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SEPARATION METHODS FOR THE STUDY OF COLLAGEN AND TREATMENT OF COLLAGEN DISORDERS

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SUMMARY

Liquid chromatographic and electrophoretic methods applicable to the separation of collagen and its fragments are reviewed. Special attention is paid to the separation of both stabile and labile cross-linking elements. Identification procedures exploiting the mapping of either collagen α-chains or of cyanogen bromide fragments are discussed. These methods can be used for diagnosing inborn errors of collagen metabolism using bioptic or necroptic samples. Analysis of urinary hydroxyproline-containing peptides or the determination of peptidically bound pyridinoline is suitable for measuring the intensity of collagen metabolism.

THE FAMILY OF COLLAGEN PROTEINS

Separation procedures based on different separation mechanisms are an inevitable step in almost any work on proteins. Some of these procedures have become routine, others involve specific tricks that are essential for obtaining the desired result. In practice one still meets low-to-medium pressure column liquid chromatographic procedures that are purposefully combined (sometimes with electromigration techniques) in order to obtain purified proteins, in addition to high-performance liquid column chromatography. In protein chemistry there is not only a need to separate intact proteins, but also their fragments (e.g., cyanogen bromide peptides for identification purposes) and marker amino acids. In this review we have tried to demonstrate the various approaches adopted for collagen; the choice of this protein is two-fold: first it offers all the different aspects out-

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lined above, and second we have worked in this area sufficiently long to have sufficient personal experience for the purpose.

In spite of their complex structure, collagens represent a phylogenetically very old category of proteins. Also in ontogeny they occur at a very early stage. Collagens constitute about one third of all proteins present in the living body and their importance for the organism's development and its proper functioning is beyond any doubt. Alterations in collagens originating during biosynthesis demonstrate themselves as pathological disturbances which can be measured by some clinically applicable methods. Chromatographic and electromigration procedures play a substantial role in this field (for a review, see ref. 1).

Currently twelve different collagen types are recognized [2]. The collagen structure is typical in containing a repeating tripeptide sequence (Gly–Pro–Hyp) which causes the formation of a triple helical structure. The high content of Pro and Hyp is the reason for the high rigidity of the structure. Further, the helical parts of collagen molecules are typical in being devoid of Tyr and Trp and in containing a small amount of His. Of the lysyl residues, some are hydroxylated and some of the Hyl residues are glycosylated [3]. Collagens are synthesized as procollagens. With some types the terminal peptides are cleaved off after the molecules have migrated from the cell into the extracellular space [4]. In some collagen types the helical structure is interrupted by non-collagenous (i.e., non-helical) regions which are accesible to cleavage by non-specific proteases (e.g., pepsin) (types IV, V, VII, VIII and IX). Other collagens bear on one or both ends of their polypeptide chains a globular domain. In those collagens which are lacking these globular domains the polypeptide chains are terminated with a non-helical region formed by 15–25 amino acids. These regions (telopeptides) are typical in containing some tyrosine residues.

The helical structure is stabilized by hydrogen bonds derived from the Hyp hydroxyl groups and by inter- and intramolecular covalent bonds (aldimines, aldols and their condensation products). These covalent bonds are derived from oxidized lysines in which, owing to the action of lysyl oxidase, the α-amino groups are oxidized to aldehydes.

Each connective tissue is composed of a characteristic pattern of one or several collagen types [5]. Generally, all collagens can be extracted from tissues after limited proteolysis and partly separated by fractional salt precipitation (Table I). An exception in this respect is collagen type I, a small percentage of which (about 10%) can be extracted from connective tissue with salt or acidic buffers [6].

Historically the oldest collagen type discovered is collagen type I, obviously because of its partial solubility. It is found exclusively in bone and, associated with other collagen types, also in tendon, skin, vessel walls, dentine and in most other connective tissues except hyaline cartilage. Its molecule (similarly to collagen types II, III, V and IX) is 300 nm long with a relative molecular mass of 300 000.

Type III collagen is generally codistributed with type I, with which it forms hybrid fibrils. Similarly to other collagen types (except types I and II), collagen type III is stabilized by disulphide bonds [7].

TABLE I

SURVEY OF MOLECULAR PROPERTIES AND PRECIPITATION CONDITIONS OF DIFFERENT COLLAGEN TYPES

Type	Molecular formula	Relative molecular mass of α-chain	NaCl (M)	
			Precipitation at acidic pH	Precipitation at neutral pH
I	$[\alpha_1(I)]_2\alpha_2$	95	0.7–0.9	2.6
I trimer	$[\alpha_1(I)]_3$		0.7–0.9	4.0
II	$[\alpha_1(II)]_3$	95	0.7–0.9	3.5–4.0
III	$[\alpha_1(III)]_3$	100– 95	0.7–0.9	1.5–1.7
IV	$[\alpha_1(IV)]_3$ $[\alpha_1(IV)]_3[\alpha_2(IV)]_3$	180– 75	1.2	1.7–2.0
V	$[\alpha_1(V)]_2\alpha_2(V)$ $[\alpha_1(V)]_3$	200–130	1.2	3.6–4.5
VI	$\alpha_1(VI)\alpha_2(VI)\alpha_3(VI)$	240–140	2.0	
VII	$[\alpha_1(VII)]_3$	>170		
VIII	$[\alpha_1(VIII)]_3$	61		
IX	$\alpha_1(IX)\alpha_2(IX)\alpha_3(IX)$	85	2.0	
X	Unknown	59	2.0	
XI	$[\alpha_1(XI)]_2\alpha_2(XI)$	95	1.2	
XII	Unknown			

Type II collagen can be obtained from hyaline cartilage after the majority of proteoglycans have been removed, e.g., with guanidinium chloride or other chaotropic agents or by DEAE-cellulose chromatography. A typical feature of this collagen is its high degree of glycosylation [8].

Collagen type IV is present in basement membranes. With this type procollagen propeptides are not removed during biosynthesis and fibrils are not formed. Instead, collagen type IV constitutes a mesh-like structure, where four collagen molecules are bonded by multiple disulphide bonds. Also, this collagen type contains a high proportion of carbohydrate side-chains [9].

The first collagen type which occurs in ontogeny is collagen type V; it constitutes fine, pericellularly located fibrils [10].

In the last decade a number of minor collagen types have been described. Of these, that with the best elucidated structure is collagen type VI, which is formed of polypeptide chains of relative molecular mass 30 000 and 40 000. These chains are terminated with globular domains and assembled in a special way. It occurs in interstitial connective tissue, in intervertebral disc and in hyaline articular cartilage. Originally it was isolated from the lamina intima of the vessel wall [11].

Collagen type VII is sometines referred to as long-chain collagen as its polypeptide chains are 450 nm long. Its molecules are arranged in antiparallel dimers with a 60-nm overlap. Its function is in anchoring connective tissue stroma to basement membranes [12].

Collagen type VIII is produced by endothelial cells and therefore it is also occasionally called EC collagen. Its polypeptide chains have relative molecular masses larger than 100 000 and they are frequently interrupted by non-helical segments [13].

From the pepsin digest of hyaline cartilage it is possible to isolate two additional collagen types, namely types IX and XI [14]. Of these two, the structure of collagen type IX is the better elucidated. The molecule of this collagen type is composed of three different polypeptide chains, each of which contains four non-helical regions. The polypeptide chains of this collagen are relatively short. As a specific attribute, collagen type IX possesses a glycosaminoglycan side-chain bonded to a non-helical region located inside the molecule. Because of this it is sometimes also called "light proteoglycan". It is believed to participate in the organization of higher structural assemblies of collagen type II. Collagen type XI is composed of three polypeptide chains, two of which are similar to collagen type V and the third to collagen type II [15].

In the calcifying enchondral cartilge it is possible to find a short collagen designed type X. It has a relative molecular mass of 59 000 with a C-terminal globular domain of 14 000. Its function is related to calcification processes and, in contrast to other collagens, it appears to be rapidly metabolized. During synthesis it is associated with cell membrane and it is released to the extracellular space only after limited proteolysis [16].

METABOLIC PATHWAYS OF COLLAGEN AND THEIR DISORDERS

Collagen, as all other proteins, is synthesized on ribosomes. In the post-translational phase it is subjected to a number of modifications, namely proline and lysine hydroxylation, glycosylation of some residues, cleavage of the signal peptide, formation of disulphide bonds, cleavage of the terminal extension peptides (propeptides) and formation of covalent cross-links [17,18] (Table II). At any anabolic stage collagen molecules are degraded. Collagen molecules excreted from the cell are aggregated into fibrils.

During the formation of collagen structure and during its degradation, a number of disturbances may appear which demonstrate themselves as pathological alterations. Thus, e.g., an increased proportion of type I results in decreased tissue elasticity (atherosclerosis) and in disturbances in transport mechanisms (liver cirrhosis, lung fibrosis, nephrosis). The presence of collagens type I and III in

TABLE II

SURVEY OF POST-TRANSLATIONAL MODIICATIONS IN COLLAGEN

Type of modification	Needed for
Proline 4-hydroxylation	Triple-helix stabilization through hydrogen bonds
Proline 3-hydroxylation	Unknown
Lysine hydroxylation	Unknown
Hydroxylysine glycosylation	Stable cross-link formation
Triple-helix formation	Extrusion of procollagen molecules from cells
N-Propeptide cleavage	Normal fibril formation
C-Propeptide cleavage	Normal fibril formation
Oriented aggregation	Normal fibril formation
Cross-link formation	Structure stabilization

articular cartilage disturbs its friction and transport properties. In principle, the following synthetic steps can be affected: (a) on the ribosome, (1) an improper collagen type is expressed [19], (2) the particular gene is defective [20] and (3) the synthesis of a particular collagen type is quantitatively changed [21]; (b) in the post-translational phase, any step of the post-translational processing may be affected, which generally leads to a stability defect of the newly formed structure.

If one or a few amino acids are interchanged within a single polypeptide chain, no clinical manifestation occurs. If, however, a deletion of more than 200 amino acids takes place, the foetus will not survive. Shorter deletions manifest themselves by a decrease in tensile strength and as alterations in other qualities of the arising connective tissue (osteogenesis imperfecta). Here it should be emphasized that some collagen polypeptide chains are coded by two different genes and therefore in addition to defective molecules also unaffected molecules can occur side by side [20].

Another alteration that occurs on the ribosome is a change in the gene expression. This means that a different collagen type than that which is typical for a particular tissue is synthesized (osteoarthrosis). Alternatively, the collagen types proper to the particular tissue is synthesized in an excessive amount (cirrhotic liver, fibrotic lung, arteriosclerotic vessels, Ehlers–Danlos syndrome type III, silicosis) [22–24]. On the other hand, decreased collagen synthesis also causes problems (slow wound healing).

While the above alterations demonstrate gene expression, the next category of deviations is represented by defective stabilization of collagen structure that occurs in the post-translational phase. Thus, decreased proline hydroxylation hinders the formation of collagen triple helix at body temperature owing to the inability to form the necessary number of hydrogen bonds. As the triple helical structure is a prerequisite for collagen extrusion under such circumstances, the amount of extracellular collagen decreases. Proline hydroxylation is dependent on ascorbic acid and therefore such a defect can be observed in scorbut [17].

Another defect of stability is caused by a change in cross-link concentration. In the first place cross-link formation is influenced by the way collagen molecules aggregate into fibrils. Disturbances here may arise if defective molecules are synthesized, which prevent collagen from undergoing proper aggregation and consequently cross-links are not formed for steric reasons [25]. Alternatively, if the activity of collagen peptidases is decreased or blocked then the uncleaved propeptides cause fibril formation where, from the steric reasons, cross-links can also not be formed (Ehlers–Danlos syndrome type VII, dermatosparaxis) [25,26]. Aggregation of collagen molecules into fibrils can be further affected by the presence of glycosidic side-chains (mucopolysaccharidoses). Blindness occurring in Hurler's syndrome reflects erroneous aggregation of collagen molecules in the cornea and may serve as a typical example [27].

The formation of covalent cross-links depends on the activity of lysyl oxidase, which converts definite lysyl residues into the δ-semialdehyde of α-aminoadipic acid (allysine, lysinal). The activity of lysyl oxidase dependent on copper; decreased absorption of this metal from the intestine results in a decreased concen-

tration of covalent cross-links in different connective tissue proteins, including collagen (Menke's kinky hair syndrome) [28].

When lysinal is blocked by a reaction with, e.g., homocysteine, covalent cross-links cannot be formed [29]. As hydroxylysine-derived cross-links are more stable than those derived from lysine, under-hydroxylation of collagen leads to its decreased stability. Every decrease in collagen stability makes the structure more prone to catabolic processes. In contrast the number of covalent cross-links can also be increased. This occurs either physiologically during ageing or after the administration of some drugs (gold complexes) [30].

The concentration of collagen structures in tissues is determined by the proportion of anabolic and catabolic processes. The less the structure is stabilized, the more susceptible it is to breakdown. Disorders affecting the anabolic phase lead finally to increased degradation of collagen structures. This is particularly true above the denaturation temperature, when non-specific proteinases come into action [31].

In order to understand what pathological changes emerge from alterations of collagen metabolism, it is necessary to remember all of its functions. In this respect its immunological properties are very important. These, however, may demonstrate themselves only if soluble collagen forms are available. Thus, e.g., on immunization with collagen type II and XI, arthritis of small paw joints is observed [32,33].

Another category of disorders can result from the ability of collagen to aggregate platelets. This occurs particularly when the vascular wall is affected by inflammation or by trauma [34].

METHODS FOR THE ISOLATION AND DETERMINATION OF MACROMOLECULAR COLLAGEN

As has been mentioned before, apart from collagen type I none of the other types can be isolated from adult tissues under non-denaturing conditions. Embryonal tissues represent an exception in that it is possible to extract small amounts of other collagen types by making use of neutral salt solutions [6]. Therefore, limited proteolysis is an inevitable step in obtaining collagen types II–XI. Pepsin is most commonly the enzyme of choice but there have been reports of the use of papain or pronase for the same purpose [35]. Such crude collagen extracts are fractionally precipitated with sodium chloride in the subsequent steps. Because collagen types differ in their amino acid composition, the size of the molecule and the content of glycosidic components, they can be precipitated at different salt concentrations; these concentrations change with pH (Table I). Fractions of individual collagen types obtained in this way have to be purified further by chromatographic procedures, as the products obtained by fractional precipitation are never sufficiently pure. It should be emphasized that definite tissues are formed by definite collagen types; thus, e.g., hyaline cartilage is composed of collagen types II, IX and XI.

Electrophoretic procedures are exploited mainly for characterization purposes and less frequently for the determination of the proportion of different collagens

in tissues. In the latter instance, however, the separation capabilities of electrophoresis are limited to distinguishing only selected collagen types.

Chromatographic procedures

DEAE-cellulose chromatography

It should be said initially that ion-exchange separations are carried out under low-to-medium pressure conditions and advanced high-performance procedures are not yet sufficiently developed for collagen separations. The chromatographic separation of collagen types I and III on DEAE-cellulose is generally accomplished by using columns of an appropriate size with respect to the weight of the loaded sample. Because collagens contain a small amount of tyrosine, they are routinely detected by UV monitoring in the range 220–235 nm.

In the first chromatographic step collagenous proteins are separated from the carbohydrate moiety-containing entities (glycoproteins and proteoglycans). For this purpose the column is equilibrated with 0.2 *M* sodium chloride in 0.05 *M* Tris–HCl (pH 7.5). The sample is applied in the same buffer and the column is eluted until no further UV-absorbing material (230 nm) is detected in the effluent. Then the eluting solvent is changed abruptly to 1.0 *M* sodium chloride in 0.05 *M* Tris–HCl (pH 7.5) and elution is continued with this buffer until an additional peak is eluted from the column. The first fraction contains purified collagen and is usually freed from non-volatile solutes by dialysis against 1% acetic acid and lyophilized [36] (Fig. 1).

Purified collagen may be fractionated further in a subsequent DEAE-cellulose step. In this instance the column is equilibrated with 0.02 *M* sodium chloride in 0.05 *M* Tris–HCl (pH 7.5), 2 *M* with respect to urea, and the sample is dissolved in the same buffer. The column is eluted with this buffer until the UV absorbance at 230 nm decreases to zero, then elution is continued by using a linear gradient to 0.3 *M* sodium chloride in 0.05 *M* Tris–HCl (pH 7.5)–2 *M* urea until the second

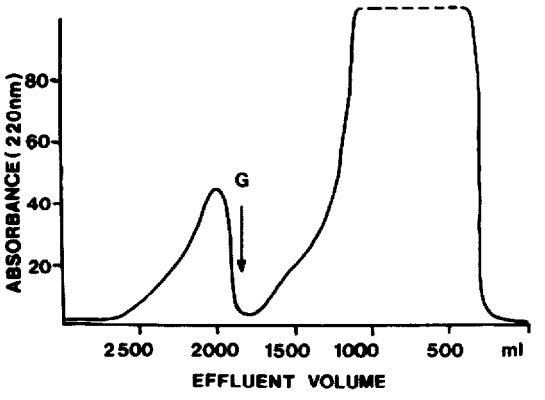

Fig. 1. DEAE-cellulose chromatography of the pepsin digest used for the removal of proteoglycans. The arrows (G) indicates the change from 0.2 to 1.0 *M* sodium chloride. The peak with a low retention volume represents collagen.

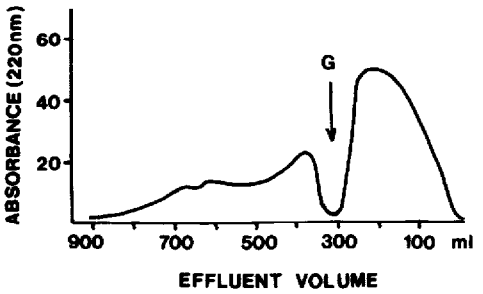

Fig. 2. DEAE-cellulose chromatography of purified collagen obtained by the separation shown in Fig. 1. First peak (low retention), a mixture of collagen types I and III. Second peak (high retention), mainly collagen type V. The arrow (G) indicates the change from 0.02 to 0.3 *M* sodium chloride.

peak is completely eluted. The first peak represents a mixture of collagen types I and III and the second is composed mainly of collagen type V [37] (Fig. 2).

This strategy is used for the separation of collagens present in pepsin digest of soft tissues. If the sample is obtained from tissue culture then the second peak in the second chromatographic step may also contain procollagens. Similarly, if the collagen preparation is obtained from pathologically altered tissues, e.g., dermatosparactic calves, procollagens may be present in this peak also.

DEAE-cellulose chromatography can also be used for the separation of macromolecular collagenous fragments arising during basement membrane digestion with proteases. If the system described above is applied then basement membrane collagen (type IV) fragments emerge in the first peak of the first chromatographic step [38]. Alternatively, the DEAE-cellulose column can be eluted with a linear sodium chloride gradient from 0 to 0.4 *M* in 0.05 *M* Tris–HCl (pH 8.6)–2 *M* urea; in this instance the collagenous proteins occur as two fast-moving peaks followed by two additional peaks of non-collagenous basement membrane proteins [38].

CM-cellulose chromatography

CM-cellulose chromatography can be used for the separation of both different collagen types and constituent polypeptide chains. In other words, these separations can be carried out both under denaturing and non-denaturing conditions depending on whether molecules or individual chains are to be separated. CM-cellulose chromatography under denaturing conditions was historically the first that offered the possibility of isolating α- and β-chains of collagen type I.

For the separation of collagen polypeptide chains, heated columns are applied. The separation can be carried out with a linear gradient from 0.0 to 0.12 *M* sodium chloride in 0.02 *M* sodium acetate (pH 4.8)–4 *M* urea. The separation is run at 42 °C and four peaks are usually seen on the chromatogram, namely α_1, $\beta_{1,1}$, $\beta_{1,2}$ and α_2 (Fig. 3). When this procedure is used with collagens composed of a single chain, then only one peak occurs on the chromatogram provided that the sample was pretreated with pepsin in order to depolymerize the sample by abolishing the terminal cross-links [35,39–41].

CM-cellulose chromatography under non-denaturing conditions can be done

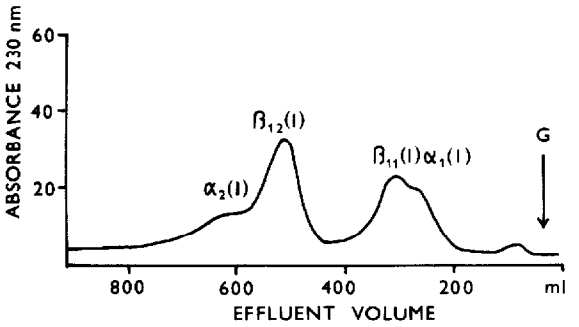

Fig. 3. CM-cellulose chromatography under denaturing conditions (42°C) of single collagen chains. For details of the separation, see text. G indicates the beginning of the gradient.

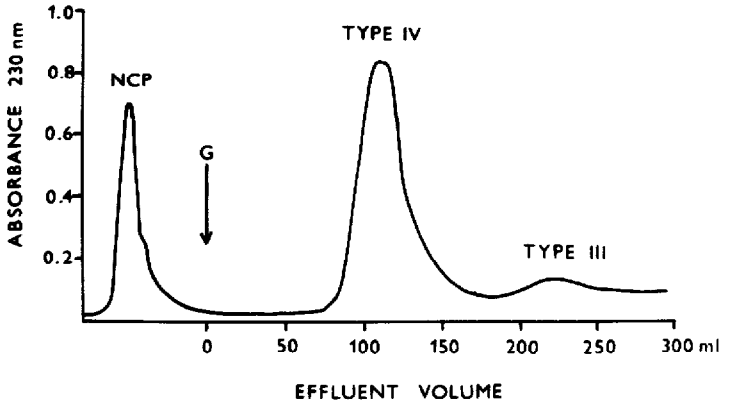

Fig. 4. CM-cellulose chromatography under non-denaturing conditions of pepsin-solubilized type IV collagen. The column is cooled with tap water (8°C); G indicates the beginning of the linear gradient from 0.0 to 0.4 *M* sodium chloride at pH 4.8. NCP = non-collagenous proteins. For further details, see text.

at either acidic or alkaline pH. The procedure using acidic pH has proved useful in the resolution of type IV collagen from relatively acidic non-collagenous contaminants and other collagens [42].

Separation at acidic pH is performed with 0.04 *M* sodium acetate in 2.0 *M* urea (pH 4.8) with a superimposed sodium chloride gradient from 0.0 to 0.4 *M*. The column is thermostated at 8.0°C. In this way collagen type IV is separated from collagen type III. This separation is particularly important because in the preliminary steps collagen types III and IV precipitate at similar ionic strength. On chromatography collagen type III has a higher retention time (Fig. 4).

Separation at alkaline pH is carried out with 0.02 *M* Tris containing 6.0 *M* urea (pH 8.0) using a linear gradient from 0.0 to 0.25 *M* sodium chloride. The importance of this purification step is based on the fact that collagens type I and V precipitate at a similar sodium chloride concentration. The peak of collagen type V follows that of collagen type I [43] (Fig. 5).

Separations using CM-cellulose are also applicable for the fractionation of individual macromolecular species of collagen type IV (Fig. 6). For this purpose a CM-cellulose column is equilibrated with 0.02 *M* sodium acetate–0.005 *M* di-

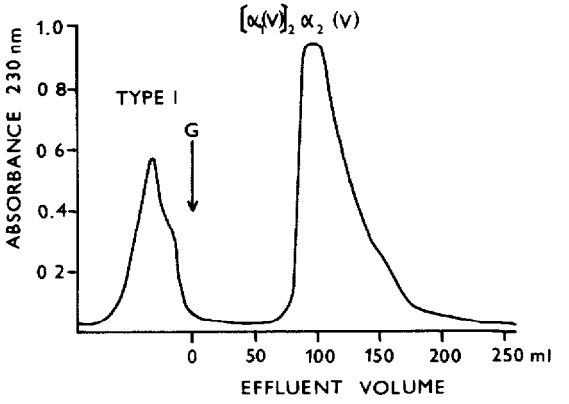

Fig. 5. CM-cellulose chromatography under non-denaturing conditions of pepsin-solubilized type V collagen. The column is cooled with tap water (8°C), G indicates the beginning of the linear gradient from 0.0 to 0.25 *M* sodium chloride at pH 8.0. For further details, see text.

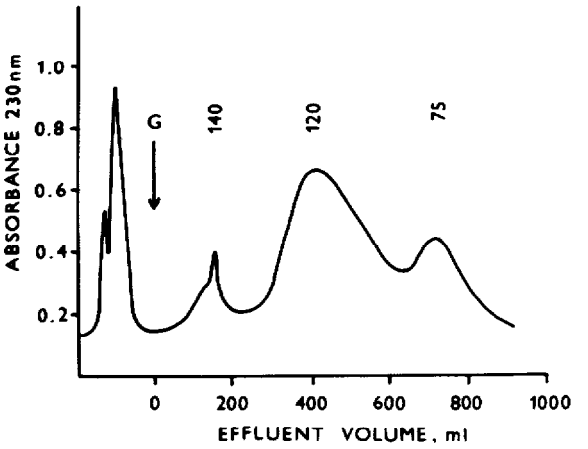

Fig. 6. Separation of different fractions of collagen type IV on CM-cellulose. G indicates the beginning of the gradient from 0.0 to 0.1 *M* sodium chloride in the presence of 0.005 *M* dithiothreitol. The numbers above the peaks indicate molecular masses in kDa. The separation is run at 44°C and pH 8.5 [44].

thiothreitol–1.0 *M* urea (pH 4.8) at 44°C. The sample is dissolved in 0.01 *M* dithiothreitol–8 *M* urea (pH 8.5) and heated at 45°C for 2 h. Then the sample is applied and the column is eluted with a gradient from 0.0 to 0.1 *M* sodium chloride in the equilibration buffer. This procedure allows the separation of high-molecular-mass fragments, i.e., α_1(IV) (140 kDa) and α_2(IV) (120 kDa). The separation of α-size components from a pepsin digest of type IV collagen can be effected under conditions similar to those described above except that dithiothreitol can be omitted from the equilibration and elution buffers [44]. This separation offers the possibility of isolating the α_1(IV) (95 kDa), α_2(IV) (95 kDa) and α_2(IV) (75 kDa) chains. This procedure is also applicable to the separation of α_1(IV) (50 kDa) and α_2(IV) (50 kDa) chains. The reason for grouping the in-

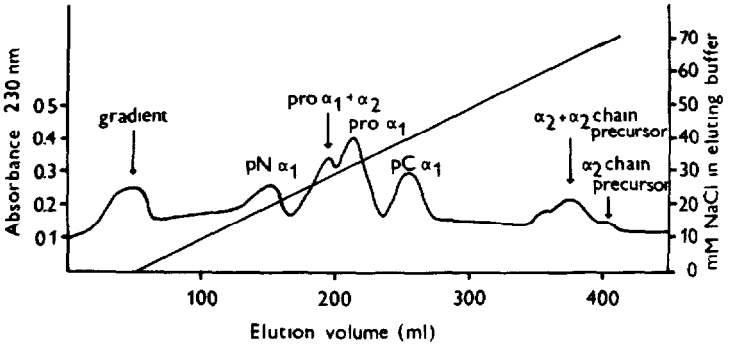

Fig. 7. CM-cellulose chromatography under non-denaturing conditions of procollagen and p-collagen chains (type I). 8 *M* Urea, 50 m*M* sodium acetate (pH 4.8), 25 °C, salt gradient.

dividual fragments into the above categories is a consequence of preliminary fractionation of the total pepsin digest on a molecular sieve column, typically agarose Bio-Gel A-5 m, on which each of these categories is represented by a single peak. Owing to the peak overlaps in gel permeation chromatography the α_2(IV) (75 kDa) chain may occur together with the 140 and 120 kDa fragments as the last peak in such a CM-cellulose run.

CM-cellulose chromatography conducted under conditions employed for the separation of individual collagen chains is also applicable to the separation of procollagens. However, the resolution of procollagen and p-collagen chains from collagen α-chains of an identical collagen type is often incomplete. Typically, such a separation can be carried out in 50 m*M* sodium acetate (pH 4.8)–8.0 *M* urea using a salt gradient from 0.0 to 60 m*M* at 25 °C. The following chains can be resolved, in order of increasing ionic strength: pN-α_1(I), pro-α_1(I), α_1(I), pC-α_1(I) and pN-α_2(I). The last peak of the chromatogram is pro-α_2(I), which appears at the very end of the gradient and is usually contaminated with pC-α_2(I) [45,46] (Fig. 7).

Molecular sieve chromatography

Molecular sieve chromatography is a suitable method for evaluating the molecular mass distribution of collagen denaturation products and offers the possibility of isolating the primary constituents. Typically, the separations are carried out in 1.0 *M* calcium chloride + 0.05 *M* Tris–HCl (pH 7.5) or in 2.0 *M* guanidinium hydrochloride + 0.05 *M* Tris–HCl (pH 7.5). The latter solvent appears more suitable as it exhibits an increased capacity for solubilizing collagen denaturation products. Agarose beds with a relative molecular mass operating range of $4 \cdot 10^3$–$6 \cdot 10^3$ for random coil polypeptides are applicable for this purpose [47] (Fig. 8).

As collagenous proteins display a higher hydronamic volume than globular proteins, calibration of molecular sieve columns has to be effected by using collagen molecular mass standards, i.e., α-chain monomers, dimers and trimers [46].

From the nature of the method emerges its applicability to the separation of types I and III collagen in a pepsin digest. After limited proteolysis collagen type I is predominantly present in the form of α- or β-chains, whereas collagen type

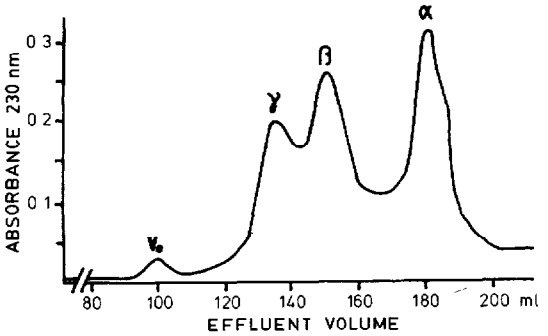

Fig. 8. Bio-Gel A-5 m chromatography of denatured type I collagen. Elution with 1 *M* calcium chloride solution (pH 7.5). The γ-fraction represents a mixture of type I and type III.

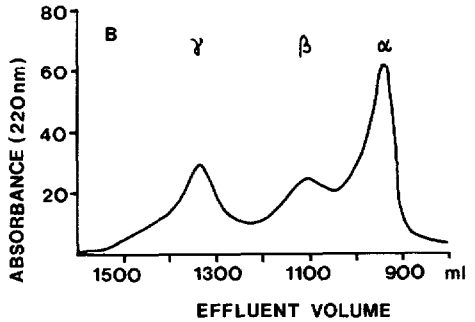

Fig. 9. Bio-Gel A-1.5 m chromatography of reduced and alkylated γ-fraction obtained by the run shown in Fig. 8 (identical separation conditions). Fractions α and β originate from collagen type III.

III is disulphidically bonded to chain trimers, which can be converted to chain monomers if the sample is treated in the presence of reducing agents, e.g., dithiothreitol.

Molecular sieve chromatogaphy also proved effective for the purification of procollagen from non-disulphide-bonded aggregates, collagen α-chains and contaminating non-collagenous proteins. The avoid undesirable sorption and to avoid aggregation of different collagenous polypeptide chains, it is recommended that the separation is run under denaturing conditions. Typically, the calcium chloride buffer mentioned above is used for this purpose, supplemented with 2.5 m*M* EDTA. The sample is heated at 45 °C for 15 min prior to application. If samples are to be chromatographed in the reduced state, reduction and alkylation are performed before the sample is loaded on the column [46] (Fig. 9).

Bioaffinity chromatography

Bioaffinity chromatography has been applied for further purification of procollagens obtained by DEAE-cellulose chromatography (see above). Concanavalin A–Sepharose is eluted with 0.4 *M* sodium chloride–0.05 *M* Tris–HCl (pH 7.5) containing 10 m*M* MEM (Eagle's minimal essential medium) and 0.3 *M* PMFS (phenylmethylsulphonyl fluoride). The sample is dissolved in the same buffer, which is made 5 m*M* in calcium chloride. The column is eluted until the absorbance at 230 nm returns to the initial level and then elution is continued

with 0.5 *M* α-methyl-D-mannoside in the same buffer. In the next step procollagen can be eluted by using a buffer containing 1.0 *M* calcium chloride–0.05 *M* Tris–HCl (pH 7.5). This procedure is particularly suitable for removing non-glycoprotein material from type III procollagen [48].

Thiol-activated agarose allows disulphide bonded components to be separated, i.e., pro- and pC-collagen from cysteine-lacking material, in particular pN-collagen and collagen. The method is used with procollagen type II as follows: the sample is dissolved in 0.03 *M* sodium chloride–1 *M* urea–1 m*M* EDTA–100 m*M* Tris–HCl (pH 8.0). Dithiothreitol is added to a final concentration of 5.0 m*M* and the solution is heated at 60 °C for 30 min. In the next step dithiothreitol is removed by passing the sample through a Sephadex G-25 column using the sample buffer as mobile phase. Reduced procollagen is then mixed with activated thiol–Sepharose and shaken gently for 2 h at 42 °C. Next, the suspension is poured into a prewarmed column and unbound type II pN-collagen and collagen are eluted with the starting buffer. In the next step the column is washed with the starting buffer containing 1.0 *M* sodium chloride and subsequently equilibrated in starting buffer containing 5.0 m*M* dithiothreitol. At this stage the flow of the mobile phase is stopped for 40 min to allow a complete sulphydryl exchange. When elution is continued type II pro- and pC-collagens emerge from the column. In addition to type II procollagen, this procedure is also applicable to type I procollagen but not to materials related to type III procollagen, as disulphide bonds link both pro-α and α-chains in this type of collagen [49].

Zone precipitation chromatography

As individual collagen types can be separated by fractional salt precipitation, the application of zone precipitation chromatography can represent another useful technique for the initial isolation of individual collagen types in their native configuration. The analytical applicability of this technique is, however, limited

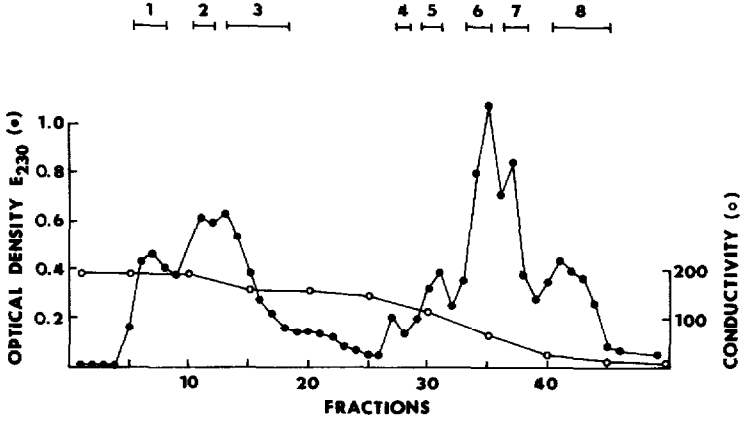

Fig. 10. Chromatographic separation of pepsin-solubilized 17-day-old chick embryo whole-body collagen by zone precipitation chromatography on Sephadex G-200. Temperature, ambient. Discontinuous decreasing salt gradient (for details, see text). Peaks: 1 = non-collagen protein; 2 = salt and small peptides; 3 = type V collagen; 4 = type II collagen; 5,6,7 = type I collagen; 8 = type III collagen [50]. Conductivity in ms.

as the emerging collagen fractions are always contaminated with other constituents of the separated mixture. Typically a 40×2.5 cm I.D. column is packed with Sephadex G-200 to a bed volume of 150 ml (other packings such as Bio-Rad P-2 or P-4 can be used). A column temperature of 21–23°C is sufficient for good separations; with thermostating of the column at 4°C no improvement in either recovery or separation of individual collagen types was found. The column is equilibrated with 100 ml of 30% sodium chloride solution at pH 7.5. The sample is applied in a volume of less than 10 ml of Tris buffer containing 150 m*M* sodium chloride and the column is eluted stepwise with a discontinuous gradient of 50-ml volumes of 20%, 15% and 10% sodium chloride solution. Finally, the column is eluted with 300 ml of 1% sodium chloride solution (Fig. 10). About 12-ml fractions are collected and the amount of eluted collagen is monitored at 230 nm. The conductivity of each fraction is also recorded, which helps in the identification of the fractions [50].

High-performance liquid chromatography

There is some scattered information in the literature on the separation of individual collagen chains and collagen chain polymers by high-performance liquid chromatographic procedures. They can be separated on a bed of Separon (a styrene–divinylbenzene polymer) within 30 min [51].

Alternatively, it is also possible to use reversed-phase chromatography on macroporous C_{18} silica gel, with which about 80 min are needed to separate α-chain monomers, dimers and trimers [52]. Typically 500 μg of heat-denaturated collagen are loaded on a 25×0.46 cm I.D. column packed with C_{18} silica (50 nm pore size). The column is eluted with a gradient created by mixing 0.1% trifluoroacetic acid (pH 2.2) with tetrahydrofuran as indicated by the broken line in Fig. 11. As shown, the gradient is 20% tetrahydrofuran for 20 min, 20–23% of tetrahydrofuran over 15 min, 23% of tetrahydrofuran over 20 min, 23–25% of tetrahydrofuran over 15 min and the run is finished with 25% tetrahydrofuran for 20 min. Similar results can be obtained with phenyl-bonded phases using a 0.1% trifluoroacetic acid–acetonitrile gradient over 1 h. It is also possible to achieve a reason-

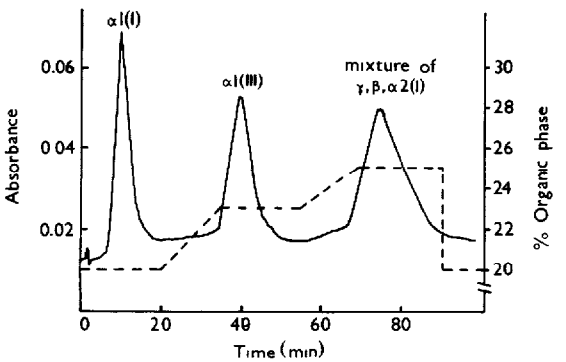

Fig. 11. Reversed-phase HPLC separation of denatured type I and III collagen chains on a silica gel C_{18} column. The broken line represents the percentage of tetrahydrofuran in the mobile phase. For further details, see text [52].

able separation of collagen chain polymers by using a linear gradient of 20–30% acetonitrile in 0.1% trifluoroacetic acid on C_{18} silica, provided that the aqueous phase is made about 0.02 *M* with respect to the ammonium hydrogencarbonate [53]. The nature of the organic modifier is of secondary importance and similar results are obtained with methanol, 2-propanol, acetonitrile and tetrahydrofuran. With nitrile-bonded phases the separation of collagen chain polymers can be achieved using a nearly identical elution system. Elution is started with 0.05 *M* ammonium hydrogencarbonate–0.4% trifluoroacetic acid (pH 3.2) for 10 min, followed by a gradient to 20% tetrahydrofuran over 10 min and finishing the run at 30% tetrahydrofuran for 15 min [54]. Another possibility with nitrile-bonded phases is seen in the application of pyridine–acetate buffer (pH 4.63) using propanol as organic modifier [55,56]. The course of the gradient is indicated by the broken line in Fig. 12.

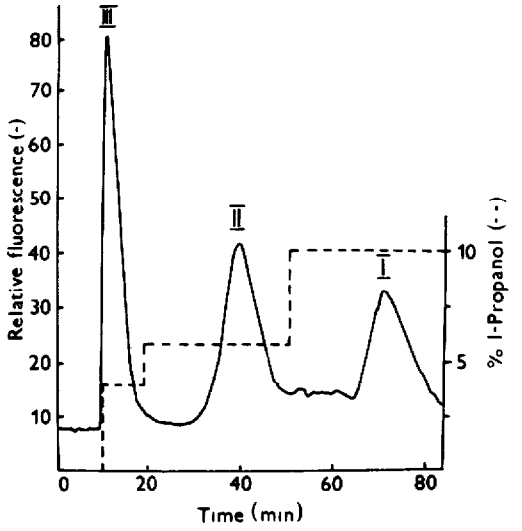

Fig. 12. Separation of a mixture of native type I, II and III collagens (as indicated) on a cyano-bonded phase. For further details, see text [55].

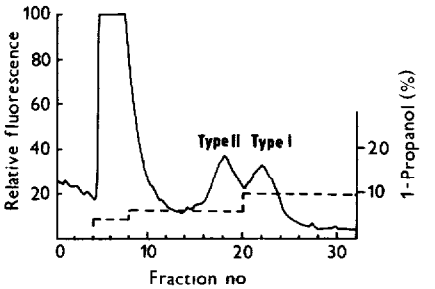

Fig. 13. Preparative separation of a crude extract of lathyritic chicken embryo cartilage containing (native) type I and type II collagens on a cyano-bonded column. For details, see text [55].

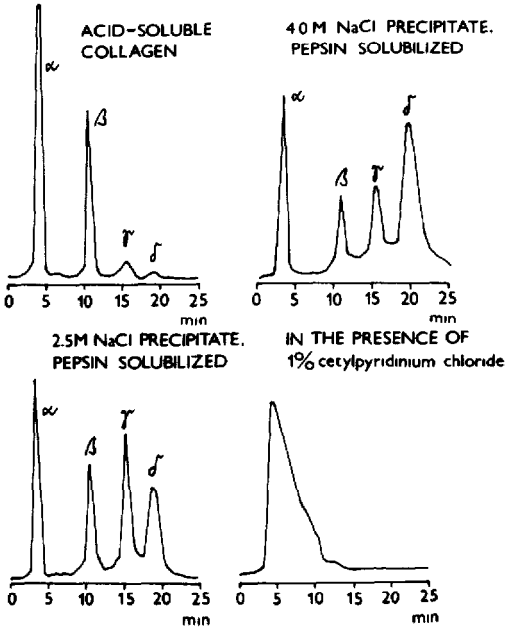

Fig. 14. Separation of acid-soluble collagen and solubilized insoluble collagen fractions on sepiolite. Mobile phase, 0.1 *M* sodium acetate buffer (pH 3.4); temperature, ambient.

Cyano-bonded phases can also be used for the separation of native type I and II collagens. The elution system is similar to that just described (Fig. 13) [55].

None of the described procedures is capable of separating chain polymers beyond trimers. This can be achieved by using sepiolite, a magnesium silicate with only a minor aluminium component, as sorbent [57]. It was demonstrated that rod-like collagen molecules interact with this sorbent with the formation of a collagen–clay complex that is more stable with collagen chain polymers. For this purpose the column is packed with sepiolite particles 15–30 μm long with a diameter of 1–3 μm. The mobile phase is 0.1 *M* sodium acetate buffer (pH 3.4). Lyophilized samples obtained by limited pepsin digestion are heated to 100°C, cooled, briefly centrifuged and aliquots are applied to the column (Fig. 14).

Electromigration procedures

Electrophoretic separations of collagen proteins in polyacrylamide gel can be carried out in either acidic of alkaline media. The latter procedure exploits the properties of protein sodium dodecyl sulphate (SDS) complexes and is mainly used for the determination of the size of collagenous proteins and their fragments.

Sodium dodecyl sulphate gel electrophoresis

In this section we shall focus attention on the unique behaviour of collagens and their derivatives in SDS electrophoresis. These proteins behave anomalously in comparison with typical globular proteins, e.g., the α_1 collagen chain of relative

molecular mass 96 000 migrates at a slightly lower rate than serum albumin dimer (relative molecular mass 132 000) and thus behaves as if it had a molecular mass 40% higher than the true value. It was found by Butkowski and co-workers [58,59] that this anomalous behaviour is mainly due to the interpretation of the data. The electrophoretic mobility of globular and collagenous proteins is better correlated with the number of residues in a polypeptide chain than with relative molecular mass. This relationship is demonstrated in Fig. 15, where type I collagen α-chains are characterized with respect to both molecular mass and residue number using globular proteins as standard markers. Whereas the collagen α-chains show an erroneously high relative molecular mass, the observed number of residues is 1156 and 1060 for the α_1- and α_2-chains, respectively, compared with the true value of 1052. The reason for this difference is not well understood. One plausible explanation is based on the low mean residue weight of collagen. This explanation, however, does not reconcile the different mobilities of α_1- and α_2-chains of collagen type I. This difference may result from the localized conformation effects due to the restricted rotation of polypeptide chains about imino acid residues, as proposed by Furthmayr and Timpl [60].

In practice two systems are widely used. Both are adaptations of previously published procedures, one being a continuous and the other a discontinuous buffer system. In addition, particularly for collagen fragments, gradient gels are used. Historically the first electrophoretic procedure used for the separation of collagen polypeptide chains was polyacrylamide gel electrophoresis carried out in the ab-

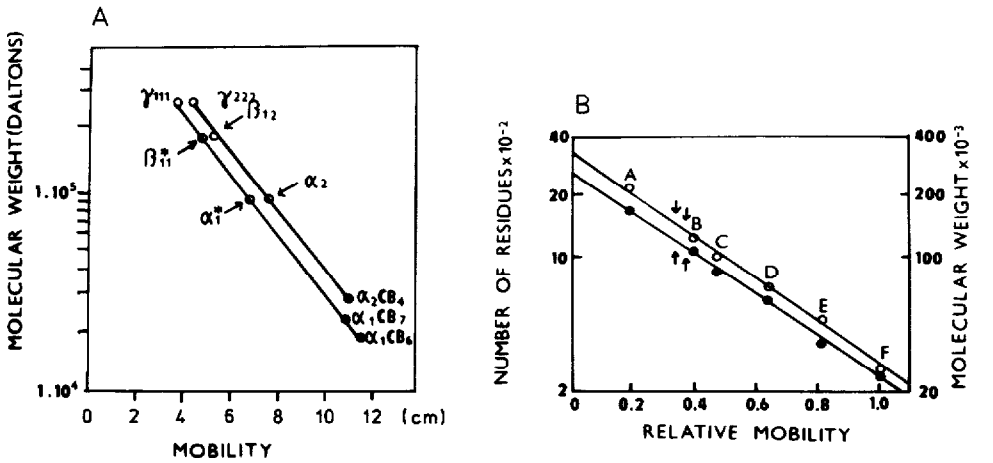

Fig. 15. Electrophoretic behaviour of collagen polypeptide chains. (A) Semi-logaritmic plot showing the migration of collagen polypeptide chains and chain polymers vs. relative molecular mass as compared with standard globular proteins. (B) Comparison of calf skin collagen α-chains with standard globular proteins with respect to the number of amino acid residues. The top line and right-hand ordinate refer to the evaluation of results according to relative molecular mass; the bottom line and left-hand ordinate refer to the evaluation according to the number of amino acid residues (A = myosin, B = α-galactosidase, C = phosphorylase A, D = bovine serum albumin, E = ovalbumin, F = chymotrypsinogen). Solid arrows in B indicate the position of collagen α_1(I) and α_2(I) chains, respectively. Both separations on 5% gel.

sence of SDS and in an acidic medium (pH 4.8). This latter approach has, however, long been abandoned and is mentioned here for completeness only.

Continuous buffer system [60]. The following solutions are needed: (a) 30% acrylamide–0.08% N,N′-methylenebisacrylamide; (b) 0.6 *M* sodium phosphate (pH 7.2); (c) 10% (w/v) SDS; (d) 8 *M* urea, deionized; (e) 0.45% (w/v) ammonium persulphate–0.3% (v/v) N,N,N′,N′-tetramethylethylenediamine (TEMED)–0.6% (w/v) SDS; (f) sample buffer, 0.01 *M* sodium phosphate (pH 7.2)–0.2% SDS–5.0 *M* urea–0.01% bromophenol blue; (g) water-saturated isobutanol; (h) electrode buffer, 0.1 *M* sodium phosphate (pH 7.2)–0.1% SDS; (i) staining and fixing solution, 2.5 g of Coomassie Brilliant Blue-R 250 in a solution consisting of 200 ml of distilled water, 500 ml of absolute methanol and 100 ml of glacial acetic acid; and (j) destaining solution, consisting of 100 ml of absolute methanol, 100 ml of glacial acetic acid and 800 ml of distilled water.

Generally, 5% gels are used for separating collagen chain monomers, dimers and polymers. The same procedure can be used for separating collagen fragments (e.g., cyanogen bromide or tryptic peptides); however, denser gels (typically 10%) are used for this purpose. Immediately after delivery of the gel solution, water-saturated isobutanol should be layered over the surface to prevent interferences arising from the diffusion of air oxygen. Separation can be carried out in either tubes or slabs; the latter are more frequently used. The bottom electrode chamber serves as an anode and the power supply is adjusted so as to give 3 mA per tube or 15 V per gel slab for 10 min until the bromophenol blue has penetrated the gel. Then electrophoresis is continued at 6 mA per gel or 35 V per slab until the tracking dye reaches the end of the gel. Finally, the power supply is disconnected and the zones are stained in a routine way.

Discontinuous buffer system [61,62]. For this procedure the following buffers are needed: (a) 35% acrylamide–0.8% N,N′-methylenebisacrylamide; (b) separating buffer gel, 2.25 *M* Tris–0.6% SDS (pH 8.8); (c) stacking gel buffer, 0.125 *M* Tris–0.2% SDS; (d) sample buffer, 0.063 *M* Tris–2% SDS–10% glycerol–0.01% bromophenol blue (pH 6.8); (e) 0.045% ammonium persulphate–0.15% (v/v) TEMED; and (f) electrode buffer, 0.25 *M* Tris–0.5% SDS–1.92 *M* glycine; solutions (g), (i) and (j) are identical with those described above.

Electrophoresis in the discontinuous buffer system involves stacking gel layered over the separation gel. The latter is prepared as described above. The stacking gel is prepared by mixing 1.0 part of solution (a), 2.7 parts of solution (c), 5.0 parts of solution (e) and 1.3 parts of distilled water.

Samples should be prepared by dissolving 4 mg/ml collagen material in solution (d), heat denaturated at 50°C for 0.5 h and reduced with mercaptoethanol, if desired.

Mapping of collagen α-chains and their fragments

Two-dimensional electrophoresis of collagen α-chains is performed in principle by the method described by O'Farrell et al. [63] modified to apply to the basic isoelectric point of collagen. Isoelectric focusing in the first dimension is carried out in 4% gel containing 2% Pharmalyte and 6 *M* with respect to urea. This separation is run in 130×2.5 mm I.D. tubes using 0.02 *M* sodium hydroxide and

0.02 *M* phosphoric acid as catholyte and anolyte, respectively. The isoelectric focusing is run by applying voltage stepwise: 200 V for the first 0.25 h, 300 V for the next 0.25 h and finally 400 V for 4 h. The voltage changes are essential in order to prevent overheating. The isoelectric focused gel is extruded into 5 ml of 10% glycerol–2.3% SDS–0.0625 *M* Tris–HCl (pH 6.8) and incubated at 40°C for 30 min. If necessary, reduction of the sample is carried out at this stage.

The second dimension is in principle identical with that described by Weber and Osborn [64]. Running gel (1×140×120 mm) composed of 5% acrylamide containing 3 *M* urea is used for this purpose. Two-dimensional mapping techniques can also be applied for mapping of collagen peptides. For this purpose collagen polypeptide chains are cleaved by a suitable protease such as proteinase K (EM Biochemicals) at pH 8.0. In addition, collagen samples have to be radioiodinated. Thin-layer electrophoresis is performed on 10×10 cm precoated cellulose plates without fluorescent indicator in acetic acid–formic acid–water (15:5:80) at 15°C and 400 V for about 60 min. The plates are dried at room temperature for at least 1 h and placed in a chromatography tank, where they are developed with butanol–pyridine–acetic acid–water (32.5:25:5:20) containing 7% (w/v) 2,5-diphenyloxazole. ϵ-DNP-Lysine is spotted above the level of the solvent and the run is terminated when the dye has migrated 5.5 cm from the bottom of the plate. Next, the plates are dried at room temperature and exposed to sensitive film for detection [65].

ANALYSIS OF CROSS-LINKING COMPOUNDS [66]

Methods of stabilization

The first procedure applied to the stabilization of the cross-linking components was borohydride reduction [67]. In this procedure carbonyl compounds are converted to the corresponding alcohols and aldimine bonds into secondary amines. From the analytical point of view the main advantage of this procedure is that by using tritiated borohydride it is possible to obtain labelled cross-links with one tritium atom incorporated per cross-linking element. At elevated pH borohydride reduction yields a considerable amount of α-amino alcohols. Reduction of peptide bonds is negligible; however, application of free amino group-containing buffers should be avoided as these participate in reductive amination of aldehyde groups in collagen. In practice, the reduction is carried out at pH 7.5–8.0 in 0.15 *M* sodium chloride buffered with 50 m*M* sodium phosphate. Solid potassium or sodium borohydride is added to give a dry mass ratio of borohydride collagen of 1:15–30. If the pieces of the tissue are small enough (about 2 mm in size) the reduction is completed within a few minutes. However, usually the reaction mixture is left to stand for about 30 min, after which the excess of borohydride is destroyed by the addition of 4 *M* acetic acid. The reduced material is purified by filtration and washing or alternatively by dialysis. When the reduction is carried out with radioactive borohydride, the reaction mixture should contain about $74 \cdot 10^7$ Bq/mmol and the commercially available radioactive borohydride (usually $1850 \cdot 10^7$ Bq/mmol) should be diluted accordingly with a non-radioactive boro-

hydride solution. In order to minimize the risk of contamination it is preferable to dissolve the radioactive and non-radioactive borohydride in 10 m*M* potassium hydroxide solution. If only comparative analyses are needed then reduction of a series of samples with the same borohydride solution would suffice; if, however, quantitation is required, then standardization of the labelled borohydride solution can be achieved by measuring the specific radioactivity of model compounds after reduction; 4-*p*-nitrobenzamidobutanal could be recommended for this purpose.

At acidic pH reduction can be effected by means of cyanoborohydride, which reduces carbonyls at pH 3–4 very effectively, however, no effect regarding carbonyl reduction occurs at neutral pH [68]. In contrast, aldimines are reduced fairly rapidly by this reagent also at neutral pH. These differences in susceptibility to reduction can be purposefully exploited for more detailed studies on the proportion of different types of cross-links. Reduction with sodium cyanoborohydride at pH 7.5–8.0 involves the same procedures as described above for sodium borohydride.

Another means of stabilizing labile cross-links is based on Strecker's reaction. The method involves reaction of the protein with sodium [^{14}C]cyanide, whereupon aldehydes are converted to α-aminonitriles; aldimines are converted into substituted aminonitriles. On acid hydrolysis, the aminonitriles produce the corresponding amino acid, which can be assayed chromatographically. The practical applicability of this method was demonstrated with collagen and elastin samples into which additional carbonyl groups were introduced by periodate oxidation [69]; however, no systematic investigation regarding the analysis of modified cross-links is available and no optimization of the procedure has been reported.

Sample preparation and preliminary fractionation of hydrolysates

In the preliminary stage, as much as possible of contaminating proteins, glycoproteins and glycosaminoglycans should be removed, but the original structure of the collagenous components should be preserved. Soft tissues such as tendon or skin specimens should be thoroughly washed at 4 °C with 0.15 *M* sodium chloride–5 m*M* sodium phosphate (pH 7.4). Extraction of the tissue with dilute acetic acid should be avoided as it cleaves the aldimine cross-links. If the so-called insoluble collagen is to be analysed and pre-purified, the extracted tissue must be equilibrated for several hours with 0.15 *M* sodium chloride at pH 7.4 in order to allow complete re-establishment of the cross-links. With hard tissues such as bone or dentine the tissue is first pulverized in liquid nitrogen and decalcified with 0.5 *M* EDTA (pH 7.4) for several days. Reduction of the pulverized material is certainly possible, but care must be taken to remove calcium salts, which would interfere with the subsequent chromatographic step. Cartilage samples are reduced directly after washing with 0.15 *M* sodium chloride at pH 7.4 [70–72].

Hydrolysis of reduced tissues

Routinely hydrolysis is carried out in sealed tubes under nitrogen at 108 °C for 24 h, then hydrochloric acid is removed under vacuum [73]. Under these condi-

tions, however, some of the cross-links are destroyed. Thus, e.g., hydroxynorleucine is converted into a chlorinated derivative, which can be minimized by using 3 *M* hydrochloric acid for hydrolysis. Alternatively, hydrochloric acid can be replaced with toluene-*p*-sulphonic acid (3 *M*) [74]. Acid hydrolysis also causes a complete loss of the reduced aldol. Instead, several ninhydrin-positive decomposition products appear in the chromatogram [75]. All forms of acid hydrolysis cause partial conversion of the hexitollysines to corresponding anhydro derivatives [76]. On the other hand, dehydrohydroxynorleucine and hydroxylysinonorleucine are stable under the conditions of acid hydrolysis and no correction of the results for hydrolytic losses is necessary.

Hydrolysis under alkaline conditions is best carried out in sealed polypropylene tubes in an autoclave at 118°C for 16 h. Preliminary flushing with nitrogen is necessary to avoid losses of glycosylated compounds. Before analysis the sample is neutralized with acetic acid, diluted accordingly and applied to the analyser. If necessary the excess of sodium acetate present in the sample can be removed by passing the sample through a small cation-exchange column [77]. If the hydrolysis is carried out in 2 *M* potassium hydroxide solution, titration of the sample to pH 4.0 with perchloric acid helps to remove the excess of salts as potassium perchlorate precipitates. After the desalting procedures have been completed, the sample is usually evaporated to dryness. The advantage of this procedure is the possibility of preserving reduced aldol and also no anhydro forms of hexitollysines are formed. Apparent losses of other cross-linking components reported in the literature result from the presence of glycosylated derivatives [76].

Preliminary fractionation of hydrolysates

Anion-exchange chromatography can be used for the preliminary fractionation of the hydrolysate on both preparative and analytical scales. For analytical purposes, preliminary removal of most of the acidic and neutral amino acids can be recommended, which is achieved by passing the sample through a small Amberlite CG-120 column which is eluted with 0.5 *M* pyridine [78,79].

For preparative purposes gel permeation prefractionation is preferred. This is effected in the conventional way on a Sephadex G-10 column (90×5 cm I.D.) eluted with 0.1 *M* acetic acid. The pH of the samples introduced to such a column has to be adjusted to 3 with either 4 *M* sodium hydroxide solution or 6 *M* hydrochloric acid, depending on the method of hydrolysis [80]. The hydrolysates prepared are usually removed as a precipitate by mild centrifugation. With preparations that were reduced prior to hydrolysis the higher molecular mass fraction contains about 80% of radioactivity and about 1–2% of all amino acids. This fraction is evaporated to dryness and subjected to further separation. It is necessary to stress that chloronorleucine is strongly retarded on Sephadex chromatography and elutes after the salt peak. After separation the column is strongly contaminated with humic material which must be removed by washing with distilled water, 0.2 *M* sodium hydroxide solution and water before it is re-used. For semi-preparative and analytical purposes it is recommended to carry out prefrac-

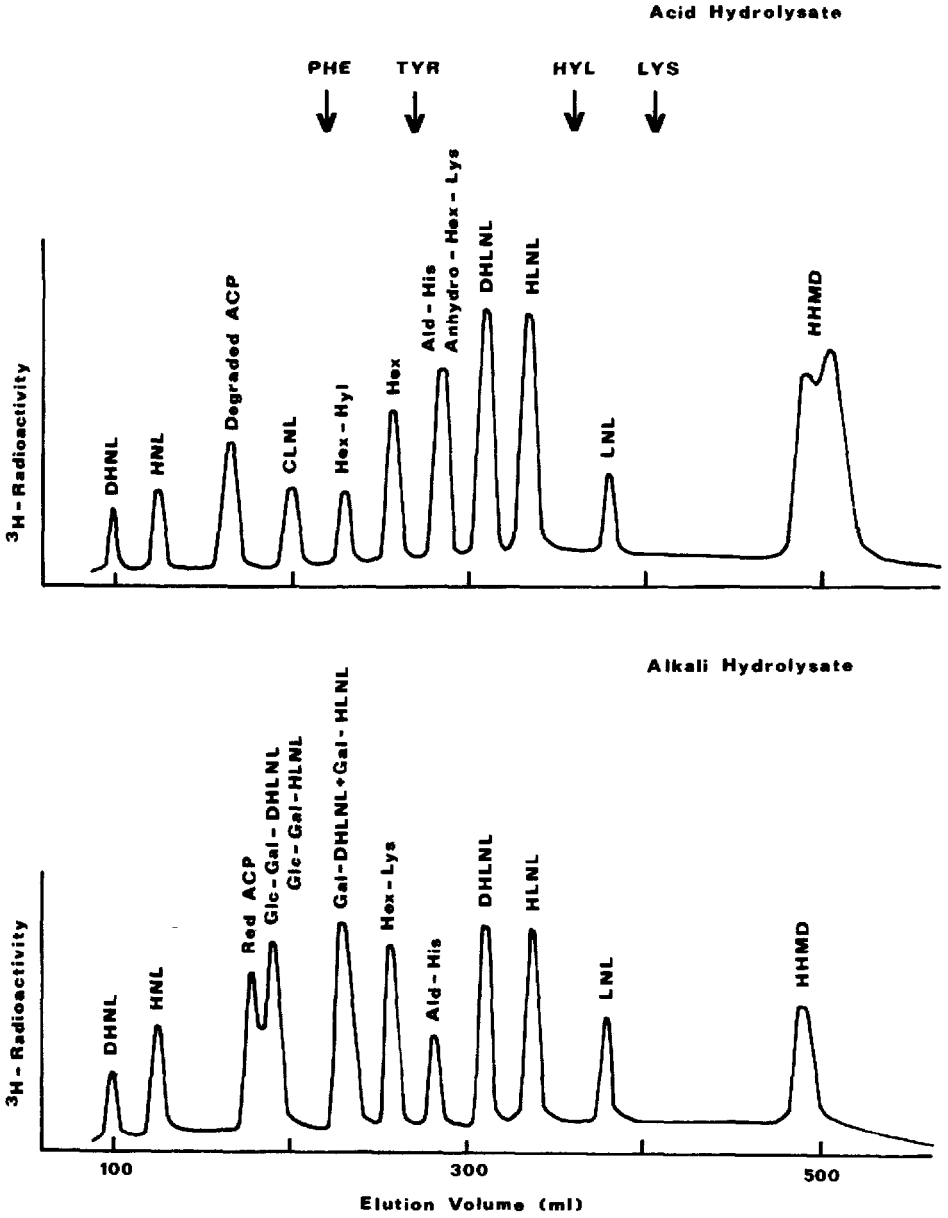

Fig. 16. Elution profiles of the reducible components in acid and alkali hydrolysates of borohydride-reduced collagen in ion-exchange chromatography using volatile (pyridine formate) buffer as mobile phase. DHNL = dihydroxynorleucine; HNL = hydroxynorleucine; ACP = aldol condensation product; CLNL = chlorononorleucine; DHLNL = dihydroxylysinonorleucine; HLNL = hydroxylysinonorleucine; LNL = lysinonorleucine; HHMD = histidinohydroxymerodesmosine. For details, see text [82].

tionation on a Bio-Gel P-2 column (140×1.7 cm I.D.) equilibrated with 0.1 *M* acetic acid. Elution is carried out with 0.1 *M* acetic acid.

TABLE III

CHROMATOGRAPHIC SYSTEMS FOR THE SEPARATION OF CROSS-LINKS

The columns (0.9 cm I.D.) were packed with sulphonated polystyrene resin with a bead size of 6–7 μm and were operated at 56°C with a flow-rate of 32 ml/h. The 0.066 *M* buffer was prepared by titrating to the required pH with hydrochloric acid a solution containing 98.5 g of trisodium citrate (dihydrate) and diluting with water to a final volume of 5 l. The 0.12 *M* buffer, containing 171.5 g of trisodium citrate (dihydrate) in 5 l, was similarly prepared. Each buffer contained 0.1 ml/l octanoic acid and 0.4 g/l Brij 35.

System	Sodium citrate buffer concentration (*M*)	pH	Column length (cm)	Resin cross-linking (% divinylbenzene)
I	0.12	5.25	26	10
II	0.12	5.25	6	10
III	0.066	4.70	15	10
IV	0.066	4.25	15	10
V	0.066	2.90 (15 min) 3.25 (60 min) 4.25 (120 min) (stepwise)	26	8

Chromatographic separation of cross-linking amino acids

Chromatography with volatile buffers [81,82]

Pyridine formate buffers are used either for preliminary fractionation or for preparative purposes; the application of pyridine formate buffers for collagen hydrolysates was introduced in the early 1970s by Bailey et al. [81] and Robins [82]. Briefly, the hydrolysates of up to 200 mg of reduced collagen are applied to a 60×1.3 cm I.D. column of Dowex 50-X8 or Zerolit 225 (30–40 μm bead size) and eluted with a linear gradient from 0.1 *M* pyridine formate (pH 2.9) to 1.0 *M* pyridine formate (pH 5.0). Usually fractions of 5 ml are collected in this instance. The location of amino acids is most conveniently traced by spotting an aliquot of the eluted fraction on a sheet of Whatman paper and detecting the presence of amino acids with ninhydrin [ninhydrin–acetone reagent, 0.25% (w/v)]. The reducible cross-links are assayed by measuring the radioactivity in each collected fraction. The positions of reducible components in acid and alkali hydrolysates are evident from Fig. 16. In this system, aldolhistidine and anhydro derivatives of hexitollysine co-chromatograph and the reduced aldol condensation product is only partially separated from disaccharide derivatives of dehydrohydroxylysinonorleucine and hydroxylysinonorleucine.

Chromatography with sodium citrate buffers [83]

Both a continuous citrate buffer gradient and a stepped gradient can be used for this purpose; it is even possible to use a single buffer system. For continuous gradient elution the separation can be carried out typically on a 58×0.9 cm I.D. column of 8% cross-linked resin. The sodium citrate gradient starts at pH 2.91 and was introduced by Mechanic [83]. Although it is possible to identify the

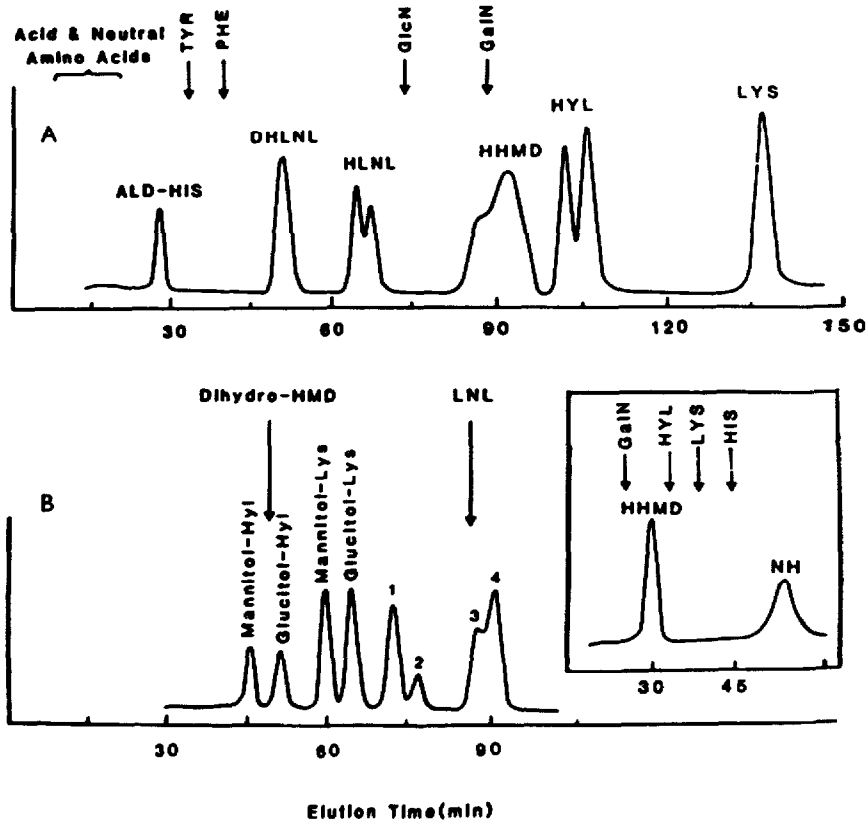

Fig. 17. Ion-exchange chromatography of reducible components with 0.35 *M* sodium citrate buffer (pH 5.25). (A) Elution positions of the major components; positions of amino acids and hexosamines is shown; (B) elution positions of hexitollysines and hexitolhydroxylysines and their anhydro derivatives. For abbreviations, see Fig. 16 [82]. Detection: ninhydrin (A_{570}).

reduced aldol condensation product in this system, the basic region of the chromatogram in which most of the cross-linking amino acids are eluted represents only a small proportion of the total running time, thereby leaving some of the cross-linking components unresolved. Therefore, gradients that expand the basic region of the chromatogram were introduced by Bailey et al. [81] and Mechanic [83].

Four single buffer systems and one stepwise gradient capable of resolving all the known cross-linking components of collagen are summarized in Table III [66]. System I separates the major cross-linking components, dehydrohydroxylysinonorleucine and hydroxylysinonorleucine, and the diastereoisomers of the latter cross-linking amino acid. Hydroxyhistidinomerodesmosine in this system gives a broad beak, the shape of which is very sensitive to small pH variations. Direct quantification of this component is best achieved by rechromatography with system II (Table III), whereupon a single peak is obtained that is well separated from the contaminating galactosamine. The positions of all individual cross-linking components are visible in Fig. 17. Interferences caused by the pres-

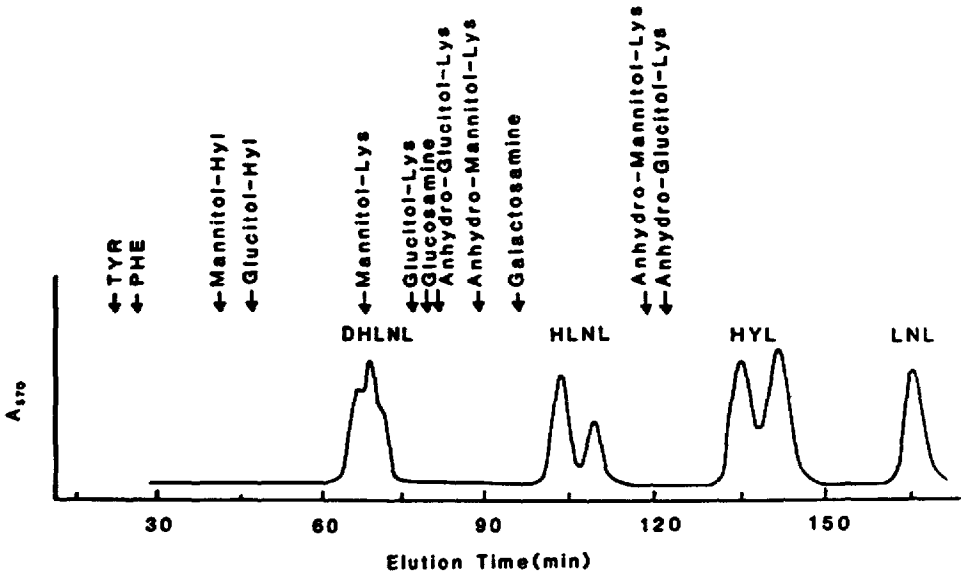

Fig. 18. Ion-exchange chromatography of cross-link components with 0.2 *M* sodium citrate buffer (pH 4.7) (system III in Table III). For abbreviations, see Fig. 16 [82].

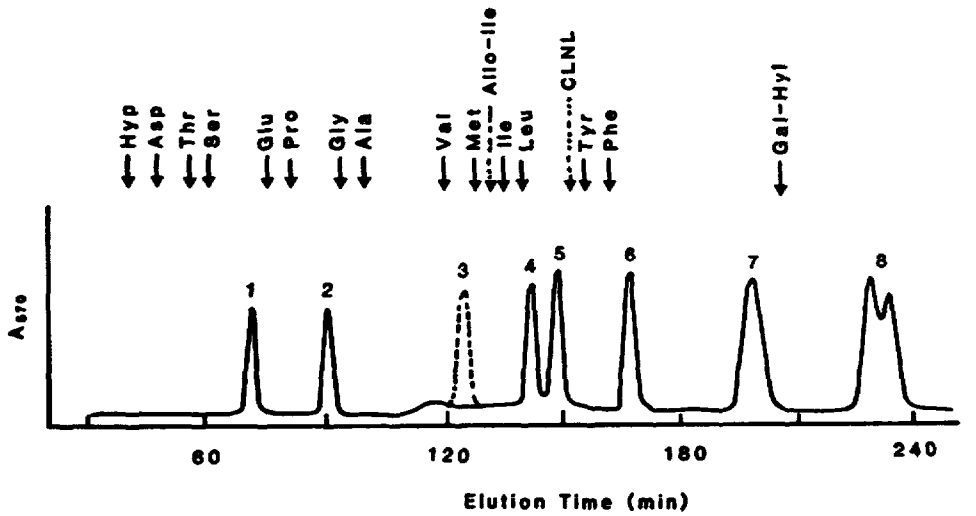

Fig. 19. Ion-exchange chromatography of reduced cross-link precursors and glycosylated derivatives with system V in Table III. The positions of some amino acids are shown. 1 = DHNL; 2 = HNL; 3 = acid degradation product of reduced ACP; 4 = reduced ACP; 5 = Glc-Gal-DHLNL; 6 = Glc-Gal-HLNL; 7 = Gal-DHLNL; 8 = Gal-HLNL. For abbreviations, see Fig. 16 [82].

ence of hexitollysines can be overcome by preliminary fractionation with the pyridine formate system in which these compounds elute as a single peak. It is also possible to fractionate the sample on an ion-exchange column with a pH 4.7 buffer (system III in Table III); the positions of the reducible components are shown in Fig. 18. A complete resolution of all basic reducible cross-links present in collagen

is offered by a combination of systems I and III. Dehydrohydroxynorleucine and dihydrohydroxymerodesmosine give multiple peaks at pH 4.7, which is the result of partial resolution of their diastereoisomers. For the purpose of quantitative analysis rechromatography should be carried out at pH 5.25 (system I) in which these compounds elute as separate peaks. Separation of the aldol condensation product and glycosylated cross-links is best achieved in system V. A typical chromatogram is shown in Fig. 19.

As evident from the previous discussion, complete resolution of all the diverse basic cross-linking amino acids in collagen cannot be carried out in a single chromatographic step. Even here separations have reportedly been achieved it is advisable to check the chromatographic behaviour of any component in at least two chromatographic systems. It may seem surprising that no data are available from any of the advanced HPLC procedures with regard to collagen cross-link separation. The reason probably lies in the fact that the separation of collagen cross-linking amino acids is not as usual as the separation of the common twenty amino acids for which the advanced procedures are designed; consequently, no information is currently available regarding even the most common derivatization procedures with, e.g., phenylthiohydantoin (PTH), dansyl and *o*-phthalaldehyde (OPA) reagents, for collagen coss-linking amino acids. Another reason may be that the proportion of the cross-linking amino acids in relation to all the others is low and frequently stabilization of the unstable components by means of borohydride treatment is necessary; therefore, in most instances detection is carried out by radioactivity monitoring, for which the necessary equipment is not commonly available with most of the advanced automated amino acid analysers. With the introduction of miniaturization into modern amino acid analysis, radioactivity measurement may also be a source of additional problems. Nevertheless, progress in this respect is highly desirable.

SEPARATION OF CYANOGEN BROMIDE FRAGMENTS

Electrophoretic procedure

Basically, all the methods described for the separation of intact polypeptide chains may be used also for the separation of cyanogen bromide (CNBr) fragments, provided that gels of appropriate density (typically 10%) are used. With 12.5% SDS polyacrylamide gels separation is also possible, giving the profiles summarized in Fig. 20. All CNBr peptides arising from collagen types I and III follow a strictly linear relationship in the Ferguson plot, except peptide α_1(III)CB-8 [84,85]. The Ferguson plot for collagen CNBr peptides differs, as expected, from a similar plot constructed for globular proteins [58]. The reasons for this phenomenon are obviously the same as those for intact collagen polypeptide chains. The reason for the anomalous behaviour of the peptide α_1(III)CB-8 is obscure and according to the available data cannot be related to anomalous SDS binding (see also Fig. 15).

Much better resolution of individual CNBr peptides can be obtained with gradient gels, as shown in Fig. 21 [61].

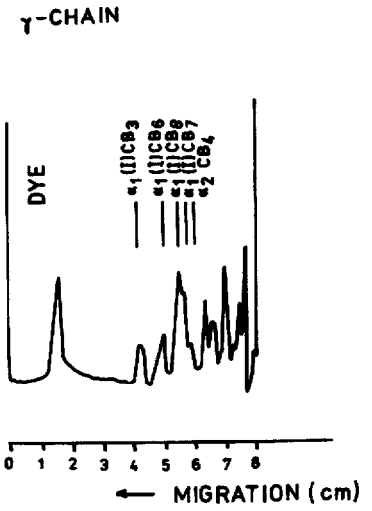

Fig. 20. Electrophoretic profile (densitometric trace) of CNBr peptides arising from type I collagen on a 12.5% polyacrylamide gel.

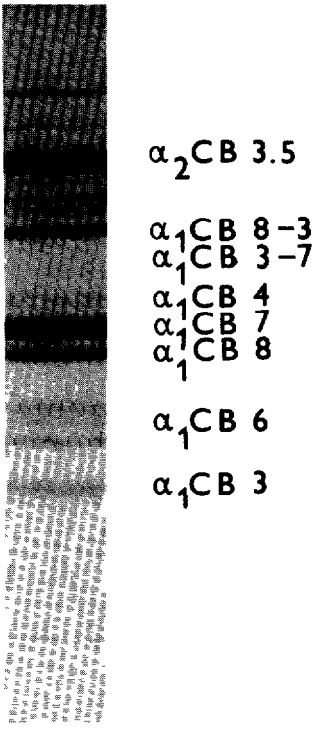

Fig. 21. Gradient polyacrylamide gel electrophoretic profile (12–18%) of CNBr peptides of collagen type I.

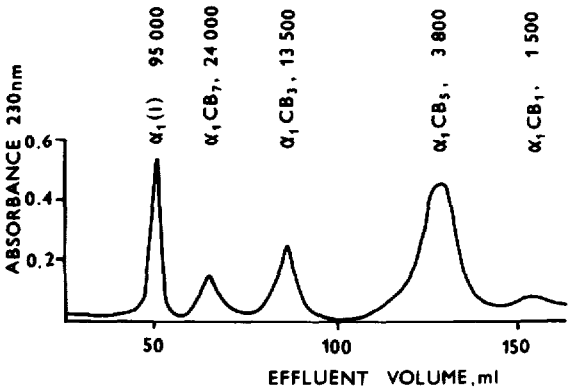

Fig. 22. Molecular sieve chromatography (Bio-Gel P-150) of α_1(I) chain CNBr peptides using 1.5 *M* calcium chloride solution (pH 7.5) [86].

Chromatographic procedures

Molecular sieve chromatography

Molecular sieve chromatography, particularly in conjunction with ion-exchange techniques (see below), is an excellent technique for resolving and isolating CNBr peptides. For these purposes most CNBr peptides can be separated on either Bio-Gel A 1.5 m or Bio-Gel P-150 columns; for peptides with molecular masses less than 2000 Bio-Gel P-6 is preferred [86]. The very separation is carried out in columns of dimensions 120–160 × 1.5 cm I.D., but the column diameter depends on the amounts of sample separated. Elution is carried out under conditions similar to those specified for intact polypeptide chains (Fig. 22).

CM-cellulose chromatography

As stated before, the column dimensions used for this purpose can differ considerably according to the amount of sample to be separated. Also, the precise composition of the gradient buffers may be varied to accommodate best the number and nature of peptides present in the separated mixture. It is advisable to specify the gradient conditions in a preliminary run. As chromatography is performed at 40–45 °C, all buffers should be deaerated.

The following starting and limiting buffers are needed [87,88]: starting buffer, (a) 0.02 *M* citrate containing 0.04 *M* sodium chloride (pH 3.6), (b) 0.02 *M* citrate containing 0.01 *M* sodium chloride (pH 3.6), (c) 0.02 *M* citrate (pH 3.6) and (d) 0.005 *M* citrate containing 0.02 *M* sodium chloride (pH 3.6); limiting buffer, (a) 0.02 *M* citrate containing 0.14 *M* sodium chloride (pH 3.6), (b) 0.02 *M* citrate containing 0.16 *M* sodium chloride (pH 3.6), (c) 0.02 *M* citrate containing 0.14 *M* sodium chloride (pH 3.6) and (d) 0.05 *M* citrate containing 0.18 *M* sodium chloride (pH 3.6). The respective pairs of starting and limiting buffers are always used in a linear gradient mode (Fig. 23).

There are situations in which it is advantageous to carry out CM-cellulose chromatography with a concave salt gradient [47,88]. The starting buffers used in this instance are (a) 0.2 *M* sodium acetate (pH 4.8), (b) 0.03 *M* sodium acetate

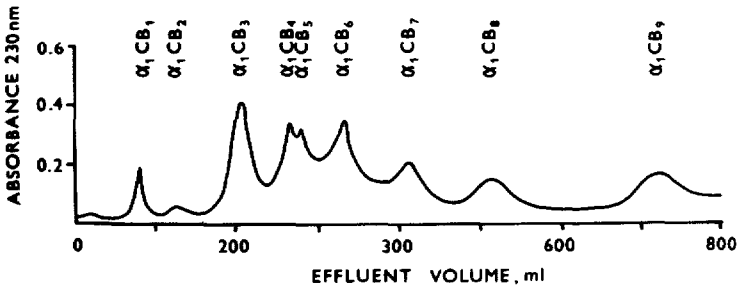

Fig. 23. CM-cellulose chromatography of CNBr peptide derived from human α_1(V) chain. Elution was achieved with 5 m*M* citrate containing 20 m*M* sodium chloride (pH 3.6) using a linear gradient of 0.02–0.18 *M* sodium chloride [87].

(pH 4.8) and (c) 0.05 *M* sodium acetate (pH 4.8), and the respective limiting buffers are prepared from the starting buffers by making them 0.12 *M* in sodium chloride. As the concave salt gradient is formed by placing 820 ml of starting buffer in the mixing chamber and 500 ml of limiting buffer in the reservoir chamber of a constant-level device, the dimensions of the column have to be kept constant at 10×2.0 cm I.D. Under these conditions certain peptides, specifically those derived from α_1(I) chains, tend to be separated into as many as three different peaks of identical amino acid composition; the reason for this phenomenon is obscure.

Phosphocellulose chromatography

For this type of separation 0.001 *M* sodium acetate (pH 3.8) is used as the starting buffer, the limiting buffer being prepared by making the starting buffer 0.3 *M* in sodium chloride. Separations are run in a linear gradient mode at 40–45 °C, typically in 8×1.8 cm I.D. columns. The gradient volume in this instance is of the order of 500 ml. When required, the separation can be optimized with particular peptides by altering the slope of the gradient [89].

Reversed-phase high-performance liquid chromatography

HPLC procedures are certainly very effective for the analytical separation of collagen CNBr cleavage products. Good results can be obtained with a 30-nm pore reversed-phase system using a μBondapak column. Elution is carried out with a linear gradient of acetonitrile–water (12.8–44.8% acetonitrile) containing 0.01 *M* heptafluorobutyric acid. Alternatively, it is also possible to use C_{18} Bondapak as the sorbent. The use of larger pores (30 nm) was shown to have a favourable effect on the resolution and the more hydrophobic counter ion markedly increases the retention time and resolution of the CNBr peptides [90].

Typical profiles obtained with the CN reversed-phase system for CNBr-cleaved collagen types I, II and III are shown in Fig. 24.

The C_{18} reversed-phase system was successfully used also for the separation of tryptic peptides [35 °C, linear gradient, 1–500 m*M* guanidine hydrochloride, 0.05 *M* Tris buffer (pH 6.8) or gradients of guanidine hydrochloride and methanol] [91].

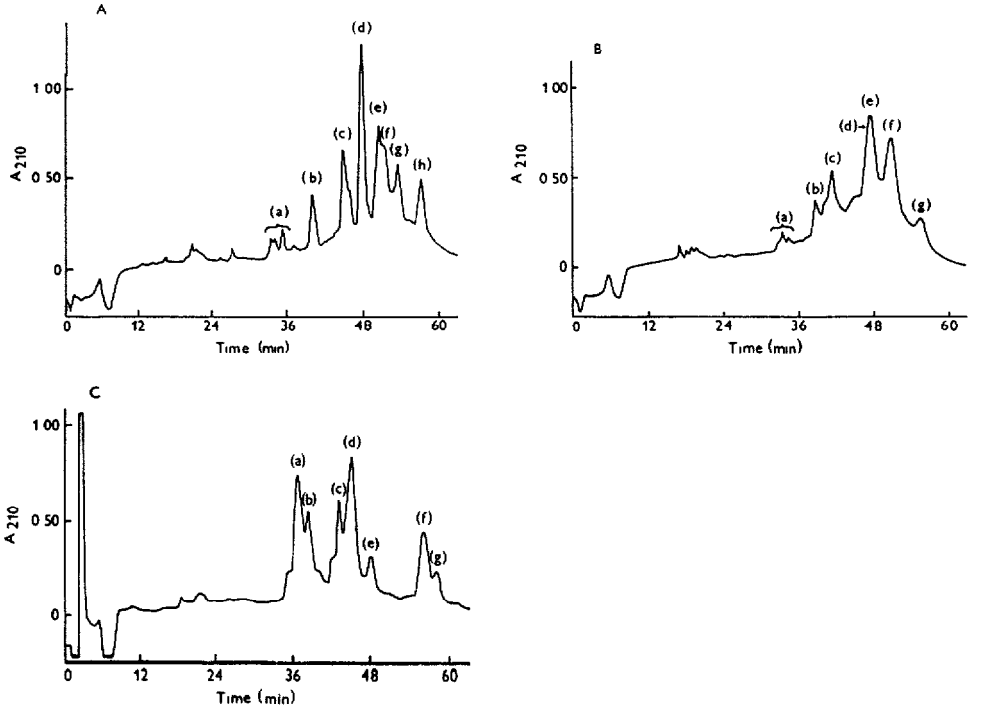

Fig. 24. HPLC separation of CNBr collagen peptides obtained with CN reversed phase. For separation details, see text. (A) Collagen type I, (a) α_1CB2, α_1CB4, α_1CB5, α_2CB2; (b) α_1CB3; (c) α_1CB6; (d) α_1CB7, α_1CB8; (e) and (f) incomplete cleavage products; (g) α_2CB4; (h) α_2CB3,5. (B) Collagen type II, α-chain fragments identified as follows: (a) CB6 and incomplete cleavage products involving CB4, CB2, CB3 and CB6; (b) CB8; (c) CB9,7, CB9,7X and CB12; (d) incomplete cleavage product; (e) CB11 and CB10; (f) incomplete cleavage product CB11-8; (g) incomplete cleavage product CB8-10. (C) Collagen type III, α-chain fragments identified as follows: (a) CB6 and CB3; (b) CB4; (c) CB8; (d) CB5; (e) incomplete cleavage product; (f) CB9; (g) incomplete cleavage product.

SEPARATION OF URINARY HYDROXYPROLINE-CONTAINING POLYPEPTIDES

The determination of total urinary hydroxyproline is non-specific as this amino acid is not only present in all collagen types but is present in some other compounds such as acetylcholinesterase and C1q. Therefore, other markers of collagen metabolism have been sought. Basically two ways were exploited. In the first, hydroxyproline-containing peptides are assayed. The second, which is much more specific, is based on the determination of peptidically bound pyridinoline.

Gel permeation chromatography of urinary hydroxyproline-containing peptides

In all analyses of urinary peptides urine must be adequately pretreated. Typically, urine is collected under toluene in the dark and stored in the cold. Aliquots of 20–100 ml are taken and the pH is adjusted to 4.8. The samples are then heated in a boiling water-bath and the precipitated protein is removed by centrifugation.

The clear supernatant is concentrated 10–25-fold on a rotary evaporator. The pH of the sample is adjusted to 3.5 and the sample is chilled. The precipitate is removed by brief centrifugation and the clear supernatant is used for gel permeation chromatography.

The first step in the chromatographic analysis is separation on a Bio-Gel P-2 column. Typically 2.5 ml of urine concentrate are loaded on a 35×2.6 cm I.D. column which is eluted with 0.1 *M* acetic acid and the eluate is monitored for hydroxyproline and for its absorbance at 230 nm. In this separation hydroxyproline-containing peptides yield two distinct peaks. Of these, the first eluted peak is subjected to further chromatography on Bio-Gel P-6. For this purpose columns 1.6 cm in diameter with an effective bead length of 400 cm (several columns in series) were recommended. Elution is carried out with 0.1 *M* acetic acid and the eluate is analysed for hydroxyproline [92–94].

Additional urinary peptide separations

The separation of hydroxyproline-containing peptides on Bio-Gel P-6 is poor and better results can be obtained by chromatography on phosphocellulose. The excluded fractions from five chromatographic runs on Bio-Gel P-2 are pooled and evaporated to dryness and the residue is dissolved in 0.01 *M* sodium acetate buffer (pH 3.8) and separated on a 30×1.6 cm I.D. phosphocellulose column equilibrated previously with the same buffer. The separation is obtained through a gradient of sodium chloride from 0.0 to 1.0 *M*.

Both of the above-described separations dealt with the peptide fraction that is not retained on the Bio-Gel P-2 column. The other fraction, which contains small peptides, can be separated on QAE-Sephadex: the fraction is concentrated, dissolved in 1.0 ml of a buffer obtained by mixing γ-picoline–morpholine–pyridine–water (80:60:40:3820, v/v) and adding a few drops of acetic acid to adjust the pH to 9.4. This sample is layered on the top of a 35×2.6 cm I.D. column of QAE-Sephadex A-25 previously equilibrated in the same buffer. The elution is effected through a pH gradient obtained with a five-chamber Autograd. Chambers 1, 2 and 3 are filled with the γ-picoline buffer, the pH of which is adjusted to 9.4, 8.4 and 6.5, respectively. Chamber 4 is filled with 140 ml of 0.5 *M* acetic acid and chamber 5 with 140 ml of 2.0 *M* acetic acid. The eluate is monitored for hydroxyproline.

Determination of peptidically bound pyridinoline [95]

As has been said above, evaluation of the separated peptides according to the content of hydroxyproline is non-specific as this amino acid may also be present in proteins other than collagen. To increase the specificity of the assay of collagen degradation products, a method has been developed that makes use of pyridinoline, which is a stable, non-reducible cross-link that is present in collagen fibres but not in other hydroxyproline-containing proteins such as acetylcholinesterase and C1q. It is abundant in adult cartilage collagen and also in bone and dentine, but is almost absent in collagen of the skin and cornea and also in newly synthe-

TABLE IV

GRADIENT PROGRAMME

Mobile phase A, water adjusted to pH 2.2 with trifluoroactic acid; mobile phase B, methanol. The gradient was corrected for the precolumn volume.

Time (min)	A (%)	B (%)
0	100	0
12	92	8
24	60	40
25	0	100
28	0	100
30	100	0
45	100	0

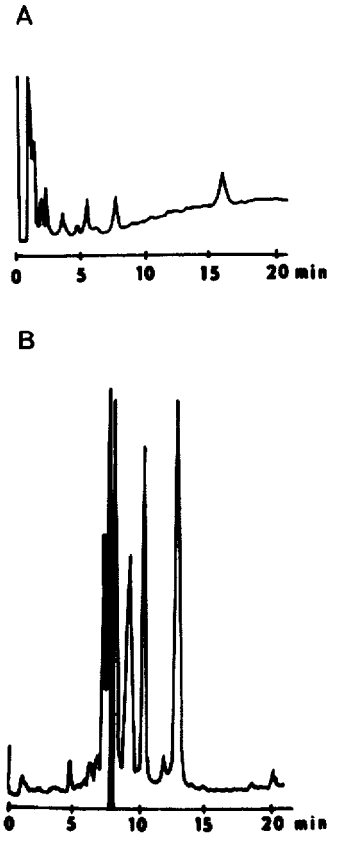

Fig. 25. HPLC separation of urinary peptides with molecular mass higher than 10 000. Fluorescence detection; excitation 270 nm, emission filter 389 nm. (A) Urine from a healthy control; (B) urine from an osteoarthritic patient. For the gradient system used for elution, see Table IV. For further details, see text [95].

sized collagen [95]. For the purpose of determining peptidically bound pyridinoline, 25 ml of 24-h urine are filtered, using a molecular mass 10 000 cut-off membrane; the urine is concentrated to 4 ml, the membrane is washed twice with 40 ml of water, the filtrate is discarded and the sample remaining above the membrane (4 ml) is used for further analysis.

The separation of high-molecular-mass peptides is carried out on an Ultrapore RPSC (particle size 5 μm, 30-nm pores) (Beckman, Berkeley, CA, U.S.A.) column (75×4.6 mm I.D.) protected with a guard column (20×4.6 mm I.D.) packed with Supelcosil LC-308 (particle size 5 μm, 30-nm pores) (Supelco, Bellefonte, PA, U.S.A.). The gradient system used for elution is described in Table IV. Separation is carried out at 40°C. For detection the typical fluorescence of pyridinoline is exploited: excitation wavelength 270 nm, excitation filter 7-54, emission filter 389 nm. Typical profiles obtained with urine from a healthy control subject and from an osteoarthritic patient are presented in Fig. 25. The separation procedure can also be used for assaying urinary peptides if the eluate is monitored at 220 nm or for the determination of hydroxyproline-containing peptides; however, no method for the on-line detection of peptidically bound hydroxyproline is available.

AMINO ACID ANALYSIS OF COLLAGEN PROTEINS

Collagen proteins undergo extensive post-translational modifications of some of their amino acids. Such modified amino acids can serve as collagen markers in tissues or body fluids (in the case of collagen degradation products). Collagen is particularly typical in containing hydroxyproline and hydroxylysine. Among the amino acids arising by post-translational modifications one can also list hexosylated hydroxylysines, products of glycation and all cross-linking elements. Here we shall limit ourselves particularly to the determination of hydroxyproline and hydroxylysine.

In the past, methods were published for hydroxyproline determination involv-

TABLE V

GRADIENT PROGRAMME

Mobile phase A, 3 g SDS + 1 ml of 1 *M* trichloroacetic acid (TCA) in 1 l water (pH 3.0); mobile phase B, 3 g SDS + 5 ml of 1 *M* TCA in 1 l water (pH 2.1); mobile phase C, 3 g/l SDS in 80% aqueous 1-propanol.

Column A				Column B		
Time (min)	A (%)	B (%)	C (%)	Time (min)	A (%)	B (%)
0	97	0	3	0	96	4
4.5	97	0	3	8	96	4
6	0	90	10	12	86	14
11.3	0	84	16	44	86	14
22.5	0	81	19	64	60	40
30	66	0	34	70	60	40
45	66	0	34			

ing ion-exchange chromatography. However, such methods possess the disadvantages of a long analysis time and a low column efficiency (for a review, see ref. 96).

Modern methods for hydroxyproline assay operate in the reversed-phase mode with an ion-pairing reagent in the mobile phase [97–99]. Eluted amino acids are detected with the ninhydrin procedure. The separation is achieved on a 250×4.6 mm I.D. column packed with Ultrasphere IP (particle size 5 μm) (Beckman) or with Separon SGX C_{18} (Tessek, Prague, Czechoslovakia). The gradient system needed for complete resolution is complex and is presented in Table V. Separations are carried out at 35°C using flow-rates between 0.5 and 1.2 ml/min, the ninhydrin reagent flow-rate being 0.2 ml/min. The eluate is monitored by measuring the absorbance at 440 nm, the wavelength being switched automatically to 570 nm after elution of aspartic or glutamic acid [100] (Fig. 26).

The above procedure is applicable in situations when all amino acids are to be assayed. If, however, the determination of hydroxyproline only is needed, e.g., for the determination of collagen in tissues, a shortened version can be applied. Dydek and Kehrer [101] developed an HPLC procedure for separating hydroxyproline from proline. The amino acids in question are detected in the fractions by spectrophotometry, which is cumbersome. Stimler [102] introduced a method for the separation of hydroxyproline from proline on a high-performance cation-exchange column with detection by absorptimetry. This method, however, has a low sensitivity (5 μg) and a long analysis time (40 min).

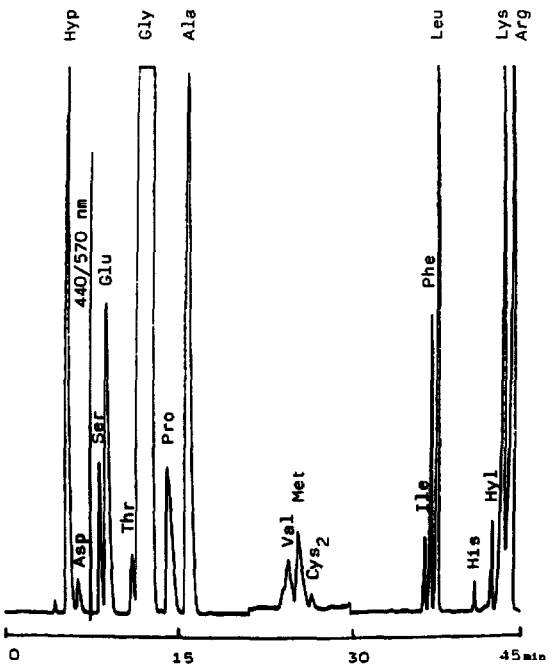

Fig. 26. HPLC of a collagen type I hydrolysate. The attenuation was 64 except for Val, Met, and Cys 2, which were recorded at attenuation 16. For the gradient system, see Table V. For further details, see text [100].

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