyme hydroxylates certain lysyl residues in the triplets Gly-X-lys and the substrate needs to be in a random form (Kivirikko et al., 1973; Ryhänen and Kivirikko, 1974). The factors that control the tissue and age differences in lysyl hydroxylation are unknown. One of the limiting factors however appears to be the rate of helix formation. The slower rate of helix formation in procollagen from cartilage (pro type II) and lens cells (pro type IV) than tendon cells (pro type I) may account for the relatively high hydroxylysine contents of cartilage and lens collagen (Miller, 1971; Kefalides, 1971; Grant et al., 1973; Schofield et al., 1974).

Selected lysyl residues in the telopeptides can also be hydroxylated (Eyre and Glimcher, 1973). Gallop and Paz (1975) in reviewing some of the features of the protocollagen lysyl hydroxylase deficient form of human Ehlers-Danlos syndrome (see chapter III) suggest that a separate enzyme may be responsible for lysyl hydroxylation in the telopeptides.

c) Glycosylation

In 1965 Butler and Cunningham showed in chick embryo tibiae that glycosylation occured within several minutes of the formation of hydroxylysine residues.

The enzymes required for this step, collagen UDP-galactosyl transferase and collagen UDP-glucosyl transferase have been isolated and been shown to require manganese as a cofactor (Bosman and Eylar, 1968; Spiro and Spiro, 1971; Myllylä et al., 1975). These enzymes are located in the rough and smooth endoplasmic reticulum (Harwood et al., 1974b; Brownwell and Veis, 1975).

Glycosylation probably occurs in nascent chains but it is apparent that the substrate specificities vary with the source of the enzyme (Myllylä et al., 1975).

Morgan et al (1970) and Aguilar et al (1973) have identified a sequence which may be a specific requirement, -Gly-X-Hyl (Gal-Glc)-Gly-X'-Arg-. Glycosylation can however occur in other sequences as shown by the carboxy telopeptide site described by Eyre and Glimcher (1973).

As with the other enzyme steps little is known about the controlling factors. The number of hydroxylysine residues available would seem to be one factor as more hexoses are found in the type II and IV collagens which contain the largest numbers of hydroxylysyl residues (Kefalides, 1971; Miller, 1973).

d) Helix formation

Triple helical collagen molecules can be formed *in vitro* but the yield is small, the process is slow and it lacks specificity in that $[\alpha 1(I)]_3$, $(\alpha 2)_3$ as well as the normal $[\alpha 1(I)]_2\ \alpha 2$ molecules can be made (Piez and Sherman, 1970). However, *in vivo* helix formation is efficient and rapid (Piez, 1970).

One of the explanations for the rapid helix formation *in vivo* was that the amino terminal extension (registration peptide) brought the three pro α chains into register so that spontaneous helix formation would occur (Sherr *et al.*, 1973). Hydroxyproline was also shown in experiments using underhydroxylated procollagen and protocollagen to be important for normal helix formation and stability (Berg and Prockop, 1973; Sakakibara *et al.*, 1973).

In 1974, Tanzer *et al.* showed that procollagen contains an extra extension at the carboxyterminal end as well. The amino and carboxy registration peptides have been recently characterized by Byers *et al.*, (1975), Fessler *et al.* (1975) and Rosenbloom *et al.* (1976). The amino peptide has a molecular weight of approximately 20,000 daltons and it contains cysteine while the carboxy peptide has molecular weight of approximately 35,000 daltons and contains tryptophan and cysteine residues but the disulphide bonds are restricted to the carboxy peptides. Fessler *et al.* (1975) described the component parts of procollagen as $[(NH_2\text{-peptide})\text{-(collagen)-(COOH-peptide)}]_3$ with interchain disulphide bonds between the carboxypeptides.

Disulphide bonding between the carboxypeptides is a relatively late event in the synthesis of procollagen and if the formation of these carboxypeptides is blocked with puromycin the chains despite normal prolyl hydroxylation will not form a triple helix and they will not be secreted (Fessler et al, 1975; Rosenbloom et al., 1976). These observations demonstrate that proper pro α chain registration and prolyl hydroxylation are required for normal helix formation and secretion.

The above description refers to the method of assembly assuming a monocistronic mRNA pattern of pro α chain synthesis. However Park et al. (1974) proposed that with a polycistronic mRNA system that in addition to producing procollagen of the correct chain composition suitable folding of the long polypeptide chain could bring the pro α chains into registration for triple helix formation.

e) Transport and secretion

Schmitt in 1960 and Speakman in 1971 suggested that the secreted form of collagen was probably longer than tropocollagen. In 1971 Layman et al. described a soluble "transport" form of collagen in tissue culture experiments and in the same year, Bellamy and Bornstein (1971) called it procollagen. Weinstock and Leblond (1974) showed in odontoblasts and osteoblasts the route by which this soluble form of collagen is secreted. Using ^{3}H proline autoradiography they showed that procollagen moved from the ribosomes to the spherical Golgi saccules where the procollagen assumed a more parallel aggregated form and then to the cylindrical portions of the Golgi saccules. In this region the procollagen was contained within presecretory and secretory

granules which were extruded from the cell surface by exocytosis. Harwood et al. (1976) reported data which supports this proposal.

f) Conversion of procollagen to collagen

In 1971 Lapiere et al. isolated from calf skin the enzyme procollagen peptidase that mediates this step. It has also been extracted from rat and chick calvaria by Bornstein et al (1972). The observations by Goldberg et al. (1975) of this enzyme in the media of fibroblasts cultures suggested that its normal site of action was outside the cells.

It is a neutral endopeptidase that requires calcium as a cofactor. It cleaves the terminal extensions (registration peptides) of procollagen in a stepwise manner (Goldberg et al., 1975). It is not clear whether more than one enzyme is required for removal of the amino and carboxypeptides.

The studies made by Bailey and Lapiere (1973) on tissue collagen in the procollagen peptidase deficient disease of cattle, dermatosparasis, clearly showed that if the registration peptides are not removed that normal packing and crosslink formation required for normal tissue function cannot occur.

The extraction of small amounts of procollagen I and III from skin by Byers et al. (1974) and Lenaers and Lapiere (1975) may indicate that there is a delay in the normal conversion of the soluble procollagen into the fibrillar collagen.

g) Fibril and crosslink formation

The major crosslinks which stabilize collagen are partly formed from certain lysyl and hydroxylysyl residues situated in the telopeptides following their conversion to the aldehydes allysine and hydroxyallysine by the enzyme lysyl oxidase (Siegel and Martin, 1970).

Lysyl oxidase was isolated and characterized by Pinnell and Martin (1968) and Siegel and Martin (1970). The enzyme requires oxygen and copper as a presumptive cooxygenase. Recent studies show that aortic lysyl oxidase is heterogenous in that it contains two antigenically distinct components (Vidal et al., 1975).

The principal intramolecular crosslink arises by the condensation of the allysines in the aminotelopeptide to form the aldol condensation product (Gallop and Paz, 1975). For normal intermolecular crosslink formation Tanzer (1967, 1968) showed that the molecules must pack together into a quarter stagger. The allysine and hydroxyallysine residues in the amino and carboxytelopeptides plus the secondary aldehyde from the aldol condensation product are the precursors of Schiff bases formed with lysine, hydroxylysine and histidine residues at specific sites in the helix. Further details are given by Gallop and Paz (1975).

CHAPTER III

PREVIOUS INVESTIGATIONS:
ABNORMAL PRIMARY STRUCTURE OF COLLAGEN

There are many sites in the biosynthetic pathway where collagen defects could arise. Most of the known human defects are inherited as recessive traits and effect the enzyme mediated post translational modifications of the collagen primary structure (McKusick and Martin, 1975). Other defects involve the types of collagen synthesized (Penttinen et al., 1975).

In Table II the features of the seven types of human Ehlers-Danlos syndrome as defined by McKusick and Martin (1975) are listed. In three of the recessive forms enzyme defects have been identified.

Lysyl protocollagen hydroxylase deficiency

In 1972 Pinnell et al. observed low hydroxylysine levels in specimens of skin, fascia and bone from two siblings with the type VI Ehlers-Danlos syndrome. Associated with this anomaly the tissues showed a marked reduction in the hydroxylysine derived crosslinks (Eyre and Glimcher, 1972). The enzyme lysyl protocollagen hydroxylase which mediates the hydroxylation of lysyl residues was shown to be deficient in cultured fibroblasts from these patients (Krane et al., 1972). Sussman et al. (1974) confirmed this enzyme defect in cultured fibroblasts from one of two affected siblings from a different family.

It may be possible to increase the activity of this enzyme as an affected boy from another family has shown improved lysyl hydroxylation with the administration of the enzyme's principle reductant ascorbic acid (Elsas et al., 1974).

TABLE II

The Ehlers-Danlos Syndrome

Name	Clinical features	Genetics	Biochemical defect
EDS I, gravis type	Classic features, all severe	Autosomal dominant	Unknown
EDS II, mitis type	Classic features, all mild	Autosomal dominant	Unknown
EDS III, benign hypermobile type	Generalized marked joint hypermobility without skeletal deformity; skin features minimal	Autosomal dominant	Unknown
EDS IV, ecchymotic, arterial or Sack-Barabastype	Severe bruisability, very thin skin, rupture of bowel, rupture of large arteries, minimal joint laxity (e.g., limited to fingers)	Autosomal recessive	Deficient synthesis of type III collagen
EDS V, X-linked type	Stretchable skin striking, joint hypermobility minimal, skin fragility and bruisability variable.	X-linked recessive	Deficiency of lysyl oxidase
EDS VI, ocular type	Scoliosis severe, skin features moderate, blindness from retinal detachment or ocular rupture.	Autosomal recessive	Deficiency of lysyl hydroxylase
EDS VII, arthrochalasis multiplex congenita	Short stature, severe joint laxity with congenital dislocations, moderate skin stretchability and bruisability.	Autosomal recessive	Deficiency of pro-collagen peptidase

Procollagen peptidase deficiency

In 1973 Lichtenstein et al. showed that three patients with type VII Ehlers-Danlos syndrome had detectable amounts of type I procollagen in extracts of their skin and tendon. This abnormality was considered to be the result of a defect in the conversion of procollagen to tropocollagen. Reduced activity of procollagen peptidase which is responsible for this conversion was observed in cultured fibroblasts from these patients.

A similar enzyme defect has been observed in dermatosparaxis which is an autosomal recessive disease of cattle but the clinical features are quite different (Lenaers et al., 1971).

Lysyl oxidase deficiency

In 1975, DiFerrante et al. reported two maternal cousins with type V Ehlers-Danlos syndrome in whom cultured fibroblasts produced poorly cross-linked collagen due to reduced lysyl oxidase activity.

They also showed that the flavanoid (+) catechin which can create hydrogen bonds between collagen molecules in vitro decreased the abnormal solubility of the collagen produced by these fibroblasts. It was suggested that this agent may produce a similar effect in vivo.

This enzyme step is also affected by the action of several drugs and other genetic diseases. The activity of lysyl oxidase is reduced by the competitive inhibitor β amino proprionitile and by disorders which decrease the level of the essential cofactor, copper (Pinnell and Martin, 1968); Danks et al., 1972). The reduction in crosslink formation produced by penicillamine, homocysteine and hydrallazine is in part due to the binding of these agents to the aldehydes produced from lysine and hydroxylysine by this enzyme (Gallop and Paz, 1975).

There are also several reported defects involving the types of collagen synthesized;

1) Deficiency of type III collagen :

In 1975, Pope et al. reported five patients with type IV Ehlers-Danlos syndrome who had histological evidence of reduced amounts of tissue collagen, absent type III collagen in the skin, aorta and intestine and absent type III collagen in the cells and media of cultured fibroblasts.

2) Deficiency of type I collagen

Also in 1975, Penttinen et al. reported that cultured fibroblasts from a lethal form of osteogenesis imperfecta congenita produced less type I and more type III collagen than normal fibroblasts. It was concluded that the defect involved type I collagen production.

Meigel et al. (1974) and Müller et al. (1975) studied a boy with a new connective tissue disease which appeared to be inherited as a recessive trait. He was marfanoid with some radiological features of osteogenesis imperfecta. Fibroblasts culture studies showed a reduction in type I and an increase in type III collagen production.

It is apparent that great advances have been made since 1972 in the detection of primary structure anomalies of collagen in several inherited connective tissue diseases. This information has contributed to the understanding of normal collagen biosynthesis, the improved classification of

these diseases and has suggested possible methods of therapy. However very little information is available concerning the primary structure of collagen in the dominant forms of Ehlers-Danlos syndrome and osteogenesis imperfecta in which amino acid substitutions in the collagen chains probably constitute the basic defect (McKusick and Martin., 1975; Lancaster et al., 1975).

CHAPTER IV

AIM OF STUDY

In contrast to classical primary structure studies in which large quantities of purified collagen are used, it is necessary in studying human genetic connective tissue diseases to use techniques suitable for the analysis of collagen in small tissue samples.

The techniques of amino acid analysis, crosslink analysis, cyanogen bromide peptide analysis and polyacrylamide electrophoresis have provided valuable information regarding the collagen in small tissue samples obtained from patients with the Ehlers-Danlos syndrome (Lichtenstein et al., 1973; Penttinen et al., 1972; Mechanic, 1972; Pope et al., 1975). It is unlikely however that these techniques applied to small tissue samples would enable the detection of an amino acid substitution such as may exist in the dominant forms of the Ehlers-Danlos syndrome and osteogenesis imperfecta (McKusick and Martin, 1975; Lancaster et al., 1975).

While an amino acid substitution may not produce a detectable alteration in the amino acid content of the tissue or in the electrophoretic or chromatographic characteristics of soluble collagen or the cyanogen bromide peptides it may alter the behaviour of smaller collagen peptides. Smaller peptides, in particular tripeptides are released from collagen by clostridiopeptidase A (Michaels et al., 1958). It was reasoned that if suitable methods of peptide separation and detection were available this technique may be appropriate for the study of collagen in dominantly inherited connective tissue diseases.

Recently Paz and Gallop (1975) reported that good fingerprints were obtained when the peptides released from small amounts of collagen by clostridiopeptidase A were separated by high pressure ion exchange liquid chromatography and detected by scintillation counting. As a result of this report it appeared likely that with some modifications this technique would be suitable for fingerprinting collagen in small tissue samples.

The aims of the study were :

1. To determine if specific fingerprints could be obtained when 1 mg samples of human type I collagen were digested with clostridiopeptidase A and the released peptides separated by high pressure ion exchange liquid chromatography and reacted with ninhydrin.
2. To determine the fingerprints of human $\alpha 1(I)$, $\alpha 1(II)$, $\alpha 1(III)$ and $\alpha 2$ chains.
3. To determine if specific fingerprints could be obtained when this technique was applied to small samples of human dermis, tendon, bone and cartilage.
4. To determine from the results whether this technique would be suitable for the primary structure analysis of collagen in human genetic connective tissue diseases.

CHAPTER V

MATERIALS AND METHODS

5.1. REAGENTS

Pepsin (2 x crystallized), azocasein, clostridiopeptidase A (E.C. 3.4.4.19) (described as collagenase type III from Clostridium histolyticum), 4-hydroxy-L-proline and δ-hydroxy-lysine-HCl were purchased from SIGMA Chemical Co. (St. Louis, Mo.). The tripeptides Gly-Pro-Hyp, Gly-Ala-Ala, Gly-Pro-Ala and Gly-Pro-Pro came from FOX Chem. Co. (Los Angeles, Ca.). DEAE-cellulose (DE 52) and CM-cellulose (CM 52) were obtained from WHATMAN (Maidstove, Kent). PIERCE (Rockford, Il.) was our supplier of ninhydrin reagent (NIN-sol) and of amino-acid standard (hydrolysate, sodium citrate buffer) which was completed with weighed hydroxyproline and hydroxylysine. Reagents for gel electrophoresis were from EASTMAN KODAK Co. (Rochester, N.Y.) except for sodium dodecyl sulfate (specially pure) which was obtained from BDH Chemicals (Montreal, Canada). All the other reagents were from FISHER Scientific (Montreal, Canada). The insoluble material found in guanidine hydrochloride (purified) was eliminated by filtration before use.

5.2. GENERAL PROCEDURES.

Tissue specimens were milled for 4 min. at liquid nitrogen temperature in a freezer-mill (SPEX Ind., Metuchen, N.J.). The centrifugations to collect precipitated material were made for 30 min. at 4^{o} C and 37,000 g (r_{av} 8.25 cm) in the SS-34 rotor of the SORVALL RC-2B centrifuge. Tris-HCl buffers were prepared by weighing the required amount of Tris, dissolving

it in half of the final volume and adjusting the pH with 1 M HCl. The solution was then brought to volume. Dialyses were made with stirring at 4° C in SPECTRAPOR #2 membranes (specified molecular weight cut off : 12-14,000) (SPECTRUM MED. Ind., Los Angeles, Ca.).

5.3. PURIFICATION OF HUMAN TYPE I, II and III COLLAGENS.

Skin samples were collected at autopsy from patients and foetuses who had died from conditions not likely to have been associated with collagen abnormalities. Foetal dermis was used as the source of type III collagen and both foetal and adult dermis were used as the sources of type I collagen. These collagens were isolated and purified using the techniques described by Epstein (1974). The type III collagen obtained was found to contain a little type I collagen. When chromatographed on CM-cellulose, the type III collagen coeluted with the β12 chains of type I collagen. Pure type III collagen was precipitatied from this coeluting material by dialysis against 0.05 M Tris-HCl buffer, pH 7.5, containing 1.71 M NaCl.

Hyaline costal cartilage was obtained at autopsy from a 3-year-old child who had died of cardiac disease. After excision of the perichondrium and milling of the cartilage, the proteoglycan was extracted at 4° C with 5.5 M guanidine hydrochloride, 0.15 M potassium acetate, pH 6.3 solution (10 ml/gm wet weight) changed daily for 5 days (Rosenberg, 1975). Type II collagen was obtained by limited pepsin digestion of the residue using the techniques of Miller (1972) and of Eyre and Muir (1975,a). The type II collagen obtained was chromatographed on both CM and DEAE-cellulose.

CM- and DEAE-cellulose chromatography.

CM-cellulose chromatography was performed as described by Lichtenstein et al. (1975). The bed size was 1.5 x 20 cm and the column was eluted at 45° C with a 400 ml linear gradient from 0 to 0.1 M NaCl in 0.03 M sodium acetate buffer, pH 4.8, containing 4 M urea. The absorbance of eluting material was continuously measured at 230 nm with a SCHOEFFEL 440 spectro-flow monitor equipped with a 8 µl cell.

The technique of Trelstad et al. (1972) was used for DEAE-cellulose chromatography with the addition of 1.3 M urea to the buffers as suggested by Seyer et al. (1974,a). The bed size was 1.5 x 5 cm.

Gel electrophoresis.

Urea-sodium dodecysulfate polyacrylamide gel electrophoresis was made according to the technique of Goldberg et al. (1972) in a BÜCHLER POLYANALYST instrument. The gels were stained with 1 % amido-black in 7 % acetic acid for 30 min. Destaining was performed in a diffusion destainer (model 172, BIO-RAD, Richmond, Ca.) using a solution of acetic acid (50 ml), methanol (75 ml) and water to 1 L. Gels were scanned with a scanning microdensitometer (Model 445-50 Densicomp, CLIFFORD Inst., Natick, Mass.) equipped with an interferential filter centered at 550 nm.

5.4. PREPARATION OF TISSUES FOR CLOSTRIDIOPEPTIDASE A DIGESTION.

Samples of skin, tendon, bone and costal cartilage were obtained at operation or autopsy from patients with disorders not likely to have been associated with collagen abnormalities. Each tissue was mechanically cleaned and milled to a fine powder. The milled dermis, tendon and costal cartilage underwent limited pepsin digestion as described by Epstein (1974). Following the digestion the pepsin was inactivated by raising the pH of the mixture to 8 for 20 min. using 2 M NaOH. The mixture was dialyzed at 4^{o} C against 0.05 M Tris HCl buffer, pH 7.5, containing 0.5 M NaCl, against deionized water and lyophilized. Five mg of each lyophilized sample was used for clostrodiopeptidase A digestion.

Samples of milled bone were washed with a 0.15 M NaCl solution at 4^{o} C until the solution was clear. The powder suspended in this solution was dialyzed at 4^{o} C against a 0.6 M EDTA solution adjusted to pH 8.0 with NaOH (200 ml/100 gm of bone) (Dickson, 1974). The solution was changed each day for 7 days. The decalcified bone suspension was dialyzed against 0.05 M Tris-HCl buffer, pH 7.5 containing 0.5 M NaCl, against deionized water and lyophilized. Limited pepsin digestion of this material was made as described above. Five mg of the resulting lyophilized material was used for clostridiopeptidase A digestion.

5.5. CLOSTRIDIOPEPTIDASE A DIGESTION.

Digestion of collagen by clostridiopeptidase A was made essentially as described by Paz and Gallop (1975).

Samples of 1 to 2 mg of collagen were suspended in 0.5 ml of a 0.02 M Tris-HCl buffer, pH 7.5, containing 5 mM $CaCl_2$ and 0.01 % azocasein. 10 µl of a clostridiopeptidase A solution (2,000 units/nl) in the same buffer was added and the mixture was maintained at 37° C for 15 hours with mild agitation. 0.5 ml of a 10 % trichloracetic acid solution was then added and left to stand for 30 min. at 4° C. The suspension was centrifuged for 10 min. at 4° C and 9,200 g (r_{av} 8.25 cm). The pellet was discarded and the supernatant extracted three times with ether to remove trichloroacetic acid. The residual solution was lyophilized.

5.6. HIGH PRESSURE LIQUID CHROMATOGRAPHY OF THE CLOSTRIDIOPEPTIDASE A DIGESTION PRODUCTS.

The automatic analysis of the small peptides released by the clostridiopeptidase A digestion was performed on the DURRUM D-500 amino acid analyzer (DURRUM INST. Co., Palo Alto, Ca.). Each lyophilized sample was dissolved in the sodium citrate sample buffer, pH 2.2 (100 µl/mg), and an aliquot of 40 µl transfered into a sample holder unit. The chromatography was performed at 2,000 psi on the standard 1.75 mm bore, 48 cm long, DC-4A-resin column, using a modified DURRUM sodium citrate buffer system to produce a discontinuous pH and ionic strength gradient. The elution system

was as follows : time 0 refers to sample injection. 0-44.5 min, pH 3.25, Na^+ 0.2 N; 44.5-52 min., pH 3.75, Na^+ 0.2 N (prepared as a 1:1 mixture of the Na^+ 0.2 N, pH 3.25 and pH 4.25 standard buffers); 52-80 min. pH 4.25, Na^+ 0.2 N; 80-130 min., pH 4.50, Na^+ 0.38 N (prepared by addition of 6 N HCl to the Na^+ 0.38 N, pH 5.02 standard buffer); 130-165 min., pH 7.9, Na^+ 1.1 N. The column temperature was 50° C and raised to 65° C at 10 min. The elution time of the tripeptides Gly-Pro-Hyp, Gly-Ala-Ala, Gly-Pro-Ala, Gly-Pro-Pro, were determined with synthetic standards.

Reaction with ninhydrin was performed at 126° C under 110 psi back-pressure and the absorbance of the reaction products read with a 590 nm photometer.

Amino-acid analysis.

Samples were hydrolyzed under nitrogen in 6 M constant boiling HCl for 20 h at 110° C and analyzed with the DURRUM D-500 amino-acid analyzer using the standard 4 buffer system for collagen analysis. Hydroxyproline and proline reaction products were detected with a 440 nm photometer.

CHAPTER VI

RESULTS

6.1. CHARACTERISTICS OF THE ISOLATED COLLAGENS.

The behaviour of our type I collagen preparation on CM-cellulose chromatography (fig. 4) and polyacrylamide electrophoresis (fig. 5) conformed to the characteristics of type I collagen reported by Bornstein and Piez, 1964. Characteristic electrophoretic mobilities and amino acid contents were also observed for the eluted α1(I), β11, β12 and α2 chains. The slight heterogeneity observed in the electrophoretic pattern of type I collagen and the α1(I) chains has also been observed by others (Chung and Miller, 1974; Eyre and Muir, 1975) and attributed to the production of some shorter chains by the action of pepsin.

The cartilage collagen produced a single peak when chromatographed on CM-cellulose (fig. 6) and when chromatographed on DEAE-cellulose this peak was resolved into two main fractions (fig. 7). These fractions had identical amino-acid compositions (Table III) and hexose contents as determined by the method of Robertson and Harvey (1972). Apart from higher hydroxylysine contents their amino acid compositions were similar to the composition of human type II collagen calculated from its cyanogen bromide fragments by Miller and Lunde (1973). As a result, the major peaks obtained from the CM- and DEAE-cellulose chromatographies were considered to contain type II collagen. The presence on electrophoresis of some β chains in the second DEAE-cellulose fraction suggests that the type II collagen in these fractions differed in their state of aggregation (fig. 8). In contrast to Seyer et al. (1974a,b) who found both type I and II collagens in bovine cartilage we only found type II collagen in our isolated human material.

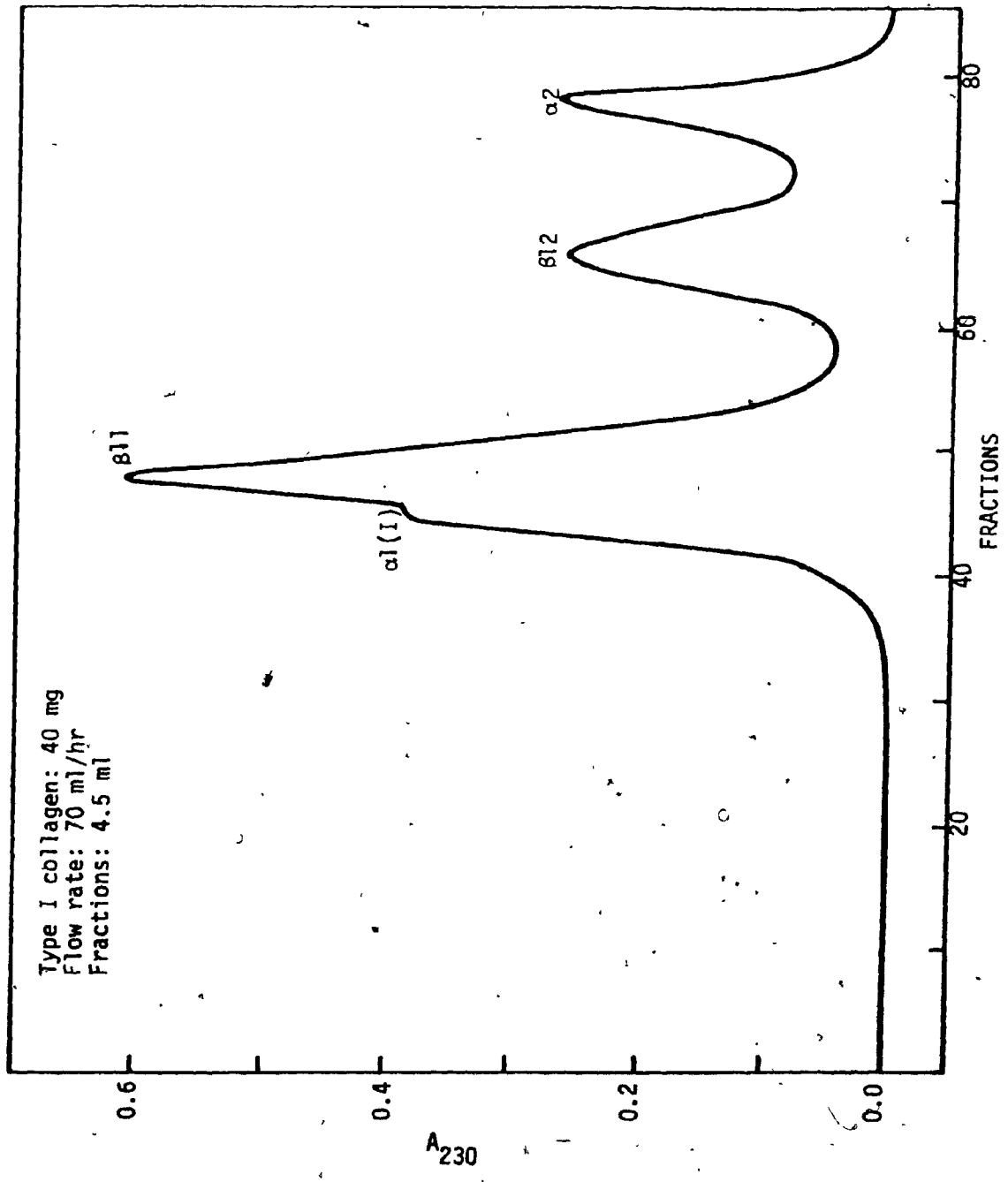

FIGURE 4. CM-cellulose chromatogram of type I collagen.

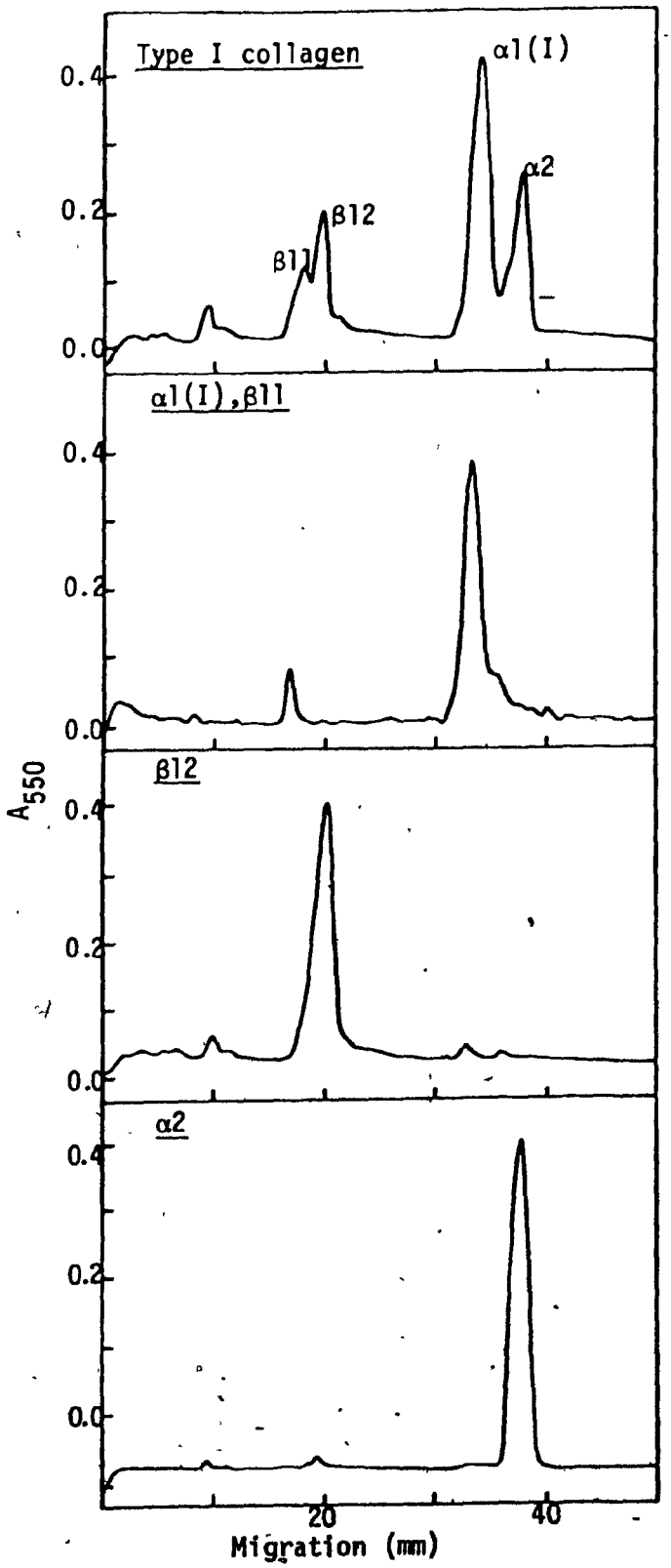

FIGURE 5. Electrophoretogram of type I collagen and its component chains.

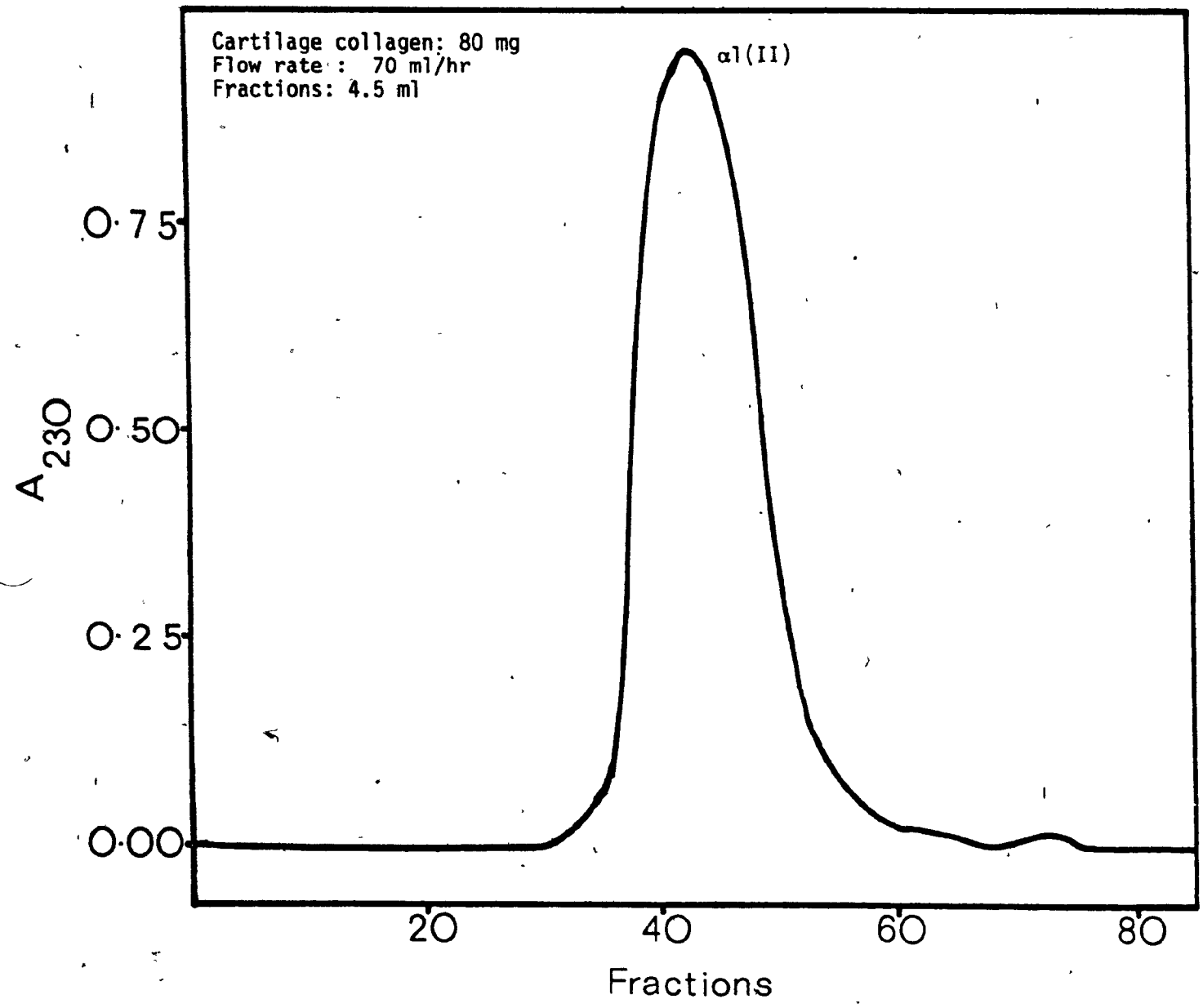

FIGURE 6. CM-cellulose chromatogram of cartilage collagen.

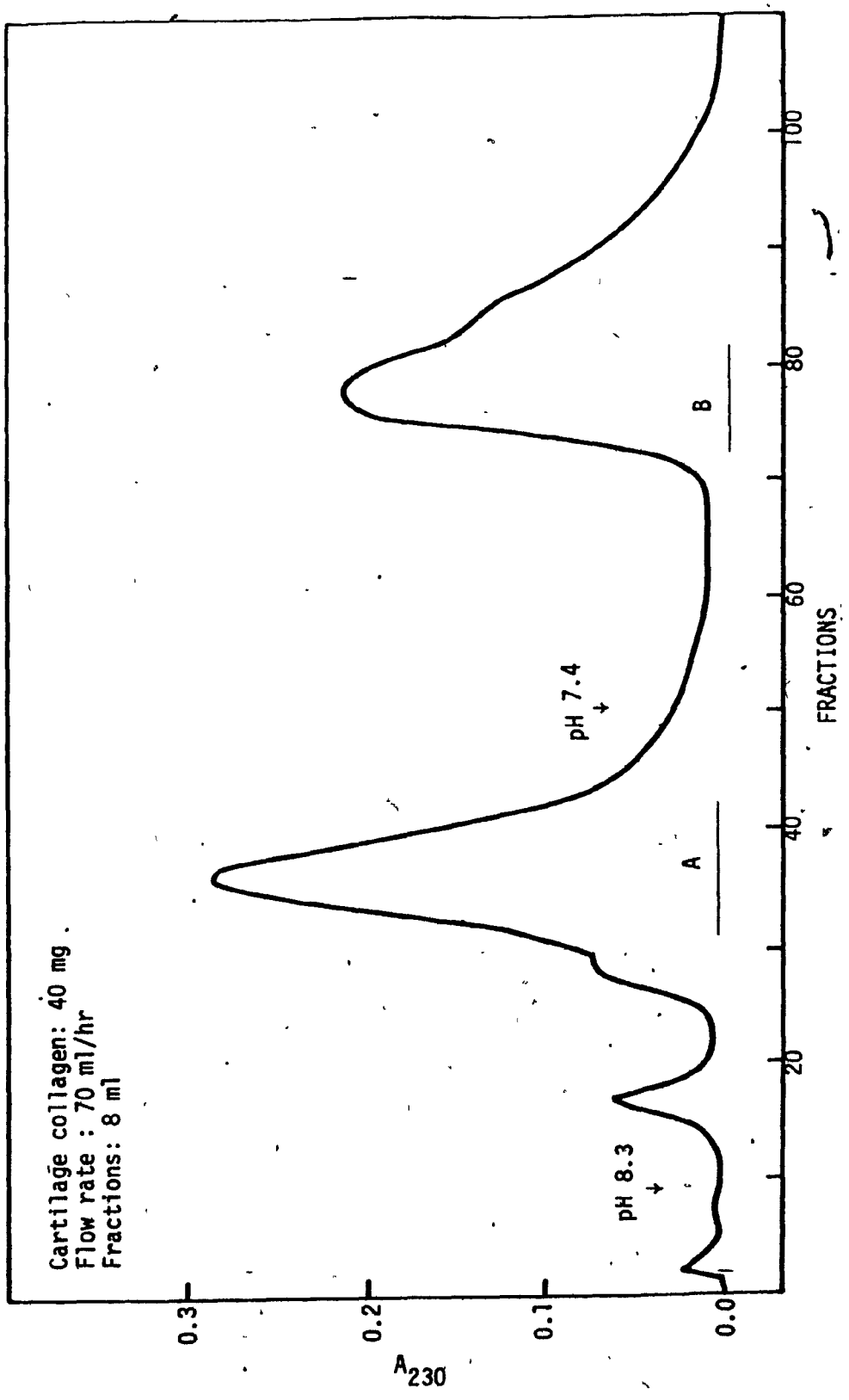

FIGURE 7. DEAE-cellulose chromatogram of cartilage collagen.

TABLE III

Amino Acid Composition of Cartilage Collagen

Cartilage collagen was chromatographed on CM-cellulose and yielded a single fraction referred to as CM-cellulose. When chromatographed on DEAE-cellulose two major fractions, referred to as Fractions A and B were obtained.

Amino Acids	Content (residues/1000 residues)		
	CM-cellulose	DEAE-cellulose	
		fraction A	fraction B
Hyp	97	96	95
Asp	40	42	41
Thr	20	22	20
Ser	27	30	29
Glu	99	98	99
Pro	119	116	118
Gly	336	332	331
Ala	101	103	105
Val	16	15	16
Met	6.0	7.9	7.0
Ile	11	9.9	10
Leu	30	28	29
Tyr	1.2	1.8	1.1
Phe	13	12	12
His	2.1	2.7	2.7
Hyl	19	19	18
Lys	17	17	17
Arg	49	49	49

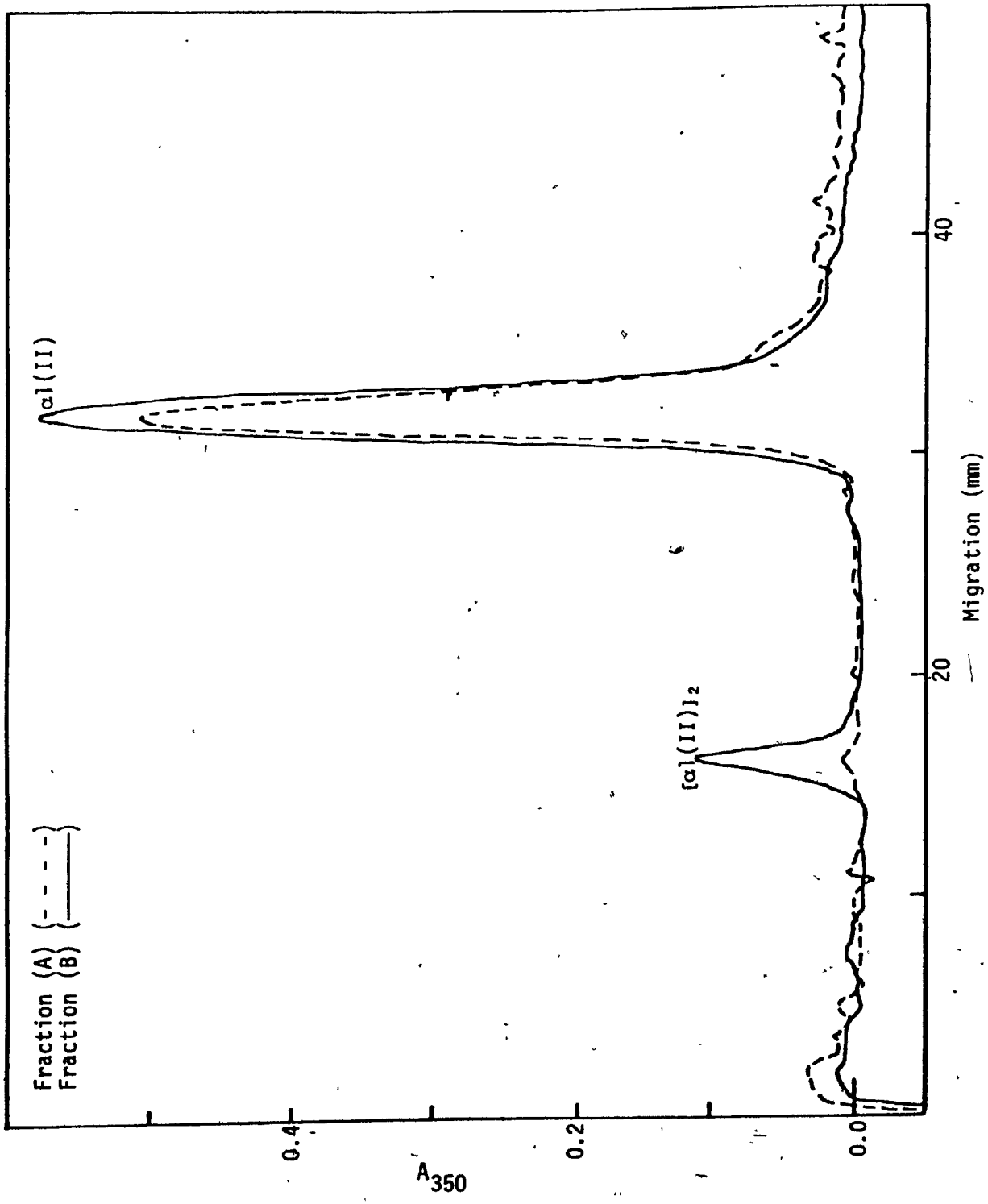

FIGURE 8. Electrophoretograms of cartilage collagen components.

A characteristic single symmetrical peak was obtained when our unreduced type III collagen preparation was chromatographed on CM-cellulose (fig. 9). On gel electrophoresis the same preparation showed a major γ band and some higher molecular weight material (fig. 10). After reduction of the disulfide bonds with β-mercaptoethanol the characteristic conversion of the slowly migrating γ chains into the faster migrating αl chains was observed (Eyre and Muir, 1975),. The presence of some dimeric (β) and trimeric (γ) material was probably due to the presence of some non disulphide crosslinks. The amino acid composition of this type III collagen preparation was also found to be similar to the compositions reported by Epstein (1974) and Chung and Miller (1974).

6.2. ANALYSIS OF THE PEPTIDES RELEASED FROM ISOLATED COLLAGENS

After digestion with clostridiopeptidase A, each type of collagen showed a specific elution profile on high pressure ion exchange liquid chromatography (fig. 11). More than 40 peaks were resolved. Peaks corresponding to the elution times of the tripeptide standards were identified (Gly-Pro-Hyp, 43 min.; Gly-Ala-Ala, 46 min.; Gly-Pro-Ala, 61 min. 15; Gly-Pro-Pro, 62 min.20). Partial resolution between the Gly-Pro-Hyp and Gly-Ala-Ala and between Gly-Pro-Ala and Gly-Pro-Pro was obtained.

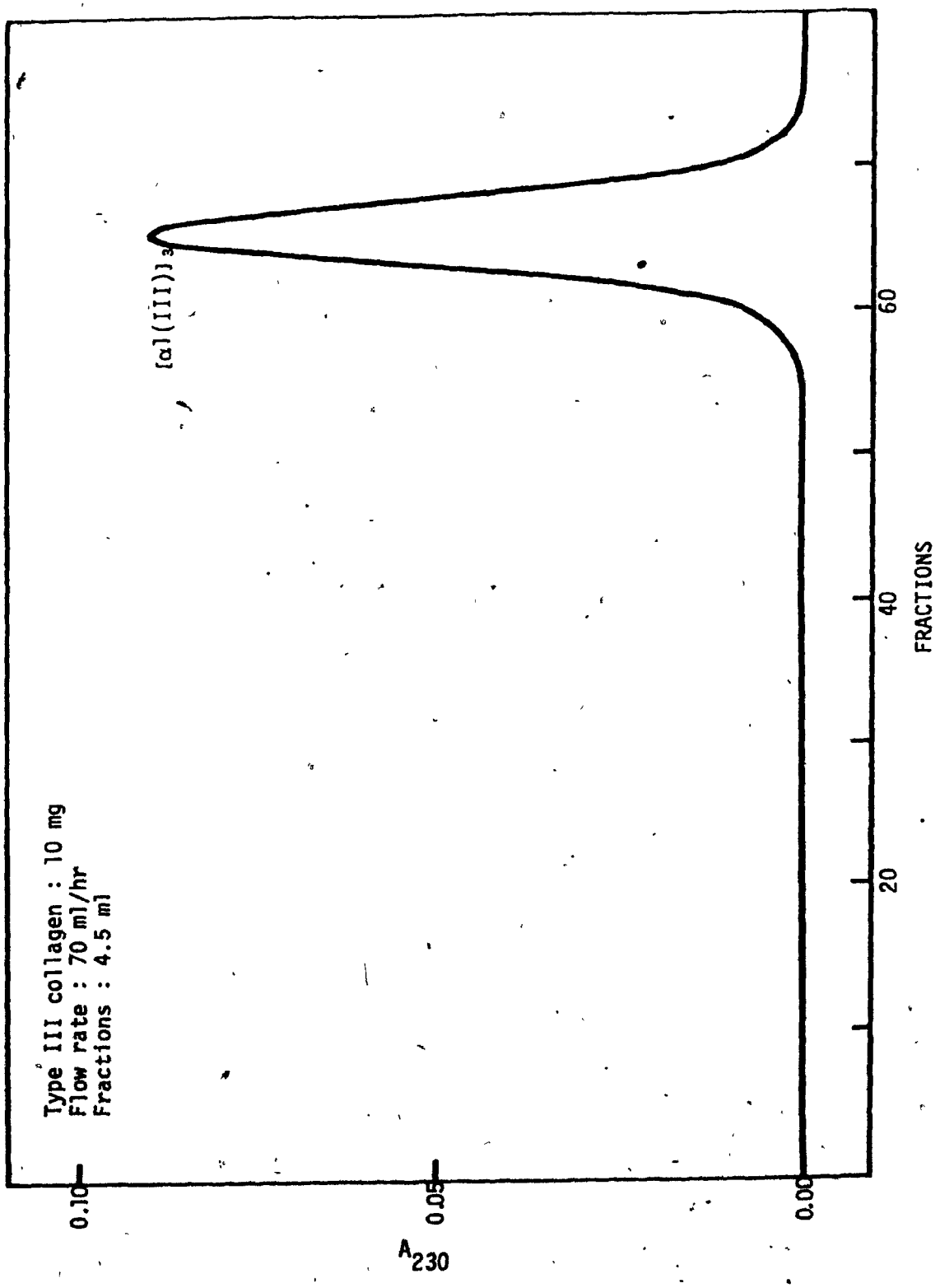

FIGURE 9. CM-cellulose chromatogram of type III collagen

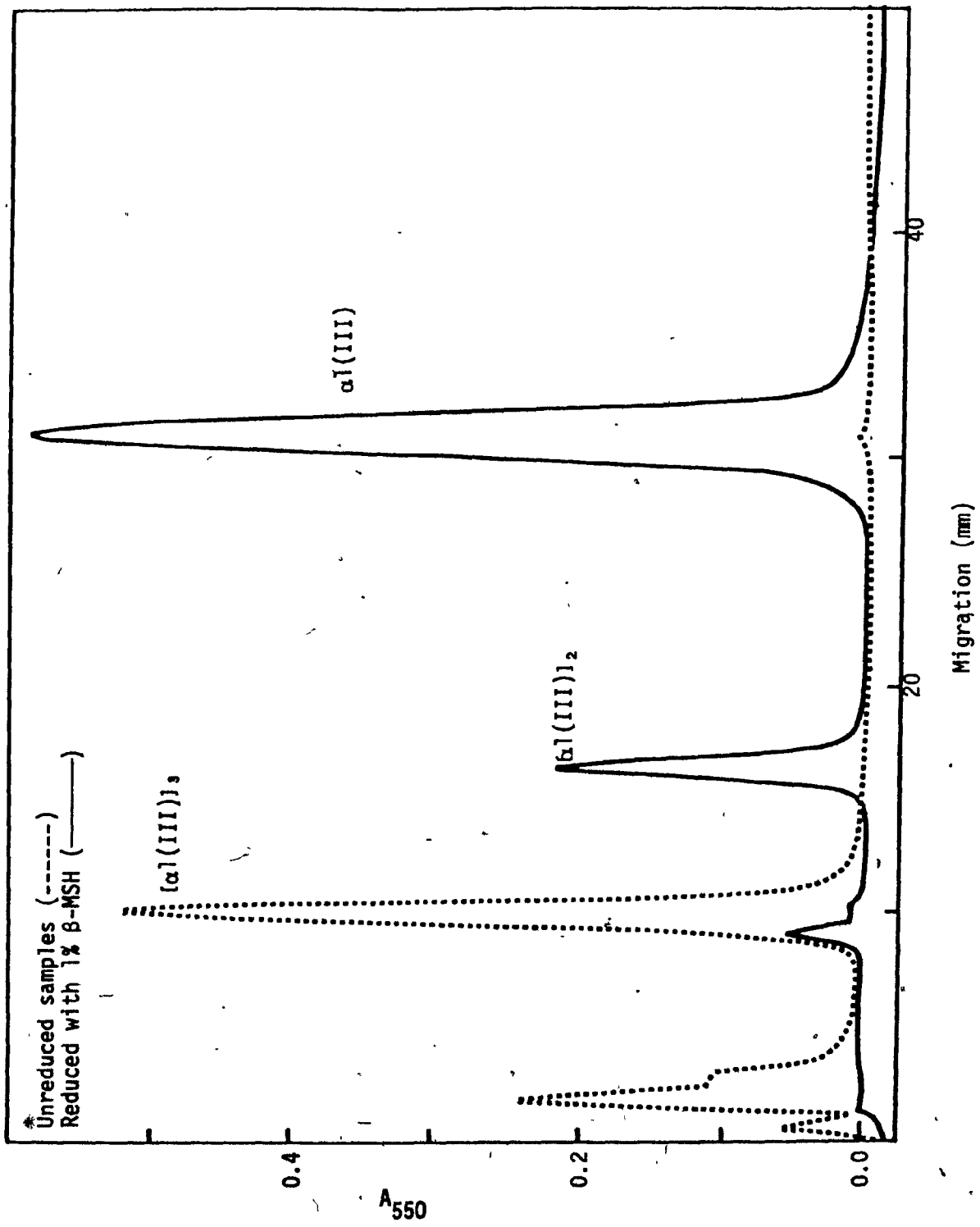

FIGURE 10. Electrophoretogram of type III collagen.

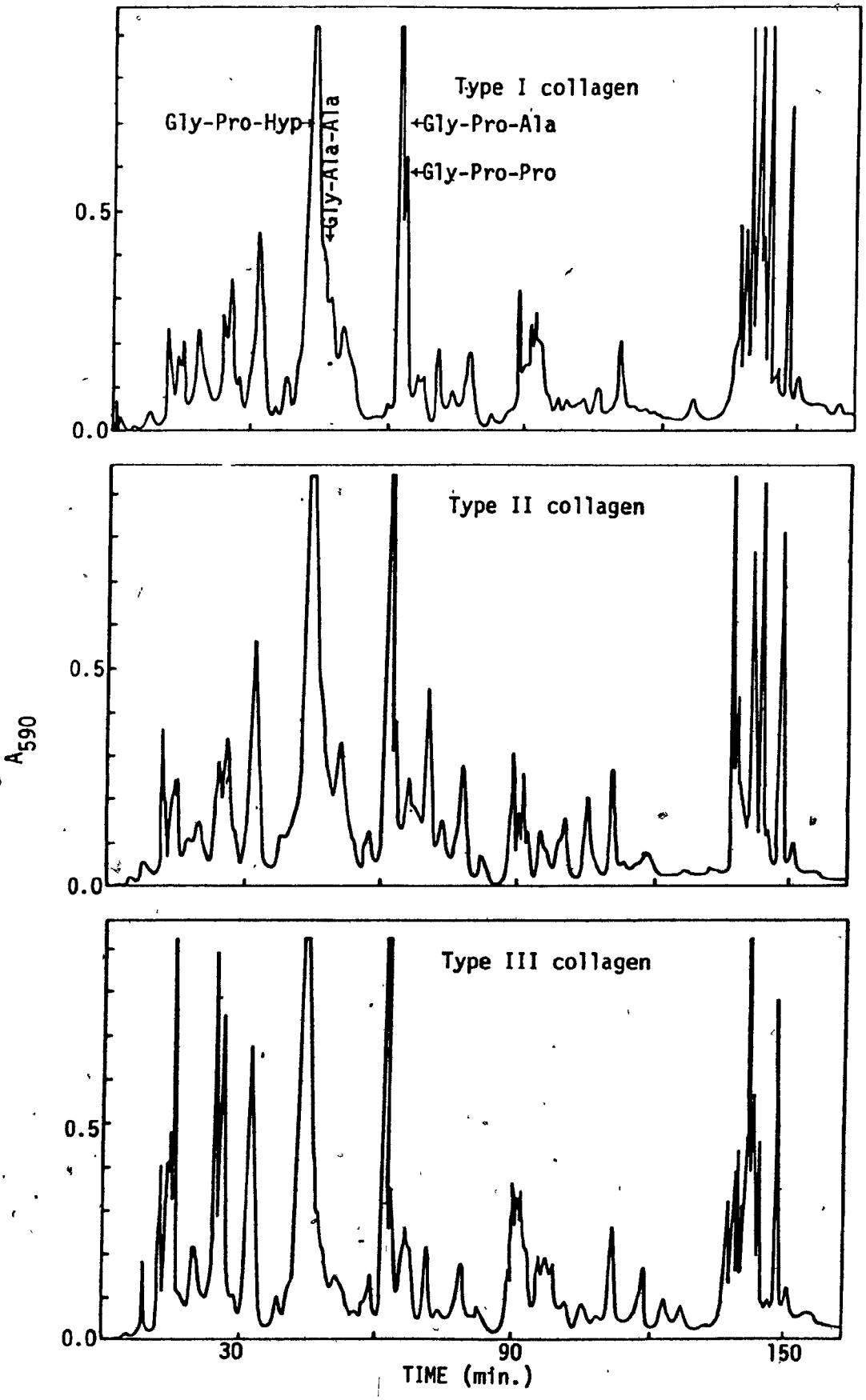

FIGURE 11. High pressure liquid chromatograms of the collagens after collagenase digestion.

Three peaks were produced by non collagenous materials. A urea peak eluting after 9 min. was found in the digests of chromatographically purified chains. The peak eluting after 78 min. was an artefact also present in blanks and the peak eluting after 111 min. was due to ammonia.

The conditions used for the clostridiopeptidase A digestion as judged by the completeness of the digestion and the lack of non specific proteolysis appeared to be satisfactory. Quantitative amino acid analysis of the collagen substrates and the 5 % trichloracetic acid soluble collagen peptides showed that the reaction was complete. In addition a comparison of the elution profiles of the collagenase released peptides with amino acid elution profiles failed to show any significant coincident peaks. This finding was considered to indicate the absence of any significant exopeptidase activity. The activity of non specific endopeptidases as assessed by the digestion of azocasein was also shown to be minimal.

The elution patterns obtained from different runs of the same peptide preparation were found to be almost identical. The elution pattern obtained from separate digests of the same substrate were also almost identical provided the same conditions, in particular the same enzyme preparation was used. We observed several differences when ADVANCE BIOFACTURE Corp. (Lynnbrook, N.Y.) form III collagenase was used instead of the SIGMA type III collagenase. These differences were probably related to the known heterogeneities of this enzyme (Miyoshi and Rosenbloom, 1974).

An assessment of the suitability of this technique for the quantitative analysis of collagen was made. Purified type I collagen, α1(I), α2 and β12 chains were separately digested with clostridiopeptidase A and the resulting peptides chromatographed. Two peaks were selected; the one eluting at 88 min. 58 sec. (peak 88/58) was considered specific for the 1(I) chain and the one eluting at 150 min. 58 sec. (peak 150/58) specific for α2 (fig. 12). Using these peaks as markers, we recalculated the chain composition of type I collagen and β12 chains. The results given in Table IV show close agreement between the experimental and theoretical chain compositions.

Using the quantitative information obtained from this experiment we mixed the peptides produced by the separate digestions of α1(I) and α2 chains in a 2:1 ratio simulating type I collagen. The resulting elution profile was almost completely superimposable on the one obtained for type I collagen (fig. 13).

6.3. ANALYSIS OF PEPTIDES RELEASED FROM TISSUE COLLAGEN.

Readily recognizable collagen fingerprints were obtained from the dermal, tendon, bone and cartilage samples (fig. 14 and 15). It will be noted that a new DURRUM DC-4A column was used for these analyses and the speed of the paper was doubled. Better resolution of the peptides was obtained.

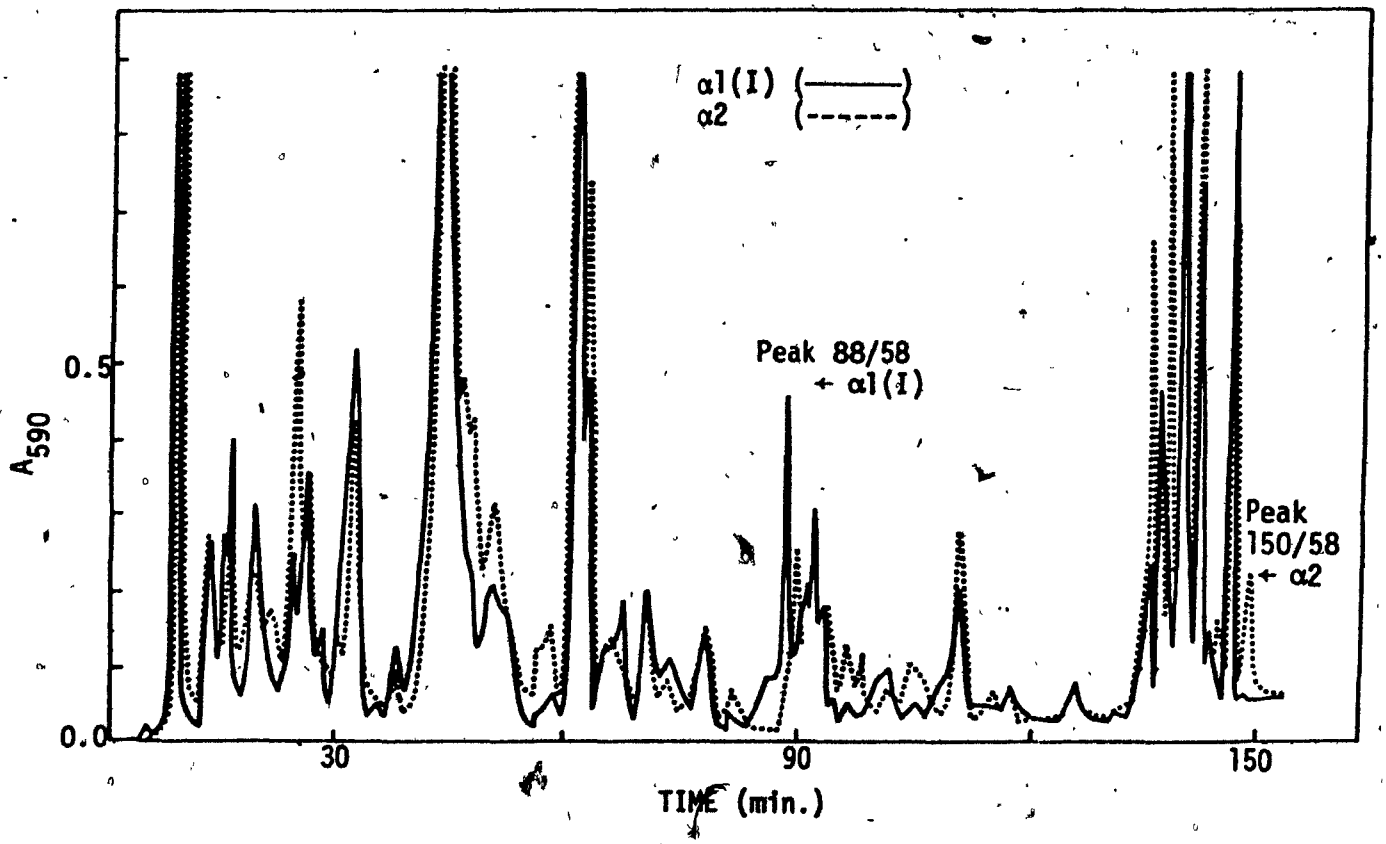

FIGURE 12. Chromatograms of α1(I) and α2 collagenase digests.

TABLE IV

Quantitative Analysis of Type I Collagen

Using the described high pressure liquid chromatography analysis of the collagenase digested collagens the proportions of α1(I) and α2 chains were evaluated from the relative area of the peak eluting at 88 min. 58 sec. considered as specific for the α1(I) chain and of the peak eluting at 150 min. 58 sec. considered as specific for α2 chain. These peaks are indicated by arrows on fig. 12.

Digested chain	Experimental values+		Theoretical values*	
	Peak 88/58 [α1(I)]	Peak 150/58 [α2]	α1(I)	α2
α1(I)-β11	25.4 (100%)	0	100	0
α2	0	14.8(100%)	0	100
$[\alpha 1(I)]_2\alpha 2$	17.0(67%)	4.9(33%)	67	33
β12	13.2(52%)	8.4(57%)	50	50

+ peak area x $10^3/\Sigma$ of integrated peaks

* expressed in % of the total collagen.

mark on original

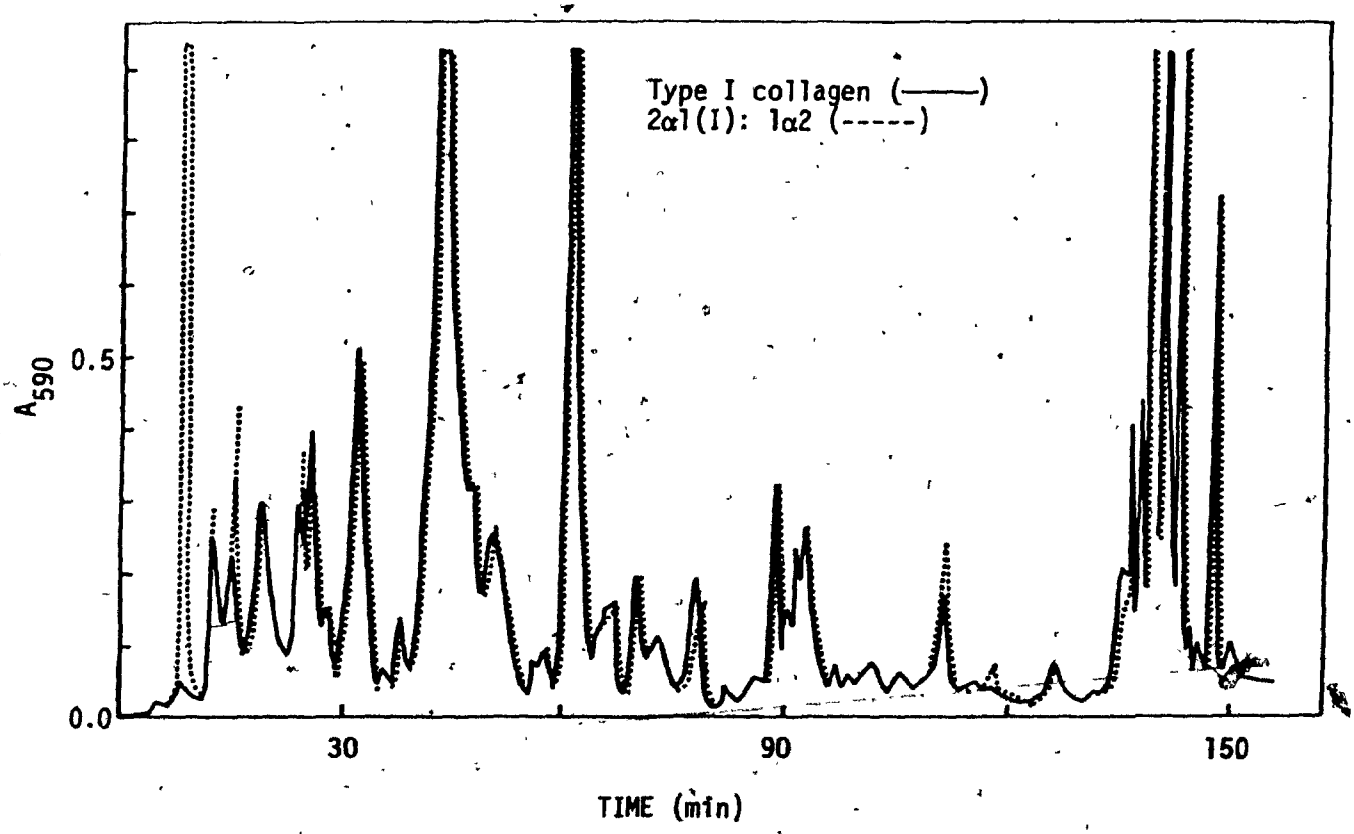

FIGURE 13. Reconstitution of the type I collagen fingerprint from the α1(I) and α2 fingerprints.

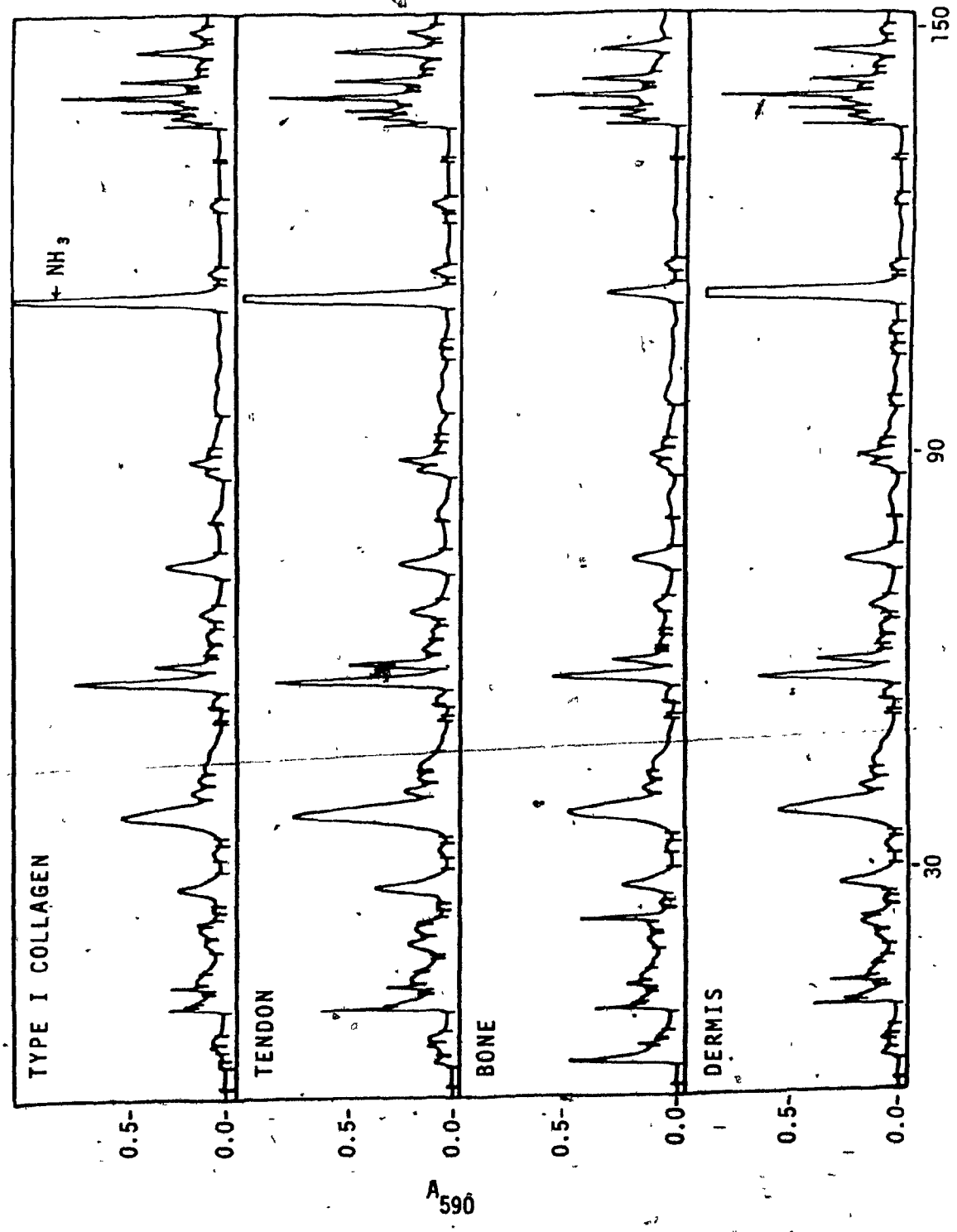

FIGURE 14. Chromatograms of type I collagen, tendon, bone and dermis collagenase digests.

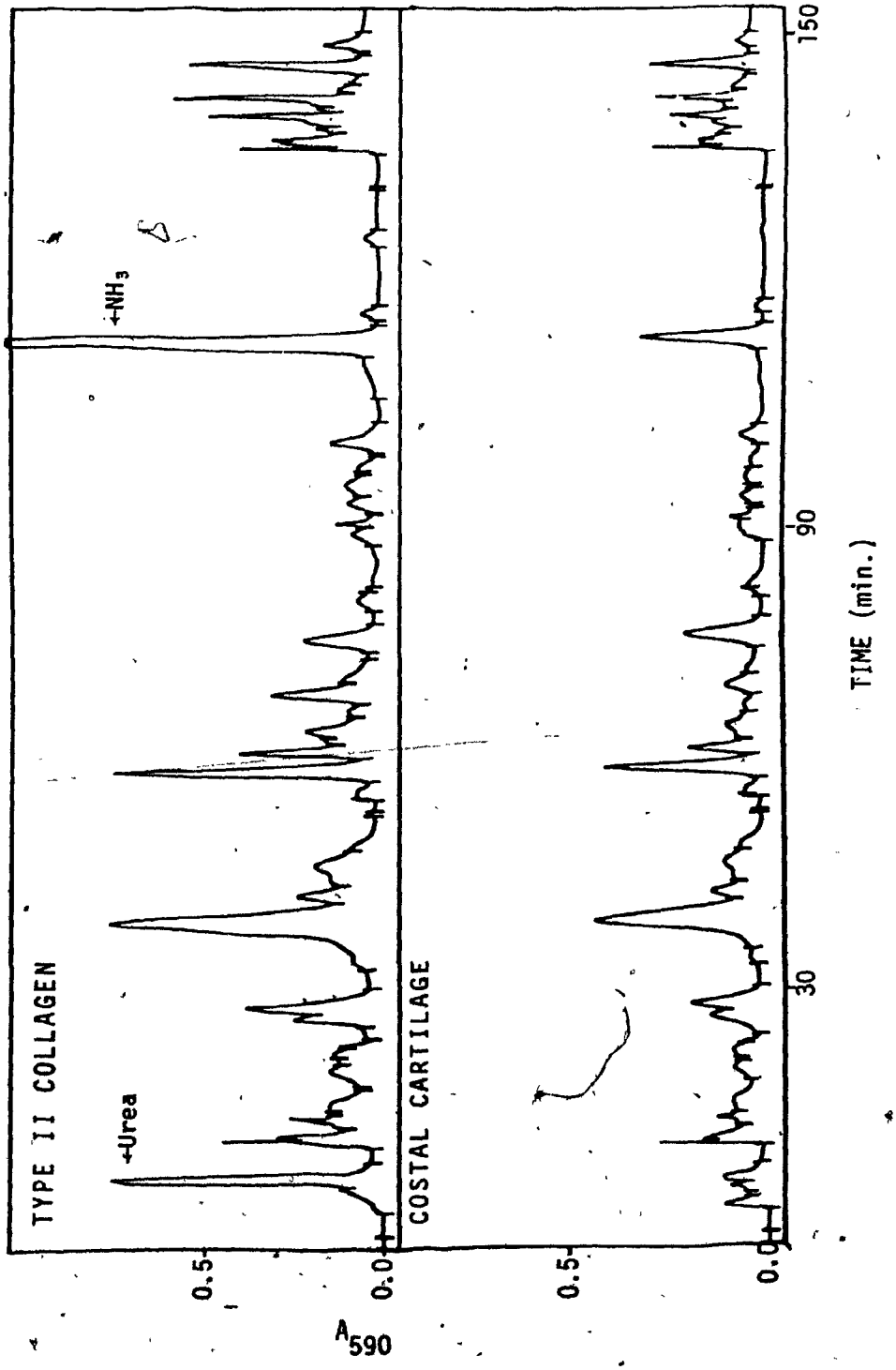

FIGURE 15. Chromatograms of type II collagen and costal cartilage collagenase digests.

The fingerprints obtained from tendon, bone and dermis were almost identical to the type I collagen fingerprint shown in fig. 14. In each tissue approximately 75 % of the collagen was converted to trichloracetic acid soluble peptides. There was no evidence of non specific proteolysis apart from the fingerprint of one bone sample which showed several additional peaks possibly produced by amino acids.

A type II collagen fingerprint was obtained from the costal cartilage digest. There were no sign of non specific proteolysis. Approximately 75 % of the cartilage collagen was converted to trichloracetic acid soluble peptides.

CHAPTER VII

DISCUSSION

DISCUSSION

The technique developed for fingerprinting human collagen was found to be satisfactory for both isolated collagen and collagen within small samples of tissue.

CLOSTRIDIOPEPTIDASE A FINGERPRINTING OF COLLAGEN

This study showed that the conditions described by Paz and Gallop (1975) for clostridiopeptidase A digestion were satisfactory. Using 1 mg samples the peptide yield for each type of collagen was essentially 100 %, the fingerprints were reproducible and there was minimal evidence of non specific proteolysis.

These observations are in agreement with the reports concerning the action of clostridiopeptidase A. This enzyme has been shown to contain two main fractions termed fraction A and B (Miyoshi and Rosenbloom, 1974). Fraction A cleaves the collagen molecule at the bond between X and Gly in the sequence -P-X-Gly-P-X- where P is proline and X is any amino acid (Harper and Kang, 1970). In addition to this site of cleavage fraction B is able to cleave at the amino end of glycine in some sequence where P is replaced by other amino acids (Bornstein, 1967; Harper and Kang, 1970). The digests contain mainly triplets and some larger peptides of 10 to 30 residues (Franzblau et al., 1964).

High pressure ion exchange liquid chormatography was a suitable method of separating these peptides. It had the advantage of requiring very small quantities of peptides (equivalent to approximately 200 µg of collagen) and of being highly reproducible. The ninhydrin detection system was sensitive so that good fingerprints were obtained without the need to use larger quantities of peptides or radioactive collagen from fibroblast cultures. It was also considerably less expensive than using radioactively labelled collagen.

FINGERPRINTS OF THE ISOLATED COLLAGENS

Specific fingerprints were obtained from 1 mg samples of human type I, II and III collagens and α1(I) and α2 chains. The differences in these fingerprints were considered to reflect differences in the primary structure of each of these collagens. This view is supported by our observation that the type I collagen fingerprint could be reconstituted from the fingerprints of its component α1(I) and α2 chains.

It may be possible to use some of these differences to assess the relative amounts of these collagens in a mixture. This was shown to be feasible for the α1(I) and α2 chains in which specific marker peaks were used.

Four tripeptide peaks Gly-Pro-Hyp, Gly-Ala-Ala, Gly-Pro-Ala and Gly-Pro-Pro were identified from the elution times of standards. Two of these peaks Gly-Pro-Pro and Gly-Pro-Hyp may be used to estimate the completeness of the post translational step of prolyl hydroxylation as shown by Paz and Gallop (1975). They suggest that in addition comparison of Gly-Ala-Hyp to Gly-Ala-Pro would provide extra information concerning prolyl hydroxylation and comparison of Gly-Pro-Hyl to Gly-Pro-Lys would provide information concerning lysyl hydroxylation. In contrast to assessing the completeness of prolyl and lysyl hydroxylation from the amino acid content of the tissue this method provides a means of evaluating the hydroxylation step in selected sequences of this molecule.

FINGERPRINTS OF TISSUE COLLAGEN.

Good peptide yields and readily recognizable fingerprints were obtained from clostridiopeptidase A digested samples of dermis, tendon, bone and cartilage.

Dermis : In our early studies untreated milled dermis was digested with clostridiopeptidase A but the resulting fingerprints while resembling the purified type I fingerprint contained many extra peaks. However when limited pepsin digestion and dialysis of the digestion mixture were made prior to clostridiopeptidase A digestion fingerprints almost identical to the type I collagen pattern were obtained. This extra step probably destroyed non specific proteolytic enzymes and removed low molecular weight ninhydrin

reacting materials. Because of this improvement limited pepsin digestion and dialysis were also used for the other tissues. The disadvantage of adding this step is that pepsin removes the non helical telopeptides of collagen (Rubin et al., 1963).

The whole pepsin digest (following the inactivation of pepsin and dilaysis) was used for clostridiopeptidase A digestion as it was considered important to obtain fingerprints from the soluble and insoluble collagen fractions. The peptide yield of approximately 75 % was a little less than can be obtained by cyanogen bromide cleavage (Epstein, 1974). However the clostridiopeptidase A technique has the advantage that good fingerprints are produced from very small quantities of collagen. The greater sensitivity of this technique is probably related to the better resolution that can be obtained using high pressure ion exchange liquid chromatography in contrast to the low pressure chromatography used for separation of the larger cyanogen bromide peptides. In addition it is likely that less than 5 mg of dermis could be used for our studies as only part of the peptide solution was used for fingerprint analysis.

The type I collagen fingerprint obtained from dermis suggests that these samples contained predominantly type I collagen. Foetal skin samples which have been reported by Epstein (1974) to contain a high proportion of type III collagen were not studied.

Because the dermal fingerprints were almost identical to those obtained from purified type I collagen it was considered that this technique could be used to study the collagen within small dermal samples without the need to isolate and purify it first.

Tendon : Similar observations were made for tendon. The tendon fingerprints appeared to only contain type I collagen peptides which agrees with the composition of tendon reported by Miller (1973).

It also appeared that this fingerprinting technique was suitable for the study of collagen in small samples of tendon.

Bone : The E.D.T.A. technique used to extract bone mineral was satisfactory. By dialyzing the bone against E.D.T.A. the small amount of collagen solubilized by this solution was retained (Dickson, 1974).

Good type I collagen fingerprints were produced and the peptide yields were comparable to those obtained from dermis and tendon. Increasing the amount of clostridiopeptidase A failed to increase the yield.

Cartilage : The yield of peptides was increased from 60 % to 75 % by using limited pepsin digestion and dialysis of the costal cartilage prior to clostridiopeptidase A digestion. The mechanism of this effect of pepsin is uncertain but it probably results from an effect on the proteoglycans allowing more collagenase to reach the collagen. To increase the yield further it may be necessary to make a more formal extraction of proteoglycans with guanidine HCl.

From the cartilage fingerprint pattern it appeared that costal cartilage contained predominantly type II collagen which is in agreement with its published composition (Miller and Lunde, 1973).

It is apparent that readily recognizable collagen fingerprints are produced when this technique is applied to samples of dermis, tendon, bone and cartilage.

SUITABILITY OF THIS FINGERPRINTING TECHNIQUE FOR THE STUDY OF HUMAN GENETIC CONNECTIVE TISSUE DISEASES.

This technique has several features that make it of potential value for the study of these diseases.

1. Collagen fingerprints can be produced from 5 mg samples of tissue.
2. Samples of this size can readily be obtained from patients by biopsy.
3. Comparison of the peptide pattern with the fingerprints of normal tissues and purified collagens may enable a peptide containing an amino acid substitution to be detected.
4. It may also be useful for the study of prolyl and lysyl hydroxylation in certain sequences of the collagen molecule.
5. It may be useful for assessing the types of collagen within tissues.

CHAPTER VIII

CONCLUSIONS

It was concluded from this study that :

1. The technique developed to fingerprint collagen was sensitive and reproducible. Specific fingerprints were produced from 1 mg quantities of human type I, II and III collagen and α1(I) and α2 chains.
2. This technique was also able to produce specific fingerprints from the collagen within small samples of dermis, tendon, bone and cartilage.
3. This technique is applicable to the study of the primary structure of collagen within small tissue samples from patients with genetic connective tissue diseases. It should provide information concerning amino acid substitutions, prolyl hydroxylation and the types of collagen.

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