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Review

Advanced separation methods for collagen parent α-chains, their polymers and fragments

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Abstract

Current techniques used for collagen α-chains and their CNBr fragments are reviewed. Ion exchange, gel permeation, reversed-phase and affinity chromatography are discussed mainly from the preparative aspects as these are both the techniques of choice to remove biological matrix contaminants always present in collagen preparations and techniques routinely used for preparative purposes. Among electromigration procedures gel electrophoresis is widely used both for intact collagen α-chains and their fragments. Recently this technique was applied also for miniaturised preparations. Immunoblotting techniques serve more specific detection of otherwise hard to distinguish different collagen polypeptide chains. Capillary electromigration techniques brought recently new aspects of understanding the behaviour of collagen proteins upon different separation modes and seem to represent a smart perspective for better quantitation of individual collagen species.

Keywords: Reviews; Collagen parent α-chains

Contents

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1. Introduction

The term collagen stands for a series of at least 19 proteins of which the most abundant ones form extracellular fibrils (network structures). Additional ten proteins contain collagen-like domains (for review see [1]). Mutations in the six different collagen types are the cause of a number of human diseases like osteogenesis imperfecta, chondrodisplasias, some forms of osteoporosis, some types of osteoarthritis, the renal disease called Alport syndrome etc. Fibrotic reactions, tissue remodelling and wound healing represent additional biomedically important areas of collagen chemistry [2,3]. Interactions with reactive metabolites result in a number of storage effects of which glycation appears the most studied phenomenon in connection with diabetes and aging (see e.g. [4–6]).

It is beyond the scope of this review to describe in detail individual collagen types (their structures are schematically presented in Fig. 1) [1].

Briefly, the collagen superfamily can be divided into several classes (each containing several collagen types) on the basis of structural features of individual members, namely fibrils forming collagens (type I, II, III and V), collagens forming networks (type IV, VIII and X), collagens found on the surface of fibrils (fibril associated collagens FACIT) with interrupted triple helices that include collagens type IX, XII, XIV and XIX), collagens forming beaded filaments (type VI), collagens forming anchoring fibrils for basement membranes (type VII), collagens with transmembrane domain (types XIII and XVII) and collagens not yet fully characterised (types XV and XVIII).

This review is devoted mainly to advances in separation techniques of fibril forming collagens, particularly collagen type I, which is the best known of this series of proteins, abundant in vertebrate tissues where it constitutes a considerable proportion of all proteins present. The building scheme of fibril forming collagens comprises a triple helical structure composed of parent α-chains winding one around the other in a superhelical manner. Individual α-chains, the sequence of which in case of type I, II, III and V is completely known [7], are of molecular mass around 100 000–150 000 (see Table 1). About one third of the sequence is formed by gly-pro-x repeats which brings about a considerable internal homogeneity of the proteins. Individual parent α-chains can be either identical (as in type III collagen) or different (as in type I collagen) or a mixture of both (as in type V collagen). In type I collagen the individual parent α-chains are called α_1 and α_2; a triple helical molecule of type I collagen is constituted of two α_1 and one α_2 chains having a chain structure of $[\alpha_1(I)]_2\alpha_2$ and is called γ-collagen (γ-fraction) while dimers of α-chains are designed as β-fraction (composed e.g. of $\alpha_1\alpha_2$ or $\alpha_1\alpha_1$ chains). There are additional specific features of fibril forming collagens: they contain hydroxyproline and hydroxylysine, one third of all amino acids present is formed by glycine residues, they are extremely resistant to common proteases (except tissue and bacterial collagenases) and they are more soluble in the random coil than in native conformation. Fibril forming collagens are devoid of tryptophan, contain little if any tyrosine residues and, except collagen type III are practically devoid of cysteine. Also the proportion of methionine is low which makes it possible to use CNBr fragmentation for the identification of a particular collagen α-chain.

Individual collagen α-chains can be polymerised either by physiological cross-links (lysinonorleucine, hydroxylysinonorleucine, aldol condensation product, pyridinoline (see e.g. [8–11])) or by non-physiological cross-linking agents (typically aldehydes (see e.g. [12–14])). Pathological crosslinking can be typically caused by the interaction with reducing sugars or oxidation products of unsaturated lipids [13] (also monotopical interactions are possible [15]).

Polymerised fibril forming collagens (no matter whether physiologically or non-physiologically) are insoluble and their solubilization is routinely done either by mild pepsinization by which short terminal regions possessing the polymerisation sites (cross-links) are cleaved off, or by CNBr cleavage which results in a limited fragmentation of the parent α-chains as mentioned already. Tissue collagenases (which unfortunately are difficult to obtain) split the triple-helical structure in two thirds from its N-end; bacterial collagenases (*Clostridium histolyticum)* are far less specific, cleave the sequence in small fragments (mostly tripeptides) and are therefore of little use in structural studies.

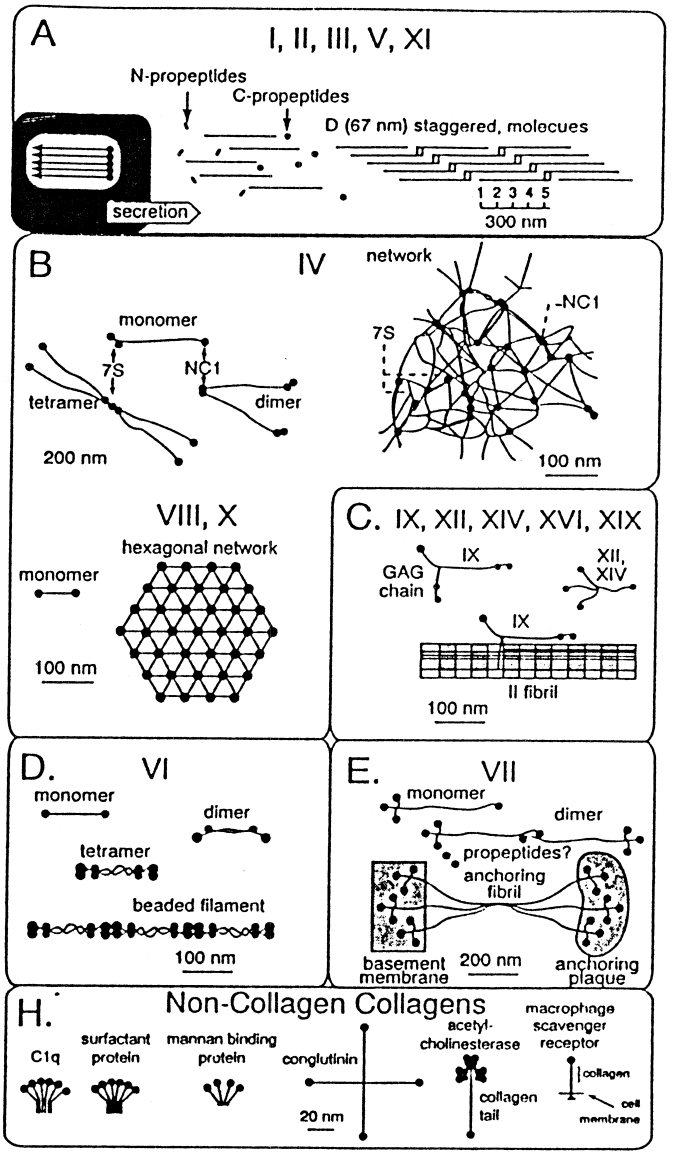


Fig. 1. Schematic structures of various collagens. Reprinted from Prockop and Kivirikko [1] with permission, from the Annual Review of Biochemistry, Vol. 64, © 1995, by Annual Reviews http://www.annualreviews.org.

2. Standard separation procedures (isolation of fibril forming collagens from tissues)

Separation of classical (low-pressure) chromatography involves quite a large number of methods. Practically all types of chromatographic operational modes have been exploited for this purpose (for a review see Ref. [17]) and frequently smart combinations of these are used exploiting e.g. the presence or absence of S–S bonds in the terminal region (or along the whole molecule as is the case of collagen type III), charge, molecular size, the presence of glycosidic residues or differences in physico-chemical properties of individual collagen species in the

native and denatured stage. The fundamental strategy about the application of individual analytical methods is presented in Scheme 1, details about actual applications exceed the scope of this survey and can be found in the quoted references and information about fractional precipitation (which may serve as a pre-purification step) is summarised in Table 1.

2.1. Examples of multiple chromatographic procedures

Examples of the purposeful combination of individual chromatographic modes are abundant. They frequently combine not only several chromatographic but almost regularly also at least one electromigration step. A nice example for many is the isolation of different species of collagen type V α-chains. In the reported procedure the first step is chromatography on Fractogel EMD SO_3^- 650(S) column with 0.04 *M* Tris–HCl buffer pH 8.2 containing 2 *M* urea and 0.05 *M* NaCl with linear NaCl gradient to the final concentration of 1 *M*. Next Bakerbond PEI Butyl Scout column was used with an NaCl gradient from 0 to 0.35 *M* over 20 min. Removal of non-volatile buffer components was done in the third step, a reversed-phase chromatography, carried out with a water–acetonitrile gradient (10–80% acetonitrile, solvents made 0.1% with respect to trifluoroacetic acid). The result was the separation of $\alpha_1(V)\alpha_2(V)\alpha_3(V)$ and $[\alpha_1(V)]_2\alpha_2(V)$ [37].

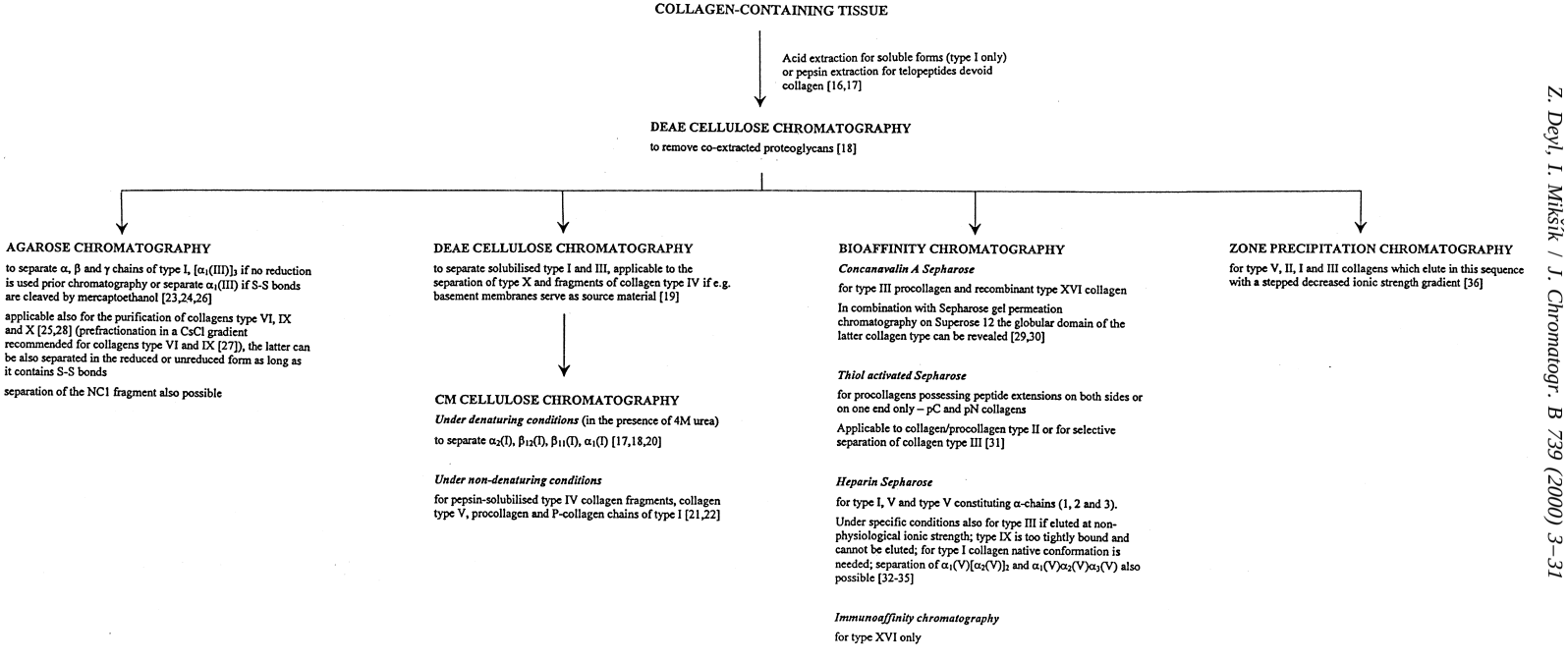
3. High performance chromatography

A number of rigid sorbents varying in polarity or capable of molecular sieving have been explored over the years.

Cyanopropyl bonded packings were shown to allow separations of human type I, II and III collagens as early as in 1981 [38]. Another possibility explored was the use of the so-called glycophases like LiChrosorb Diol, TSK-SW gels and last but not least Separon HEMA 1000 Glc (a copolymer of 2-hydroxyethyl methacrylate with ethylene dimethacrylate covalently bonded with glucose) [39–42]. While the former sorbents were successfully applied to the separation of a number of proteins, only the last one was shown to be applicable to collagen separations, in particular because of its good molecular sieving properties with relatively little of other types of interactions (though some are present as discussed later). Standard chromatographic columns (500×8 mm) packed with the Separon HEMA 1000 Glc sorbent, particle size 12–17 μm were used with 0.05 *M* Tris–HCl (pH 7.5) made 2 *M* with respect to urea as mobile phase. Skin collagen type I poly-

Table 1
Survey of molecular properties and precipitation conditions of different collagen types (according to Deyl and Adam [17])

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	$[\alpha_1(IV)]_3$ $[\alpha_1(IV)]_2\alpha_2(IV)$	180–75	1.2	1.7–2.0
V	$[\alpha_1(V)]_2\alpha_2(V)$	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	



Scheme 1. See also Refs. [16,18–36].

peptide chains yielded a clearly resolved peak of the γ-polymers followed by a peak comprised from the β-dimers and a partly resolved cluster of three peaks formed by the α_1(I) and α_2(I) polypeptide peak and some degradation products (after pepsinization) which were present in the tailing end of the α_1(I) peak (Fig. 2, Ref. [39]). Collagen type III preparations were always contamined with collagen type I (present as α_1 and α_2 chains and higher polymers) (Fig. 3). This approach allows to separate collagen type I trimers from the triple chain polymers of collagen type III. However, no separation of β_{12} and β_{11} chains can be achieved as long as dimers of type I collagen parent α-chains co-migrate with trimers. If a collagen type I preparation of collagen type III is depolymerized by performic acid oxidation, oxidised collagen type III α-chains can be separated from the rest of the mixture.

The Separon HEMA 1000 Glc separations suffer from negative peaks formation which appears always when the samples are run in 0.05 *M* Tris–HCl pH 7.5. A considerable improvement can be achieved when the eluting buffer is made 0.2 *M* with respect to NaCl (Fig. 4). Addition of NaCl to the eluting buffer not only removes the irregularities of the baseline but also allows the separation of collagen type I α-chain dimers which reflects the enlargement of the separation window, of course, at the expense of increased run time [43]. A number of collagens and collagenous chains (fragments) can be separated by this method as shown in Table 2. It is worth emphasising that the retention times of individual collagen samples/fragments decrease with increasing molecular mass, however, the decrease is not strictly linear in the semilogarithmic scale as it would be expected (Fig. 5). No separation of collagen polypeptide chains of identical rel. mol. mass but originating from different collagen types was observed. In a preceding set of experiments it was found that sorption plays an important role in high efficiency gel permeation chromatography of collagens and it was concluded that gel permeation chromatography of collagens is a mixed mode type of separation. On the contrary, in the absence of NaCl in the eluting buffer it is possible to separate e.g. collagen α-chains stemming from different collagen types, typically α_1(I), α_2(I) and α_1(III) polypeptides. This was ascribed either to differences in the hydrodynamic volumes of matching collagen polypeptide chains originating from different species, or adsorption and/or partition interactions of the separated protein molecules or, finally, to the possible weak affinity of collagen chains to glucose-coated macroporous adsorbent causing the respective retention differences of otherwise similar molecules.

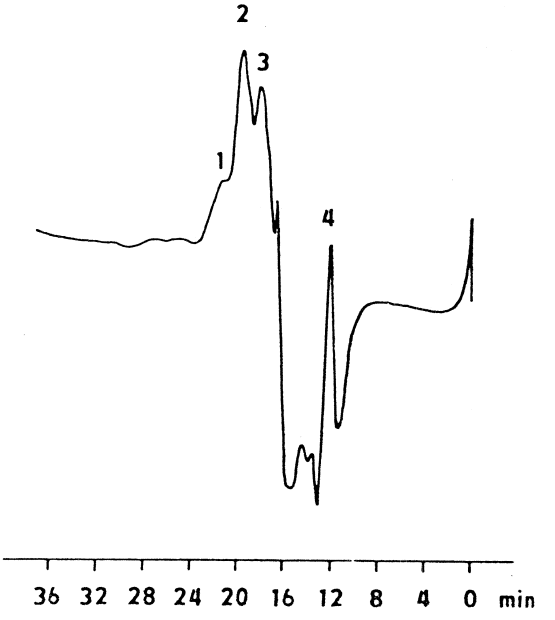


Fig. 2. Separation of collagen typ I chains by HPLC on Separon HEMA 1000 Glc column (500×8 mm). Elution: isocratic with 0.05 *M* Tris–HCl (pH 7.5) containing 2 *M* urea. Peak identification: 1, degradation products; 2, α_1(I); 3, α_2(I); 4, γ. Reprinted from Macek et al. [39].

For the separation of collagen type V $\alpha_1+\alpha_2$ chains from α_1(XI) polypeptide chain reversed-phase chromatography on C_8 phase using an acetonitrile gradient in 0.1% trifluoroacetic acid was described by Niyibizi and Eyre [44]. Under the specified conditions parent α_1 and α_2 chains of collagen type V coelute (the separation is only indicated) while the α_1(XI) polypeptide chains are clearly resolved.

A thorough study on the effect of different stationary phases (C_{18}, C_4 and diphenyl), organic modifiers (acetonitrile, isopropanol) and support pore sizes (120 Å, 300 Å and non-porous) was reported by

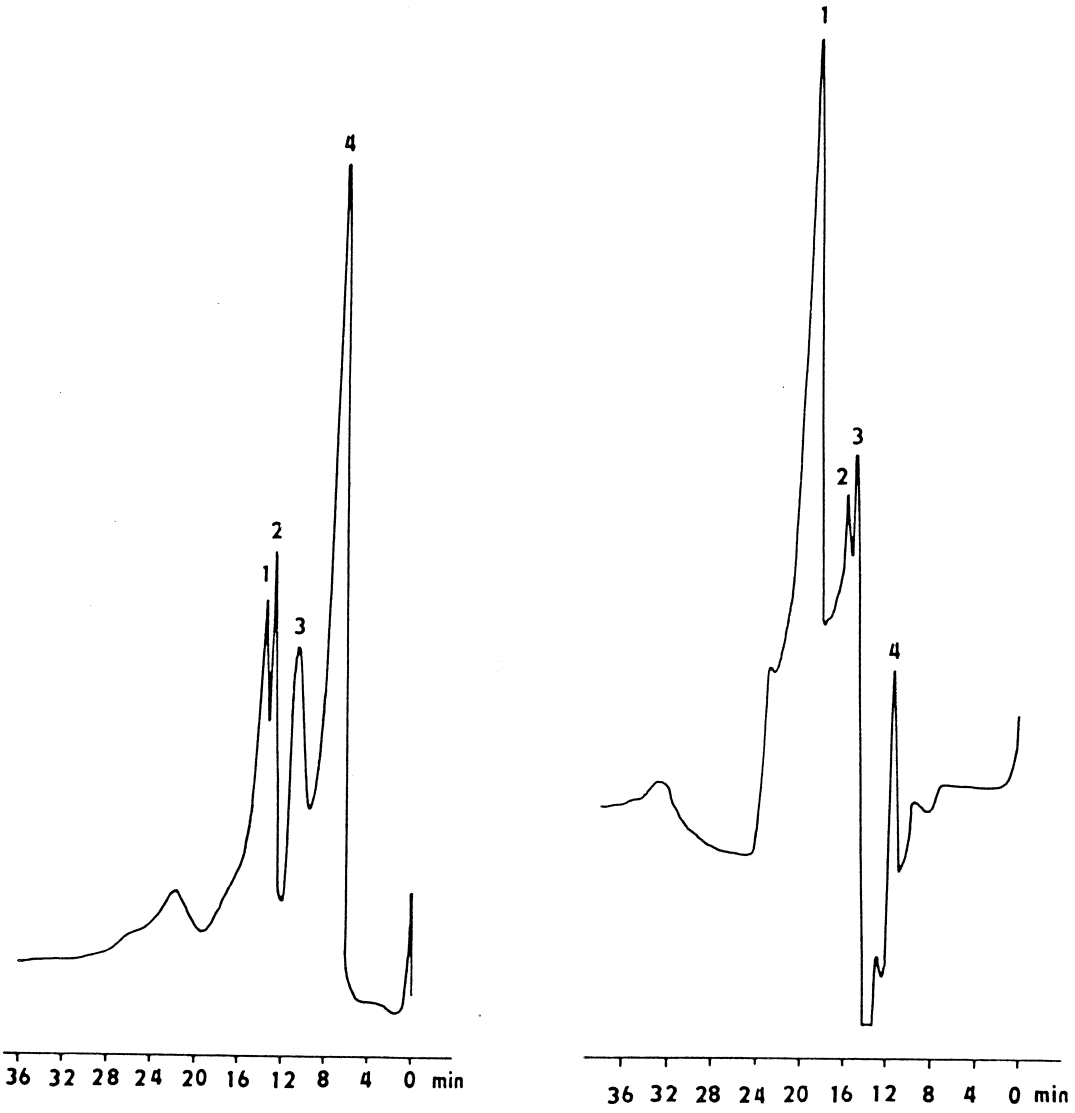


Fig. 3. Separation of collagen chains present in typ III before (**A**) and after (**B**) performic acid treatment. Conditions: column, Separon HEMA 1000 Glc (500×8 mm); elution, isocratic with 0.05 *M* Tris–HCl (pH 7.5) containing 2 *M* urea. Peak identification (A): 1, α_1(I); 2, α_2(I); 3, $[\alpha_1(I)]_2\alpha_2$; 4, γ; (B): 1, α_1(III); 2, α_1(I); 3, α_2(I); 4, $[\alpha_1(I)]_2\alpha_2$. Reprinted from Macek et al. [39].

Fields et al. [45]. It was demonstrated that large pore supports gave distorted peaks with small collagens and triple helical peptides, resulting in poor resolution. The formation of broad peaks has been ascribed to conformational instability of the separated solutes and slow cis-trans isomerisation of the peptide bonds. The best sorbents of those examined were diphenyl or non-porous C_{18} reversed-phases; as mobile phases standard water–acetonitrile gradients were recommended.

Reiser et al. [46] recommended for the separation of CNBr peptides of type I collagen (glycated in

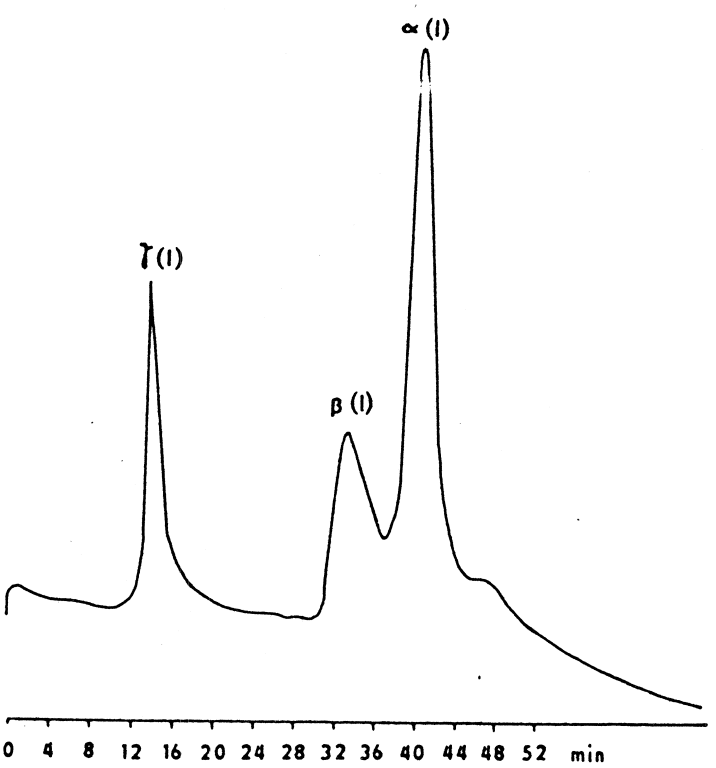


Fig. 4. Separation of collagen typ I α-chains by HPLC on Separon HEMA 1000 Glc column (500×8 mm). Elution: isocratic with 0.2 *M* NaCl — 2 *M* urea — 0.05 *M* Tris–HCl (pH 7.5) buffer. Reprinted from Deyl et al. [43].

vitro) either C_{18} reversed-phase in an acetonitrile gradient (0–40% ACN over 90 min) containing heptafluorobutyric acid as ion pairing agent.

4. Electromigration methods

4.1. Polyacrylamide gel electrophoresis

Already at the early stage of polyacrylamide gel separations of collagen type I parent α-chains it was reported that the plot of molecular mass of the collagen proteins (α-chains, their dimers and polymers) vs. relative electrophoretic mobility does not fit the analogous relations constructed for standard (commercially available) calibration sets of proteins. However, reasonable linearity can be observed with collagenous proteins both in the presence and absence of SDS. In Fig. 6A and B it is shown that α_1-chain derived entities (fragments and polymers) offer a straight line Ferguson plot which is different from a similar relationship based on α_2-chain, its fragments and polymers [47]. It is to be emphasised that both these plots differ from Ferguson dependences constructed for standard protein series of known molecular mass. This has been also observed by Furthmayr and Timpl [48] who assumed that the differences between the standard calibration series and collagen fragments reflect the rigidity of the collagen polypeptide chain (see also Ref. [49]) rather than changes in the ability of collagen chains/fragments to bind SDS.

The other unconventional fact with collagen α-chains (and their dimers as well) is the difference of relative electrophoretic mobility of the α_1- and α_2-chains; both these parent chains of collagen type I possess practically identical molecular mass. A similar difference has been published many times also for the β_{11} and β_{12} dimers. It is worth stressing that it is possible to differentiate e.g. α_1 and α_2 collagen type

Table 2
Retention time of different collagen chains separated by HPLC on Separon HEMA 1000 Glc column (500×8 mm;. elution: isocratic with 0.2–2 *M* urea–0.05 *M* Tris–HCl (pH 7.5) buffer). The α_1(I) polypeptide served as internal standard. According to Deyl et al. [43]

Type of collagen chain	Relative molecular mass	Retention time (min)	Principle of separation	
1, 11	α_2(I), α_1(II)	100 000	42.0[a]	Rat skin, cartilage and calf skin, limited pepsin digestion
2	α_1(IV)	140 000	38.5	Mouse tumour and human placenta, limited pepsin digestion
3	α_2(IV)	160 000	37.0	
4	β(I)	200 000	34.0	Human placenta, limited pepsin digestion
5	γ(I)	300 000	14.0	
5	γ(III)	300 000	14.0	Skin
3	α_1(IV) BM	160 000	37.0	EHS tumour, limited pepsin digestion
7	α_2(IV) BM	180 000	34.5	
8	α_{1-3}(V)	110 000	41.5	Human placenta, limited pepsin digestion
6, 8	C_1 fragment	120 000 (110 000 to 140 000)	41.0[c]	Minces of whole placental tissues, limited pepsin digestion
9	C fragment	95 000	43.0	
10	50 K fragment	50 000	50.0[b]	
12	7 S	360 000	4.0	Mouse tumour basement membrane, limited pepsin digestion
13	7 S coll	225 000	29.0	

[a] These collagen types yield repeatedly differing retention times when run in the pure form. In a mixture only a single peak is observed.
[b] This fragment is always fused with the 100 000 band of α-chains, see comment a.
[c] Position of this fragment varies.

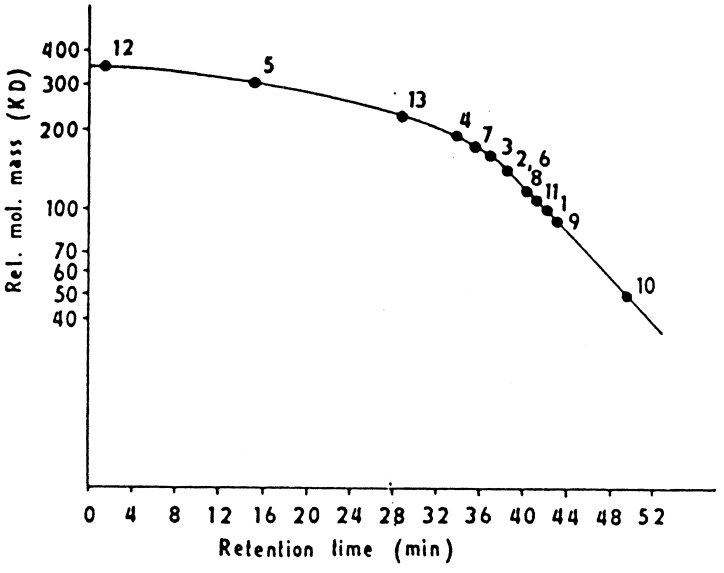


Fig. 5. Retention time vs. relative molecular mass relation for different collagen chains in the case of HPLC separation on Separon HEMA 1000 Glc column. For identification of chains see Table 2. Reprinted from Deyl et al. [43].

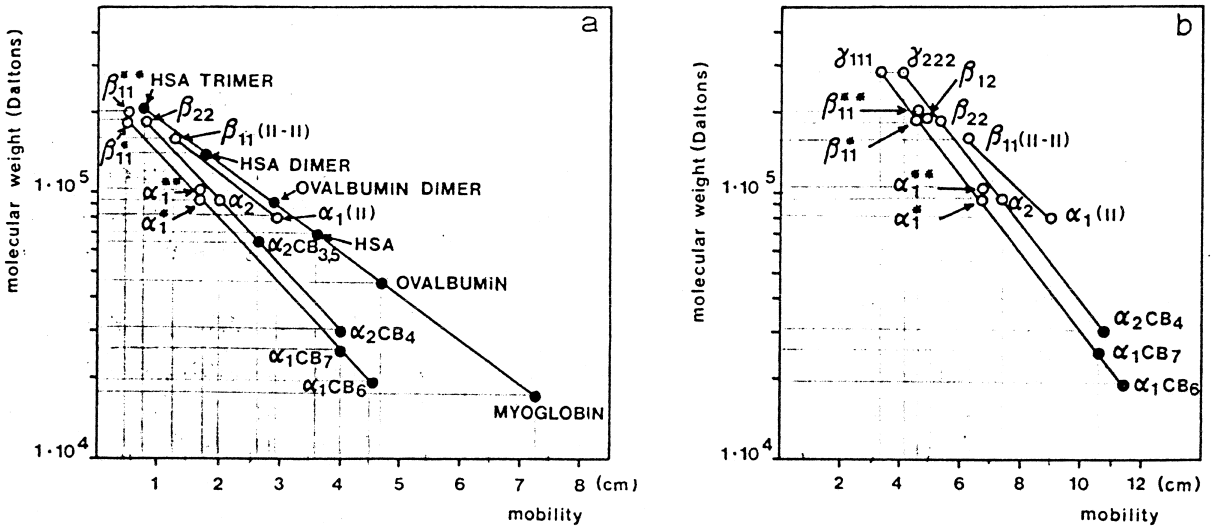


Fig. 6. Relation between mobility (migration distance) in polyacrylamide gel electrophoresis and molecular mass for collagen cyanogen bromide fragments and chains: (a) in the presence of of SDS, and (b) in the absence of SDS. Reprinted from Svojtková et al. [47].

I parent chains both in the presence or absence of the surfactant and this difference persists also with the β_{11} and β_{12} dimers. The suggestion [48] that it is the rigidity of the collagen polypeptide chains which is the cause of the different migration of collagen α-chains, polymers and fragments can be accepted when comparing the behaviour of these entities with the standard proteins calibration set, however, they are unlikely to explain the mobility difference between the α_1 and α_2 chains and their polymers as it emerges both from the amino acid composition and the total sequence of these polypeptides that they are very much alike indeed.

The plots shown in Fig. 7, left panel (constructed in the absence of SDS in the system) indicate that the straight lines corresponding to α_1- and α_2-chains (and their polymers/fragments) are parallel. It can therefore be concluded that the retardation coefficients (the slopes of these lines) are identical. This is in concert with the fact that the molecular mass of both these polypeptide chains is nearly identical.

A similar conclusion was drawn from data obtained when the separations were run in the presence of SDS (Fig. 7, right panel). It appeared therefore that the difference in electrophoretic mobility stems from the difference in a physical property of these polypeptide chains the nature of which is so far unknown. It has been demonstrated by Chrambach and Rodbard [50] that for the separation of substances with parallel lines on a Ferguson plot the optimal separation should be obtained at zero gel concentration, which in practice is approximated by 3% gel with 20% crosslinking.

There are two conclusions that emerge from the above consideration. First, dilute gels are likely to give the chance to separate chain polymers higher than trimers which in the conventional gels (5–7.5%) remain on the start (Fig. 8). The other aspect, as proven experimentally, is that for polymers composed of α_1- and α_2-chains the dependence of the relative electrophoretic mobility on the logarithm of the molecular mass falls between the extreme two lines given by the polymers consisting of α_1- and α_2-chains only, e.g. the point for the β_{12} fraction lies mid-way between the extreme lines representing the homodimers.

The above results allow a simple way for the determination of collagen polymeric structures in terms of constituting α-chains using 3% polyacrylamide, no matter whether the separation is carried out in the presence or absence of SDS. Provided that the Ferguson plot of $(\alpha_1)_n$ and $(\alpha_2)_n$ can be constructed, the polymers containing different proportion of either chain fall in between the lines obtained with pure polymers (containing a single chain only). Those closer to the $(\alpha_2)_n$ polymer line

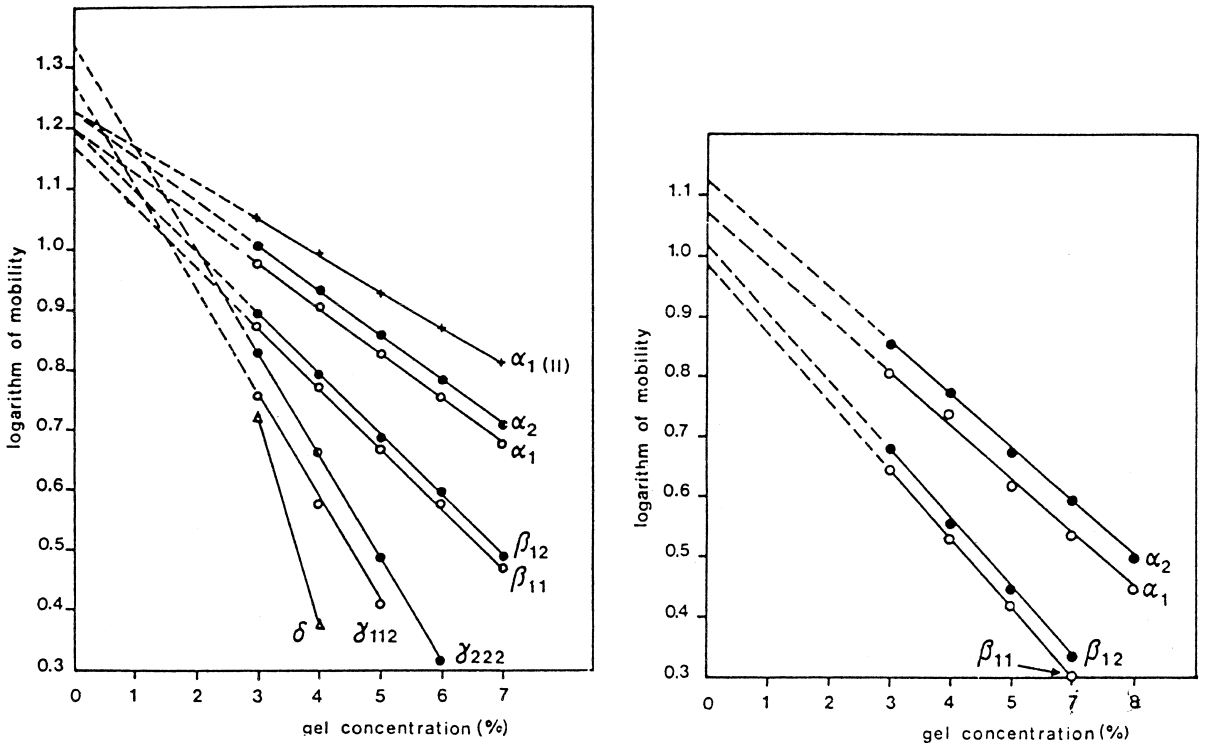


Fig. 7. Ferguson plots of polyacrylamide gel electrophoretic separation of collagen chains in the absence (left panel) and presence (right panel) of SDS. Reprinted from Svojtková et al. [47].

contain a higher proportion of α_2 while those that are closer to the $(\alpha_1)_n$ line contain a higher proportion of the α_1 chains. It can be demonstrated for instance that a point on the rel. mol. mass logarithm-migration distance plot which is in the 300 000 rel. mol. mass region and which is two thirds of the distance apart from the α_1-polymer chains plot is composed of two α_1 and one α_2 chain (see Table 3). However, it is necessary to stress that any extrapolations in the Ferguson plots may not be quite reliable [51].

As mentioned the result is the same no matter whether SDS is present or absent in the system. Acidic buffers should be preferred as they offer shorter running times. On the other hand with these buffers the chain polymers should be stabilised by borohydride reduction in order to eliminate the role of labile cross-links (their re-distribution) which reportedly occurs under these conditions [52].

Regarding the nature of the partition mechanism that causes the difference between α_1- and α_2-chain relative mobility there is one important fact that has not been discussed in our paper describing the above relations [47]; if the collagen α-chains are separated on 5–7.5% polyacrylamide gel, the band intensity for α_1 is about twice of that for α_2 (Fig. 8). However, if the same sample is run at the 3% gel concentration the intensity of the two bands is reversed, i.e. the α_2-band is much more pronounced that the α_2-zone though the latter is well recognisable. This observation contradicts the well known fact that a collagen molecule is composed of two α_1- and one α_2-polypeptide chain. This also means that the mechanism that distinguishes between α_1- and α_2-chains is less effective in dilute gels; there are two possible explanations for this phenomenon: either it is indeed the sterical hindrances that cause the differences in relative electrophoretic mobility as suggested by Furthmayr and Timpl [48], however, it also may well be a kind of another (hydrophobic) interaction of the solutes with the gel network. It is our opinion that the sterical hindrances are less likely to be involved as long as the separation was run with denatured sample in the presence of SDS (at submicellar concentration). If the concentration of SDS is in-

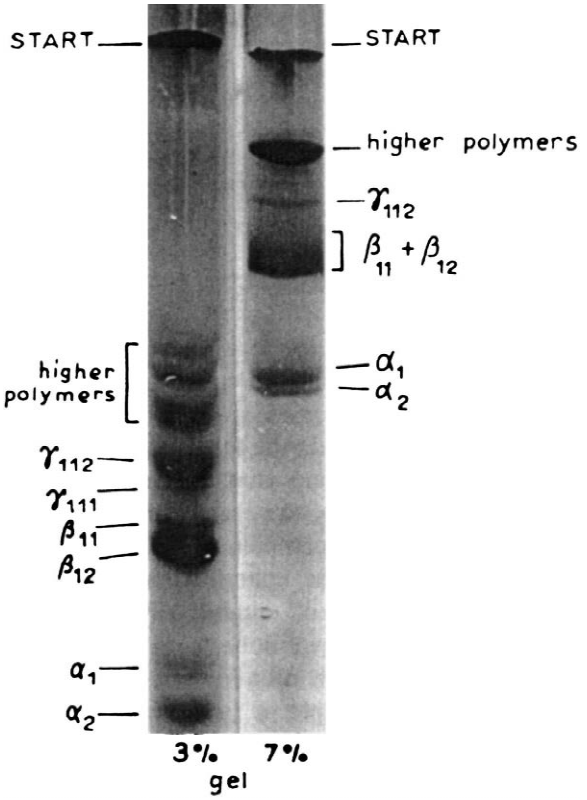


Fig. 8. Polyacrylamide gel electrophoresis of collagen chain polymers. Reprinted from Svojtková et al. [47].

creased to the supramicellar level a single zone of the mobility corresponding to α_2-zone is observed in diluted gels. This is indicative of a hydrophobic interaction between the collagen α-chains and the gel matrix. It is therefore proposed that the difference in relative migration between collagen α_1- and α_2-chains is caused by the presence or distribution of hydrophobic domains along the polypeptide chain. A supporting evidence can be obtained from the separation of α_1-collagen chain CNBr peptides; the fragments designed α_1CB_7 and α_1CB_8 exhibit both the rel. mol. mass of 24 000; still they can be differentiated in 12.5% polyacrylamide gel run in the presence of submicellar concentration of SDS (Fig. 9). By increasing the detergent concentration to the supramicellar concentration a single zone of the mobility of α_1CB_7 (the faster one) can be observed.

For type II collagen the modification of the gel electrophoresis system runs as follows: 5% separating gel, 3.5% stacking gel for uncleaved (pepsin digested only) α-chains. For CNBr peptides the recommended gel concentrations are 12.5% for the separating and 4.5% for the stacking gel. For CNBr peptides also the two dimensional version was applied, namely using non-equilibrium gradient electrophoresis in the first separation followed by molecular mass based separation in the second dimension (for details see Cole and Chan [53]). Successful separations of type II collagen α-chain was reported in 7.5% gel using the Laemmli system (for type IX α_1, α_2 and α_3 chains the recommended concentration of polyacrylamide is 6.5%). Laemmli [54] electrophoresis has been also used to show that the long believed idea of collagen type IV to be composed of two types of α-chains is not true. Johansson et al. [55] demonstrated five types of collagen α-chains and it was proposed that each of these parent

Table 3
Differences in electrophoretic mobilities between various collagen fractions at zero gel concentration (extrapolated values from Ferguson plots). According to Svojtková et al. [47]

Collagen fractions	Difference in mobility (cm) Gel with SDS	Gel without SDS	Theoretical assumption in multiples of the differences of mobility, α_2 *versus* α_1
α_2 *versus* α_1	1.18±0.20	1.14±0.25	–
β_{12} *versus* β_{11}	1.05±0.20	1.06±0.20	1
β_{22} *versus* β_{11}	2.28±0.20	2.25±0.25	2
γ_{222} *versus* γ_{112}	2.20±0.30	2.38±0.30	2
γ_{222} *versus* γ_{111}	3.30±0.15	3.25±0.20	3

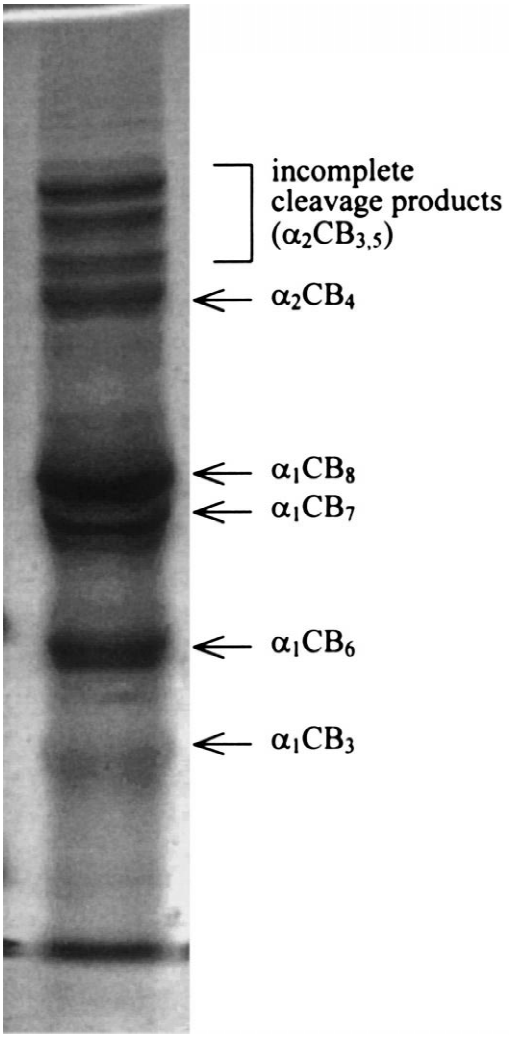


Fig. 9. Separation of CNBr peptides in 12% polyacrylamide gel; note that peptides of identical molecular mass (24.10^3–α_1CB_7 and α_1CB_8).

type IV collagen α-chains forms a specific subclass with a specific chain composition. It was concluded that type IV collagen can be categorised into two subclasses, one containing α_1(IV) and α_2(IV) chains while the other is composed of α_3(IV) and α_4(IV) polypeptide species. The final idea is that between molecules of the same chain composition polymerisation proceeds rather easily while cross-linking between different α-chains is restricted setting thus definite limits upon their collagen type polymerisation.

Type X collagen parent chains were separated by the nearly standard SDS–PAGE Laemmli procedure using 8% polyacrylamide gel under reducing or non-reducing conditions [56]. Type XI collagen of the stoichiometry α_1(XI), α_2(XI) and α_3(XI) represents a minor constituents in type II collagen chain and it is typical in undergoing complex post-transcriptional modifications. For electrophoretic separation 6% separating and 4% stacking gel are used; the sample buffer reported was 125 m*M* Tris–HCl pH 6.8, 10% glycerol, 4% SDS, 10 m*M* dithiothreitol and 0.004% Bromphenol blue. In order to allow for better identification of high molecular mass components the standard acrylamide:bisacrylamide ratio in blotting experiments 1:0.027 was changed to 1:0.006 [57].

Procollagens of type I collagen are accessible to separation in the standard PAGE arrangement (Laemmli) with 5% separating and 3% stacking gels. Two-dimensional separations of α(I) chains were described by Debey et al. [58]. Polyacrylamide gel electrophoresis can serve also for revealing α_1(I) and α_2(I) chains shortened by brief proteolysis (e.g. 5 min at 38°C) or even fragments partly treated with vertebrate collagenase (this approach was applied to reveal aminoacid substitution in a wild type of osteogenesis imperfecta with the conclusion that such replacements function as core sites stabilising the collagen helix). A similar approach of gel electrophoresis of shortened α-chains, typically α_1^S type I from human bone refers to standard conditions used for intact α-chains [59]. It is also possible to separate individual α-chains (shown for collagen type I) in e.g. 5% polyacrylamide gel, cut out the separated zones, use them for cyanogen bromide cleavage and separate the CNBr fragments in the second dimension on a 12.5% gel. For detailed description of this kind of procedures see Refs. [60,61]. The method described by Bonadio et al. [61] ran as follows: collagen proteins were separated in the first dimension on 5% SDS–PAGE under reducing conditions followed by the separation of CNBr peptides as described in the legend to the Fig. 10. Alternatively, CNBr peptides could be mapped in a two-dimensional system which employed isoelectric focusing in the first dimension. Collagens were incubated for 5 h at 37°C in 70% formic acid that contained CNBr, 20 mg/ml, under N_2 atmosphere. To terminate the reaction, samples were diluted with

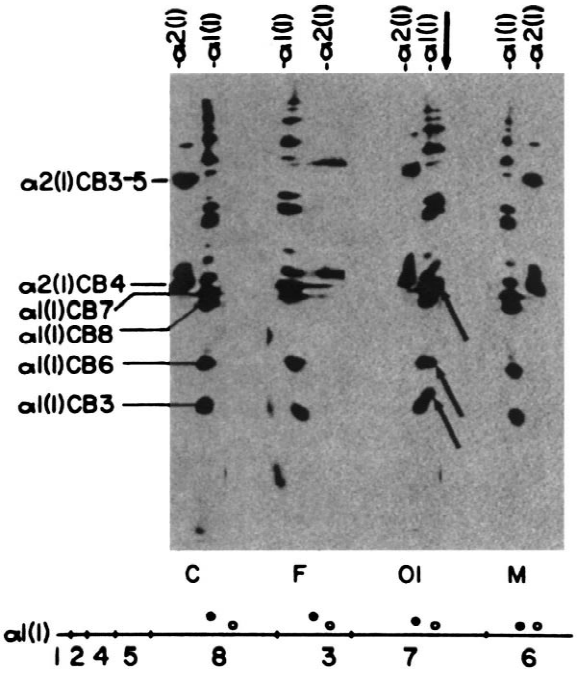


Fig. 10. Autoradiogram of medium collagens synthesized by osteogenesis imperfecta, parental, and control cells after cleavage with cyanogen bromide. For CNBr peptide mapping, α chains from medium (after pepsin) were separated in the first dimension on 5% SDS–PAGE, the lanes were cut out and proteins digested in the gel with CNBr, and the CNBr peptides were separated by SDS–PAGE in the second dimension. A schematic diagram of the α1(I) chain with its CNBr peptides indicated by numbers is presented at the bottom of the figure. The relative electrophoretic mobilities of of α(I) CNBr peptides from normal α1(I) chains (○) and overmodified α1(I) chains (●) is shown. Reprinted from Bonadio et al. [61] with permission.

water and lyophilized (×2). The peptides were dissolved in 4 *M* urea, 2.5% Triton X-100, and 4% ampholines and then separated by isoelectric focusing on 2×115 mm cylindrical polyacrylamide gels. The gels were equilibrated after electrophoresis in 0.5% ammonium persulfate and then sample buffer lacking SDS and urea then overlaid on 12.5% gels that contained no urea. Second-dimension electrophoresis was performed as described above. Procedures like these have been successfully applied to detect the biochemical background of some inherited diseases. Typically in lethal osteogenesis imperfecta an additional cysteine residue is present in the α_1(I)CB_6 cyanogen bromide fragment which results in partial recovery of α_1(I) chains in the form of disulfide bonded dimers [62].

Polyacrylamide gel electrophoresis combined with immunoblotting is widely used in protein electrophoresis to improve detection specifity. It is not surprising that examples of this technique can be found also in the area of collagen separation. For instance identification of α_{1A} and α_{1B} chains of collagen type XII and α_1 collagen chain and its dimer from collagen type XIV can be done in this way [63].

Two dimensional polyacrylamide gel electrophoresis has been applied to reveal a new form of tumour and foetal collagen typical in its capability to bind laminin. Non equilibrium polyacrylamide gel electrophoresis followed the procedure of O'Farell et al. [64] using pH 3–10 and 5–7 ampholytes with 4% gel. Denatured samples were investigated. In foetal and tumour tissue acidic components were found which upon isolation from the 2 D gels and CNBr cleavage gave a pattern differing in at least two fractions of apparent mol. mass between 24 and $29 \cdot 10^3$. It is noticeable that α_1(I)CB_6, α_1(I)CB_8 and α_1(I)CB_7 peptides were completely absent in the acidic components [65].

SDS gel electrophoresis (6.5%) under reducing conditions has been used also for revealing chain heterogeneity in type VI collagen from confluent cultures of nuchal ligament fibroblasts. 35-S methionine labelled medium proteins were concentrated by precipitation with $(NH_4)_2SO_4$ to 30% saturation (fluorographic detection). There were two interesting observations with this experiment, namely that the α_1(VI) and α_2(VI) species were more abundant in the skin samples investigated, while ligamentum nuchae samples were consistent with the idea of a heterotrimeric structure and, second, that in spite of being different in their primary structure they comigrated in the gels used (rel. mol. masses of 140 000 for $\alpha_1+\alpha_2$(VI), 260–280 000 for α_3(VI). It has, however, to be borne in mind that type VI monomer contains a relatively short triple helical region (105 nm) with large globular domains at both its C- and N-end [66]. The three polypeptides representing the major constituents of type VI collagen from bovine lens capsule can be separated by standard Laemmli procedure and identified by elec-

trotransfer blotting and immunoreaction according to the procedure described by Kyhse-Andersen [67].

For gel electrophoretic examination of collagen type XIII the samples were boiled with SDS–PAGE sample buffer (with or without reduction with 10 m*M* 2-mercaptoethanol) followed by electrophoresis and transferred onto nitrocellulose membranes [68]. The anti type XIII collagen antisera and mouse anti-human β-1 integrin antibodies were applied at dilution 1:1000 to the filters, and the affinity purified antibodies were used at concentration 5 μg/ml. The filters were washed thoroughly after 1 h of primary antibody incubation at room temperature, and then incubated with horseradish peroxidase conjugate anti-rabbit or anti-mouse secondary antibody at a dilution of 1:5000–1:10 000. The immunosignal was detected after washing using the enhanced chemiluminiscence system and film (Amersham Pharmacia Biotech.).

SDS gel electrophoresis in dilute gels (4%) can be used also to reveal α-chain aggregates. Crude collagen preparations display two α, two β and two γ-bands (which are believed to represent intramolecularly cross-linked α-chains). In addition four other bands with rather slow mobility (slower than the γ-bands) were ascribed to the presence of intermolecular cross-links [69,70].

Polyacrylamide gel electrophoresis is also applicable for tissue collagenase activity assays; an easily applicable version is that published in [71]. BioRad mini protean system was used in this case with 10% polyacrylamide gels of 10 cm high and 1.5 mm thickness. Duracryl from Millipore (containing 1 mg/ml gelatine or 0.2–1 mg/ml collagen) was dispersed in buffered solution containing 2.5 ml gel, 1.5 *M* Tris–HCl pH 8.8, 100 μl of 10% SDS, 4 ml polyacrylamide in 0.125 *M* Tris pH 6.8. The gels were polymerised by adding 50 μl of 10% ammonium persulfate and 10 μl 0.1% TEMED. Samples were diluted half in 1 *M* Tris pH 6.8 (containing 50% glycerol and 0.4% Bromphenol blue). The very separation followed the Laemmli conditions. After the electrophoresis was brought to an end the gels were washed twice in 200 ml of 2.5% Triton X100 (300 ml each) and incubated in 100 m*M* Tris–HCl, 5 m*M* $CaCl_2$, 0.005% Brij, 0.01% NaN_3 pH at 37°C for 6–48 h. Gels were then stained with Coomassie Brilliant Blue G 280 (50% methanol, 10% acetic acid) and destained in 40% methanol, 10% acetic acid. Proteinase activity was revealed as cleared (unstained) zones.

4.2. Micropreparative gel electrophoresis

A simple method for semipreparative (microscale) isolation of type I, II, III and V α-chains by sodium dodecyl sulfate electrophoresis followed by electroelution was developed by Acil et al. [72]: 4% polyacrylamide gels were used (6% for the stacking gel) and the very electrophoresis was run in 63 m*M* Tris, 2% SDS and 10% glycerol containing run buffer which was made 0.01% with respect to bromphenol blue (pH 6.8). Following the electrophoretic separation the protein bands were blotted on PVDF [poly(vinylidene difluoride)] Immobilon P membrane 0.45 μm pore size. The gels were washed in a transfer buffer (40 m*M* 6-amino-N-hexanacid, 5% methanol, 0.1% SDS pH 7.2) for 5 min (to reduce the amount of bicine and Tris. During this period the PVDF membrane was rinsed with 100% methanol for 2 min and stored in deionized water. The gels were sandwiched between a sheet of PVDF membrane and several sheets of blotting paper and assembled in the blotting apparatus. Blotting was done for 2.5 h at 100 mA (4°C). After electroblotting the Immobilon (PVDF) membrane was washed with deionized water for 5 min and stained with 0.1% Coomassie blue in 50% methanol for 5 min followed by destaining in 50% methanol–10% acetic acid for 5–10 min and air dried. The blotted protein bands were cut off, pooled in a test tube, soaked in 1 ml elution buffer (50 m*M* Tris, 2% SDS, 1% Triton X-100, pH 9) and shaken overnight. After elution of the α-chains the membrane slurry was removed by filtration (30 000 rel. mol. mass cut off) and centrifuged.

For electroelution the protein bands visualised by Coomassie Brilliant Blue were cut off, placed into an Eppendorf tube, soaked in the defixing buffer (6 m*M* urea, 192 m*M* bicine, 25 m*M* Tris, 0.2% SDS and 5 m*M* dithiothreitol) for 0.5 h at room temperature to bring the collagen α-chains into solution. The slices were then equilibrated in electrophoresis buffer (192 m*M* bicine, 25 m*M* Tris and 1.73 m*M* SDS pH 8.5) for 5 min, cut into small pieces and placed into an electroelution glass tube; electroelution was done at

10 mA per tube for 2 h at room temperature. The different α-chains were transferred from the gel slice through a frit into the membrane cap and retained by dialysis (15 000 rel. mol. mass cut off).

4.3. Detection of collagen zones by immunoblotting

Localisation of the individual collagen zones to be eluted from the polyacrylamide gel can be done by the blotting technique. To increase specificity of detection, immunoblotting procedures are gaining popularity, mainly because there is no chance to distinguish e.g. between individual collagen polypeptide α-chains by direct staining procedures.

Immunoblotting has been described in detail for type II, V and XI collagens by Ricard-Blum et al. [73] and Hartmann et al. [74]. Native collagen molecules were transferred to nitrocellulose (0.45 μm pore size, Schleicher and Schuell, Dassel) and were blotted at 0.4 A for 25 min with 0.7% acetic acid as transfer buffer.

A more detailed protocol has already been reported originally by Hartmann et al. [74]. After transfer, the nitrocellulose membrane was incubated for 1 h at 37°C to prevent non-specific binding, and probed overnight with the respective collagen type polyclonal antibody diluted 1:50 in phosphate-buffered saline–3% bovine serum albumin. Immunodetection was achieved using a biotinylated secondary antibody using avidin-biotinylated horseradish peroxidase complexes. 4-Chloro-1-naphthol was added as colour substrate.

4.4. Capillary electrophoresis

Successful separation of proteins by capillary electrophoresis stimulated attempts to use this technique also for the separation of collagens and their fragments. In spite of the wide perspectives only two papers of other authors have been published to our best knowledge so far [75,76]. Though attempts to separate different collagen constituting α-chains [77] have been quite successful at least as far as the qualitative analysis is concerned, it turned out soon that direct quantitation of parent α-chains of fibrous forming collagens suffers from strong interactions between the inner surface of bare silica capillary and the analytes involved. Therefore for quantitation of individual collagen types evaluation by the presence of CNBr marker peptides is preferred [78]. The advantage of this approach is further stressed by the fact that insoluble collagens present in tissues can be released (and assayed) by CNBr cleavage.

4.4.1. Separation of parent α-chains

Successful separations of collagen α-chains can be obtained (i) in very diluted buffers (typically 2.5 m*M* sodium borate pH 9.2) [77], (ii) in acid buffers (pH~2.5, about 25 m*M* buffers), (iii) in the presence of a neutral surfactant in the sample (but not in the background electrolyte) [80] or (iv) in acid buffers of moderate ionic strength (pH 2.5; ~75 m*M* buffers) in the presence of copolymeric liquid crystals (Pluronic F-127) [79] in the run buffer.

In an early investigation [77] it was demonstrated that collagen type I, II, V, IX and XI constituting α-chains and their chain polymers can be separated in 2.5 m*M* sodium tetraborate buffer at pH 9.2 in less than 15 min. in a 50 cm×100 μm fused-silica capillary. The separations are relatively insensitive to the voltage applied. Although separations were routinely made at 18 kV per 50 cm capillary, satisfactory results were obtained at 10–15 kV as well; naturally the run times were proportionally increased. On the other hand these separations are extremely sensitive to capillary overloading. This should be positively avoided as otherwise poorly reproducible electropherograms are obtained. It was argued that one of the reasons may be the length of the sample applied. In the view of later results it is clear that the main reason for such irregularities is the protein adherence to the capillary wall [80]. Clusters of adhering protein are under such circumstances released at a later stage of the separation owing to the endoosmotic flow, and irregular spikes emerge (as proven by separating model peptides possessing sequences similar to those found in collagens, Fig. 11; if however the amount of the sample applied is kept within specific limits and if the separation is run at pH sufficiently high (9.6) these effects can be abolished. Selectivity with respect to the separation α_1 and α_2 chains is considerably influenced by the presence or absence of (submicellar) concentration of SDS in the background electrolyte (Fig. 12). The separation of

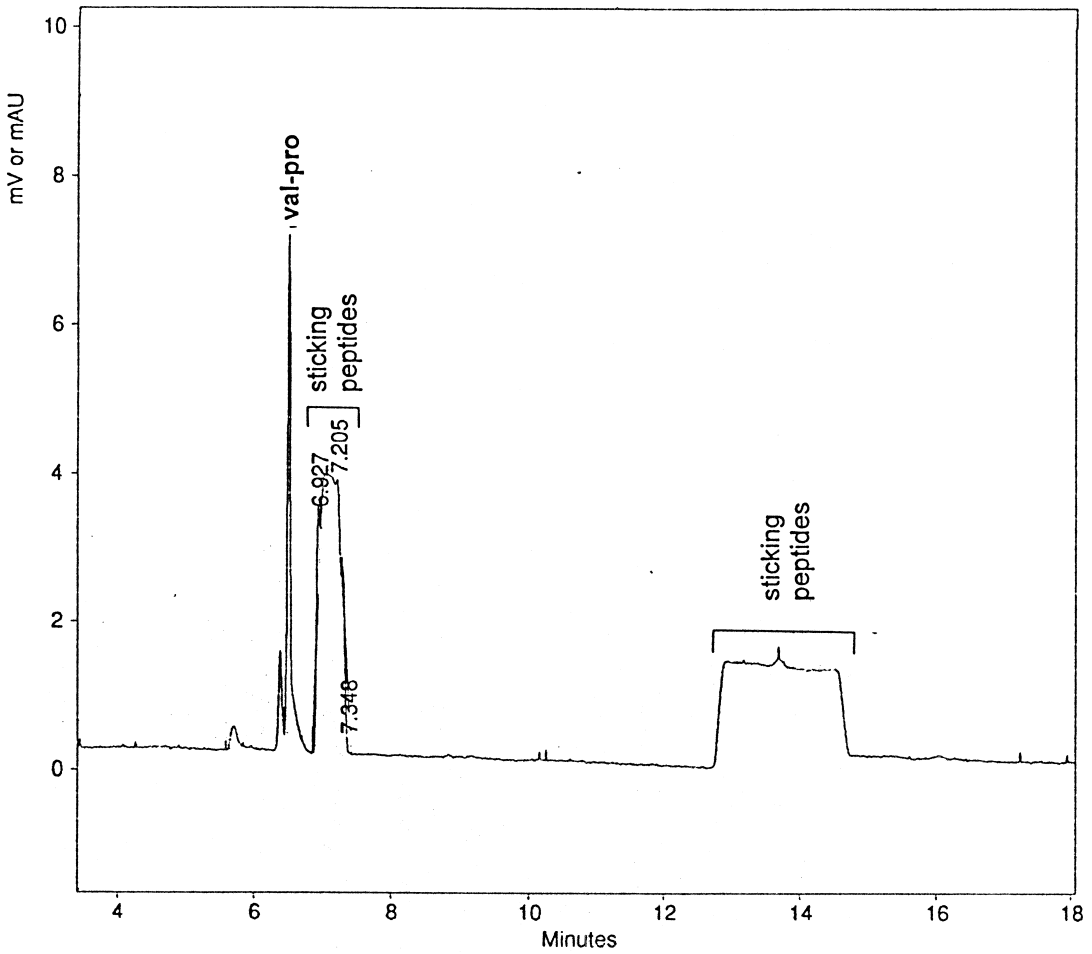


Fig. 11. Capillary electrophoretic separation of a mixture of three peptides (pro-ala, val-pro and pro-gly-gly) at pH 10.5 in 25 mmol/l sodium phosphate buffer (10 mmol/l with respect to Brij). Note the large flat zones of increased absorbancy representing step-wise elution of peptides strongly bound to the capillary wall. Reprinted from Hamrníková et al. [80].

collagen type I into α-, β- and γ-chains can be obtained with phosphate buffer of the same pH, but all attempts to separate further α_1 from α_2 chains and β_{11} from β_{12} chains were unsuccessful. A similar effect is seen if the background electrolyte is made 0.5 m*M* with respect to SDS; also here it is possible to see the peaks of α, β and γ fraction only. As turned out later this separation requires strictly the submicellar concentration of the detergent: at higher (supramicellar) concentrations of the negatively charged surfactant all fractions migrate as a single broad zone. At the early stages of collagen separations by capillary electrophoresis it was proposed that the separation of a collagen sample into α-, β- and γ-fractions reflects primarily hydrodynamic conditions based on the size of random coils of the parent α-chains and their polymers while the more delicate separation capable of distinguishing α_1 and α_2 chains (and consequently β_{11} and β_{12} chain dimers) is governed by delicate charge differences which, however, can manifest themselves only under optimised separation conditions. Separation of individual species of constituting α-chains of collagen type II, V, IX and XI is also possible (Fig. 13). These results imply, however, that the separation of collagen α-chains and their chain polymers should be

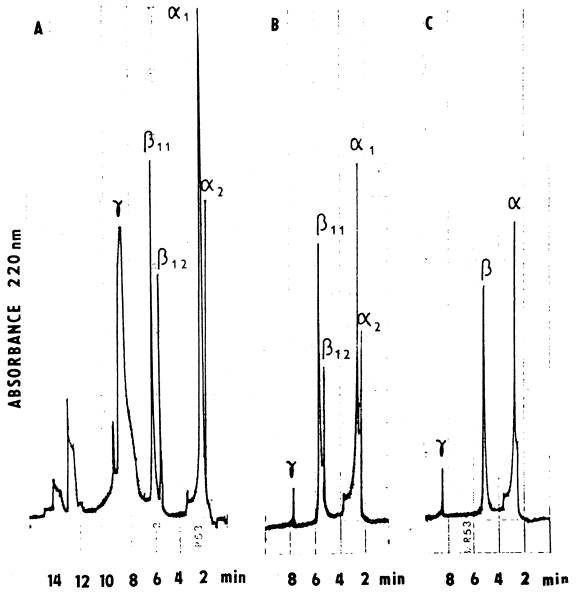


Fig. 12. Capillary zone electrophoretic analysis (2.5 m*M* sodium tetraborate buffer, pH 9.2) of (A) insoluble collagen solubilized under denaturing conditions (42°C, 30 min), (B) isolated collagen type I and (C) same sample as in B but run in the presence of 0.5 m*M* SDS. Reprinted from Deyl et al. [77].

positively influenced by adding a polymer with molecular sieving properties to the run buffer.

As shown in Fig. 14, SDS electrophoresis in capillaries containing linear polyacrylamide offers separations of constituting α-polypeptide chains as well as their polymers which are at least comparable with slab gel electrophoresis [81]. Moreover this approach offers the possibility to quantitate the area percentage of individual peaks, a procedure which with slab gel separations is less convenient.

Moreover capillary electrophoresis in non-cross-linked gels offers the possibility of separating chain polymers of rel. mol. mass 300 000 and higher. This is of considerable importance because no method offering sufficient sensitivity is available for analysing such polymers (as a matter of fact in most cases these emerge as a single broad peak). In diluted slab gels these can be separated as shown in Fig. 8; chromatographic methods don't offer sufficient selectivity.

The practical applicability of electrophoresis in linear polyacrylamide gels is shown in Fig. 15, where the separation of a glycated collagen sample is shown. There are two features in which such profiles

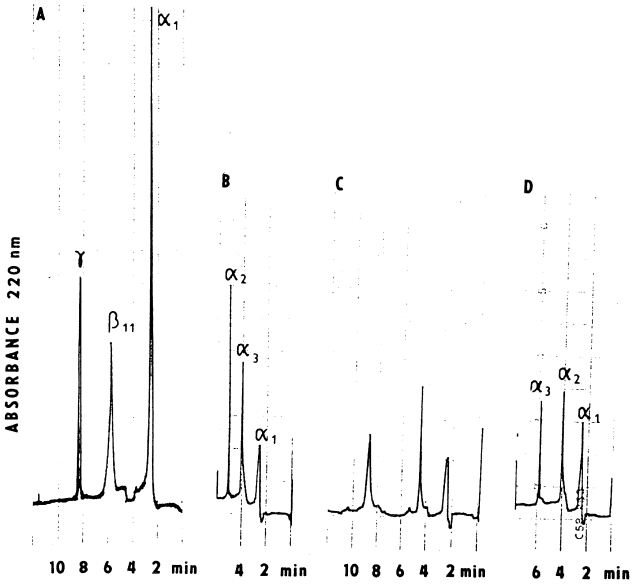


Fig. 13. Capillary zone electrophoretic separation (2.5 m*M* sodium tetraborate buffer, pH 9.2) of collagens (A) type II, (B) type V, (C) type IX and (D) type XI. Reprinted from Deyl et al. [77].

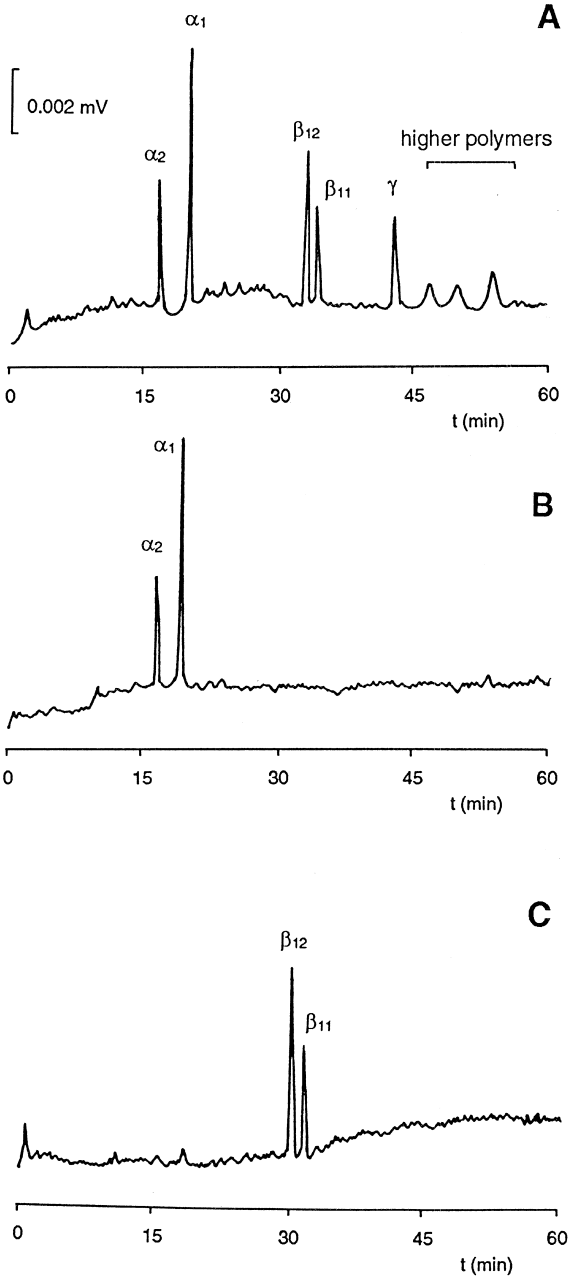


Fig. 14. Capillary gel electrophoresis profiles of collagen chains (4% polyacrylamide, Tris–glycine buffer pH 8.8). (A) Complete sample, (B) α-region proteins sampled from a preceding slab gel run, (C) as (B), but β-region excised from slab gel. Slab gel electrophoresis is shown on top of the figure (cathode on the right side of the gel). Reprinted from Deyl and Mikšík [81].

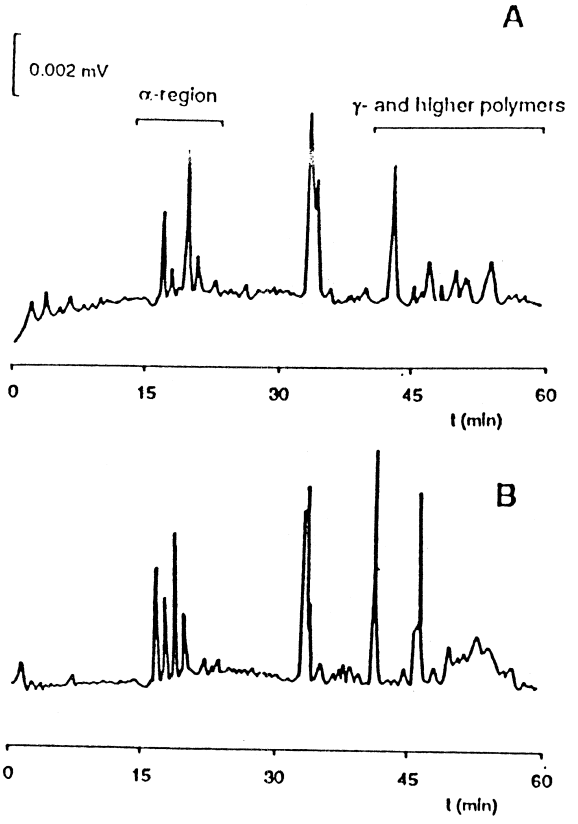


Fig. 15. Capillary gel electrophoresis profiles of collagen chains (4% polyacrylamide, Tris–glycine buffer pH 8.8) after incubation with glucose for 4 days (A) and 7 days (B). Reprinted from Deyl and Mikšík [81].

differ from untreated collagen electropherograms, namely in splitting the α-peaks into two each, and in the increased peaks of the γ-chain polymers and higher. Both these effects can be ascribed to the interaction of free lysine amino groups with glucose. It has been shown by Reiser et al. [46,82] that such interactions are non-specific, affecting several lysine residues in the collagen molecule.

Based on the above observation it is feasible to assume that splitting of the two α-chain peaks into four upon incubation may reflect monotopical binding of glucose to several sites along the collagen α-chain, which should result in a decrease of positive charges of these polypeptide chains (it does not exclude changes in the hydrophobic properties of the molecules involved and possibly some conformational alterations of the polypeptides as well). The higher

proportion of γ- and higher α-chain polymers could be explained on the basis of glucose-polymerising reactions, which eventually lead to the decreased solubility of the treated collagen samples (see [46,82]).

To explain splitting of the α-chain corresponding peaks in more detail is at the moment difficult. The anomalous behaviour of collagen α-chains in slab gel electrophoresis has been already discussed (see above). Glycation of collagen α-chains not only decreases the number of positive charges per α-chain, but also introduces a relatively large sugar molecule into the structure which may further alter the number of ionizable side chain groups (OH functionalities in the sugar moiety) and/or change the hydrophobic properties of the α-chain. It is also not clear whether or not glycated and non-glycated collagen α-chains bind the same amount of SDS; any alteration in this respect will result in changes of the electromigration properties of the protein molecules in the polyacrylamide gel matrix. It has been documented that at low gel concentrations (below 7.5) considerably higher molecular masses are revealed by Ferguson's plots for glycoproteins if the gels are calibrated with the standard protein sets and such effects can be expected for glycated proteins as well [46,83].

Though electrophoresis in linear polyacrylamide gels is well acceptable and offers results superior to all other methods, it still suffers one considerable disadvantage, namely the gels don't survive more than five runs, before being clogged and a systematic error in decreasing the peak sizes was observed with subsequent runs even if the separations were run with heat denatured samples and at elevated temperatures (50°C) to minimise renaturation and possible association of individual α-chains into higher polymers.

It has been mentioned already several times that also hydrophobic interactions with the capillary wall may contribute to the successful separation of collagen constituting chains. The evidence that it is indeed so, came from the experiments in which collagen chain fragments released were separated by capillary electrophoresis in bare silica capillaries at pH 2.5 (separations of parent collagen α-chains at this pH is impossible as no peaks can be seen in the detector window in within 1.5 h run time) and comparing these profiles with those obtained by standard reversed-phase chromatography (for more details see the part on CNBr peptide separations). These profiles exhibit a striking similarity: the larger peptides migrate more slowly than the smaller ones. This was interpreted in such a way that longer CNBr peptides, owing to the large internal homogeneity in the collagen polypeptide chains should possess more hydrophobic domains than the small ones and if it is the hydrophobic interactions that are involved in the partition mechanism the larger peptides must in electrophoresis come later before the detector's window. To bring this mechanism to usable results very delicate optimisation is needed (otherwise all solutes emerge in a single peak). Encouraged by the work of Quirino et al. [84] (on stacking of reverse moving micelles and a water plug and by the work of Lloyd and Wätzig [85] who showed that introducing an SDS wash can effectively remove all peptides/proteins sticking to the capillary wall) we finally developed a system in which no detergent was present in the background electrolyte but the sample was dissolved in the background buffer containing 33 m*M* Brij. As shown in Fig. 16 a complete separation of α_1 and α_2 chains, β and γ polymers was obtained.

The results reported so far bring about strong evidence that in capillary electrophoresis of parent collagen α-chains and chain polymers the partition mechanism involves both ionic and hydrophobic interactions and bears all the features of a mixed-mode separation process. As a matter of fact because the separation exploits also the properties of the capillary wall, it can be considered a special case of electrochromatography in open tubes (capillaries). Taking this into consideration it can be predicted that a molecular sieve capable of exerting synergistic hydrophobic and molecular sieving effects would offer good separations: such separations can be materialised by using e.g. Pluronic liquid crystals (Pluronic copolymers exhibit the general formula $[\text{poly(ethylene oxide)}]_x$ $[\text{poly(propylene oxide)}]_y$ $[\text{poly(ethylene oxide)}]_x$ [86]). Basically these copolymers are surfactants, which associate into large micelles; this self-association is favoured by increased concentration and temperature. The less polar poly(propyleneoxide) chain segments are desolvated and segregate into a hydrophobic core

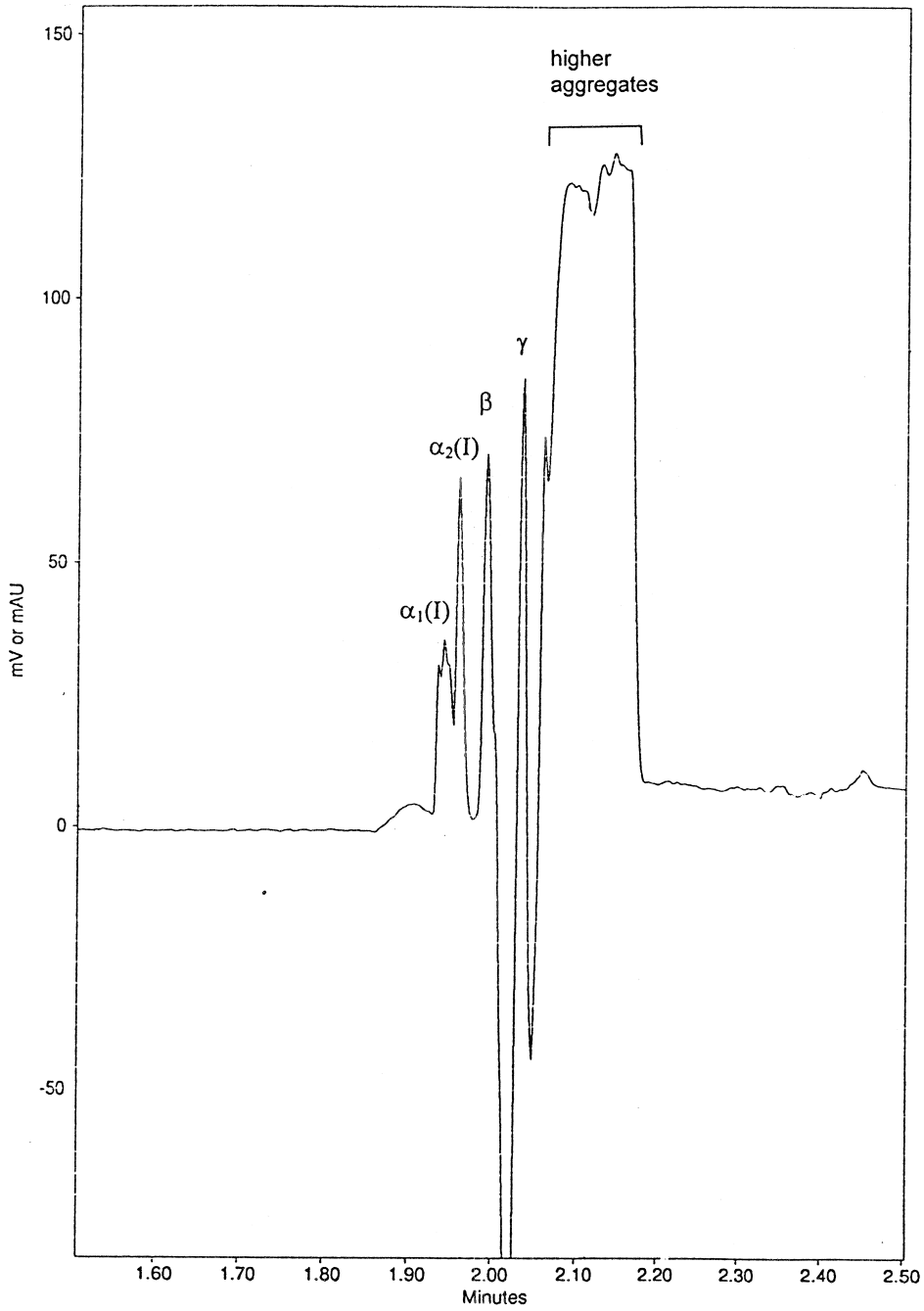


Fig. 16. Capillary electrophoretic separation of collagen type I. Constituting α-chains, α_1(I) and α_2(I), their dimers (β) and trimers (γ) along with high molecular mass aggregates. These aggregates disappear if the sample is shortly boiled (10 min) before application and if the sample is run at 50°C. Corresponding increase of α, β and γ-chains was observed. Sample applied in 33 mmol/l Brij, background electrolyte 25 mmol/l sodium phosphate at pH 10.5. Reprinted from Hamrníková et al. [80].

surrounded by highly hydrated poly(oxyethylene) chains. Indeed with collagen type I intact α-chains reasonable separations were possible [79].

5. Specific features of collagen CNBr fragments separation

Electrokinetic separation of CNBr peptides of different collagen types has been described in the section on gel electrophoresis which differs from systems used for the separation of intact α-chains in the gel concentration. In capillary electrophoresis the situation is different.

While capillary electrophoresis of intact α-chains of collagen can be under specified conditions materialised in both acid and alkaline pH, separation of collagen CNBr fragments is possible at acid pH only. Separations at pH 2.5 result in a complex profile in which small-molecular mass peptides are well resolved with short migration times. It was reported [78] that the experimental conditions may vary considerably; for instance any buffer concentration between 25 and 100 m*M*, temperature between 25 and 50°C and voltage between 10 and 25 kV all give still satisfactory results. A typical electropherogram is presented in Fig. 17. However, the increase of migration time with increasing molecular mass holds for peptides with rel. mol. mass 13 500 (149 amino acids) and larger only. Peptides having rel. mol. mass less than 4600 did not follow the rel. mol. mass-migration dependence. The same holds (or is even more distinct) with the low molecular mass peptides from type III and type V collagens (typically α_1(III)CB_2, 40 AA residues, α_1(V)CB_1, 54 residues). Fig. 17 shows collagen type I CNBr peptides profile spiked with α_1(I)CB_1, α_1(III)CB_2 and α_1(V)CB_1. As long as the peptides separated are pure and they occur in each collagen type only once per molecule, they can be used as quantitation markers (spiking was done on the level that corresponded to the expected concentration of other than type I collagen in natural samples).

In the original paper of Deyl et al. [78] a BioRad pH 2.5 buffer was used the composition of which was not disclosed at the time when the paper was published, but it was claimed that it contained a molecular sieving polymer. Later this turned to be hydroxymethyl cellulose, however, it was demonstrated that if the separation is run in a plain 25–50 m*M* phosphate buffer, the separation is practically the same as shown with the commercial buffer. The only difference reported was that the large α_2(I)$CB_{3,5}$ peptide (resulting from an incomplete cleavage) did not emerge within 90 min of running time. The separations run in 50 m*M* buffer lasted slightly over half-an-hour, the marker peptides emerging between 32 and 38 min, however, for this rather short running times it was necessary to dilute the background electrolyte to 25 m*M* phosphate concentration.

The results can be expressed as the proportion of a given collagen type (I, III and V), based on area changes of the marker peptides using them as internal standards. The proportion of individual collagen types results from peak areas of α_2(I)CB_4, α_1(III)CB_2 and α_1(V)CB_1 peptides. The applicability of this approach is demonstrated in Table 4 were the proportions of the three collagen types in various tissues obtained by the method described are compared with literary data. Spiking can be done either with purified peptides or with CNBr digests of accompanying collagen types. Obviously the latter approach simplifies the method considerably as it does not require tedious purification of spiking peptides.

The described separation of CNBr fragments of parent α-chains of fibre forming collagens by capillary electrophoresis appears satisfactory for quantitating marker peptides, however, the separation is incomplete with other members of this peptide family. There are the same types of interactions involved as with the complete α-chain polypeptides, namely ionic forces and hydrophobic interactions. The profiles obtained with CZE and reversed-phase chromatography are very much alike, supporting thereby the idea of the crucial role of hydrophobic domains.

To get a deeper insight into the mechanisms governing partition of glycine and proline/hydroxyproline rich peptides it is, perhaps, worth while to focus on model peptides of this type. As reported recently [79] there are several possibilities how to improve such separations, namely (i) by adjusting carefully the pH of the background electrolyte, (ii) to decrease the polarity of the run buffer by using the

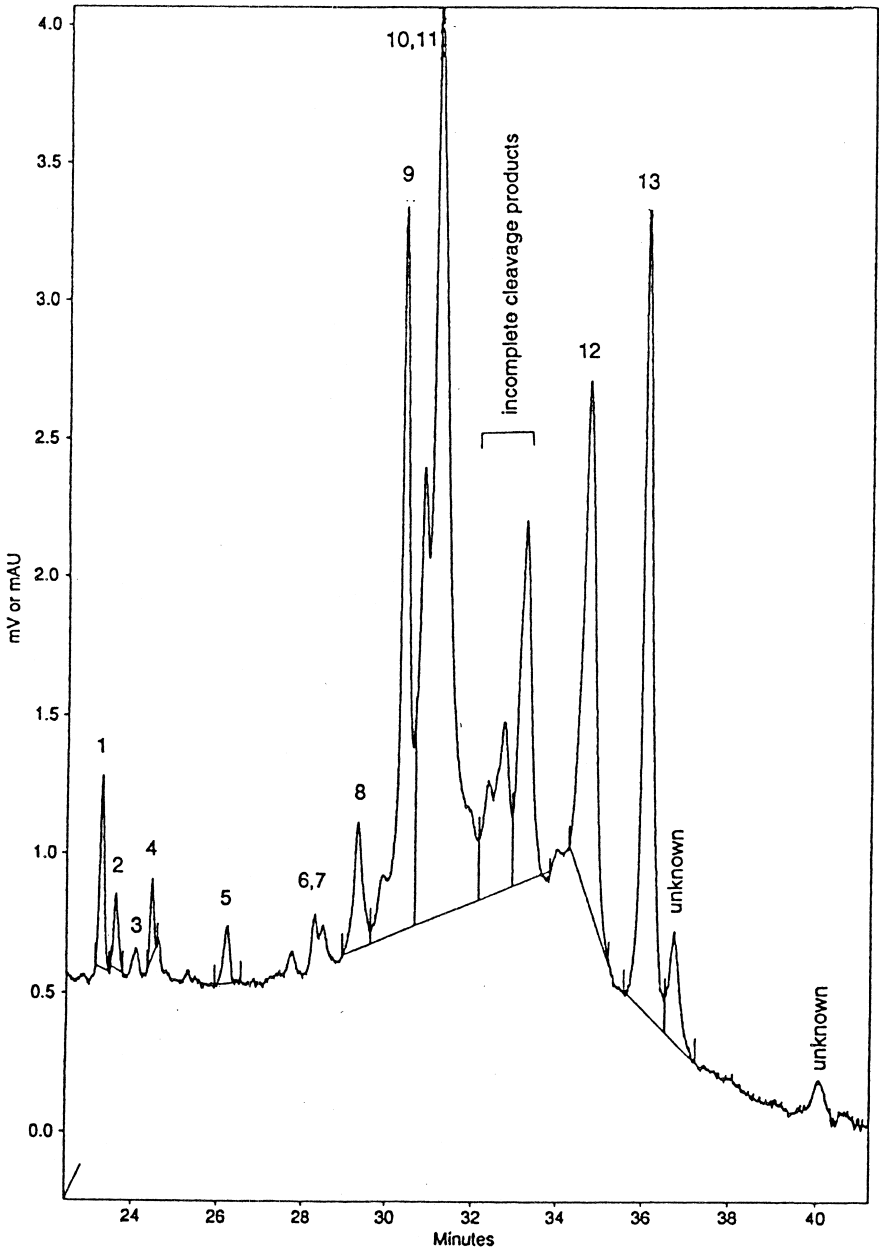


Fig. 17. Separation of collagen type I CNBr peptides obtained from a commercial preparation (Sigma) spiked with α_1(V)CB_1 and α_1(III)CB_2. Background electrolyte used was Bio-Rad pH 2.5 phosphate buffer (with polymeric modifier) diluted 1:1 with Milli-Q water. Peak identification: 1-α_1(I)CB_2, 2-α_1(I)CB_4, 3-α_1(V)CB_1, 4-α_2(I)CB_1, 5-α_1(III)CB_2, 6-α_1(I)CB_5, 7-α_2(I)CB_2, 8-α_1(I)CB_3, 9-α_1(I)CB_6, 10-α_1(I)CB_7, 11-α_1(I)CB_8, 12-α_2(I)CB_4, 13-α_2(I)$CB_{3,5}$. Reprinted from Deyl et al. [78].

addition of organic solvents (acetonitrile, methanol), (iii) by exploiting the hydrophobic domains of the separated entities using a surfactant containing run buffer (Fig. 18) [88]. The best separation of type I collagen cyanogen bromide peptides has been obtained with 7.5% Pluronic F127 in Tris–phosphate

Table 4
The proportion of collagen type I and III in artificial mixtures as determined by capillary zone electrophoresis (50 m*M* phosphate buffer pH 2.5). According to Deyl et al. [78]

Test mixture			Reveal after spiking with collagen type I and III CNBr peptide mixture			After spiking with α1(I)CB2+ α1(I)CB4 equimolar mixture for collagen type I and α1(III)CB2 for collagen type III			After spiking with α2(I)CB4 for collagen type I and α1(III)CB2 for collagen type III		
Collagen type I (μg/ml)	Collagen type III (μg/ml)	Collagen type III (%)	Collagen type I (μg/ml)	Collagen type III (μg/ml)	Collagen type III (%)	Collagen type I (μg/ml)	Collagen type III (μg/ml)	Collagen type III (%)	Collagen type I (μg/ml)	Collagen type III (μg/ml)	Collagen type III (%)
400	0	0	420	0	0	430	0	0	420	0	0
300	40	10	378 (±20)	54 (±3)	12	381 (±15)	38 (±7)	9	387 (±17)	42 (±6)	9
320	80	20	311	75	19	308	87	22	296	89	23
280	120	30	273	134	32	285	122	29	280	128	31
240	160	40	237	155	39	262	158	37	259	165	39
200	200	50	190 (±17)	197 (±12)	50	187 (±21)	203 (±18)	52	179 (±23)	204 (±12)	53

buffer (10 and 75 mmol/l respectively) at pH 2.5 (Fig. 19). Pluronic liquid crystals exhibit simultaneously a molecular sieving effect and act as surfactants as well (they are rich is spatially arranged hydrophobic domains) which leads to a smart synergistic effect of these two properties and facilitates separation of some peptides in the set (Fig. 19) [79]. However, as demonstrated by the comparison of a profile obtained by reversed-phase chromatography, some peaks are displaced indicating that the separation mechanism is quite complex. Obviously there are considerable differences in selectivity not only between HPLC and different electromigration methods but also within the set of electromigration separations as well. The critical set of peptides to be separated are those of rel. mol. mass around 20 000.

An additional possibility is to use a surfactant plug that would prevent adsorption of the peptides/proteins to the capillary wall and establish partition equilibrium between the capillary wall and the passing by micelles in the pseudophase. The latter speculation was based not only on the early comparison with reversed-phase chromatography but also on the fact that either hydrophobic coating of the capillary or using SDS micelles in the run buffer prevented binding of the peptides/proteins to the capillary wall.

With the short glycine and proline containing dipeptides the electrophoretic behaviour is rather simple [80]. Switching from acid to alkaline pH the order of migrating peptides is reversed following their pKa values; no separation is observed at the intermediate pHs. Optimum separation can be obtained either at pH 2.5 or pH 8.0 in 25 mmol/l phosphate buffer at 15 kV. Addition of either 10% acetonitrile or 20% methanol to the pH 8.0 buffer results in a complete separation of the three members of the test set. While addition of bases (hexylamine or triethylamine) leads only to a partial improvement (decreased sticking of the gly–gly dipeptide at alkaline pH), making the background electrolyte 5 mmol/l with respect to cetyltrimethylammonium bromide offers another possibility how to separate the tested peptide mixture (reversed polarity mode).

For more complex peptide mixtures, however, the above rather traditional approaches are not applicable. The best results were obtained at alkaline pH (10.5, 25 mmol/l phosphate buffer) if the sample is dissolved in the presence of a detergent (17.5 mmol/l Brij 35 or 33 mmol/l SDS). If either detergent is present in the background electrolyte, depending on the surfactant concentration the analytes emerge in a single peak (high concentrations) or are separated in more (two) broad sections (plateaus). The separation of the 10 component test mixture was never complete, however the selectivity can be adjusted by manipulating the pH of the background electrolyte or by changing the surfactant used. Generally better results were obtained with Brij 35 as compared to SDS. The main profit of these experiments is visualised in the fact that by dissolving proline and glycine rich proteins, typically α-chains of collagen and their polymers, separation not only of the α_1 and α_2 chains but also of their dimers, trimers and higher

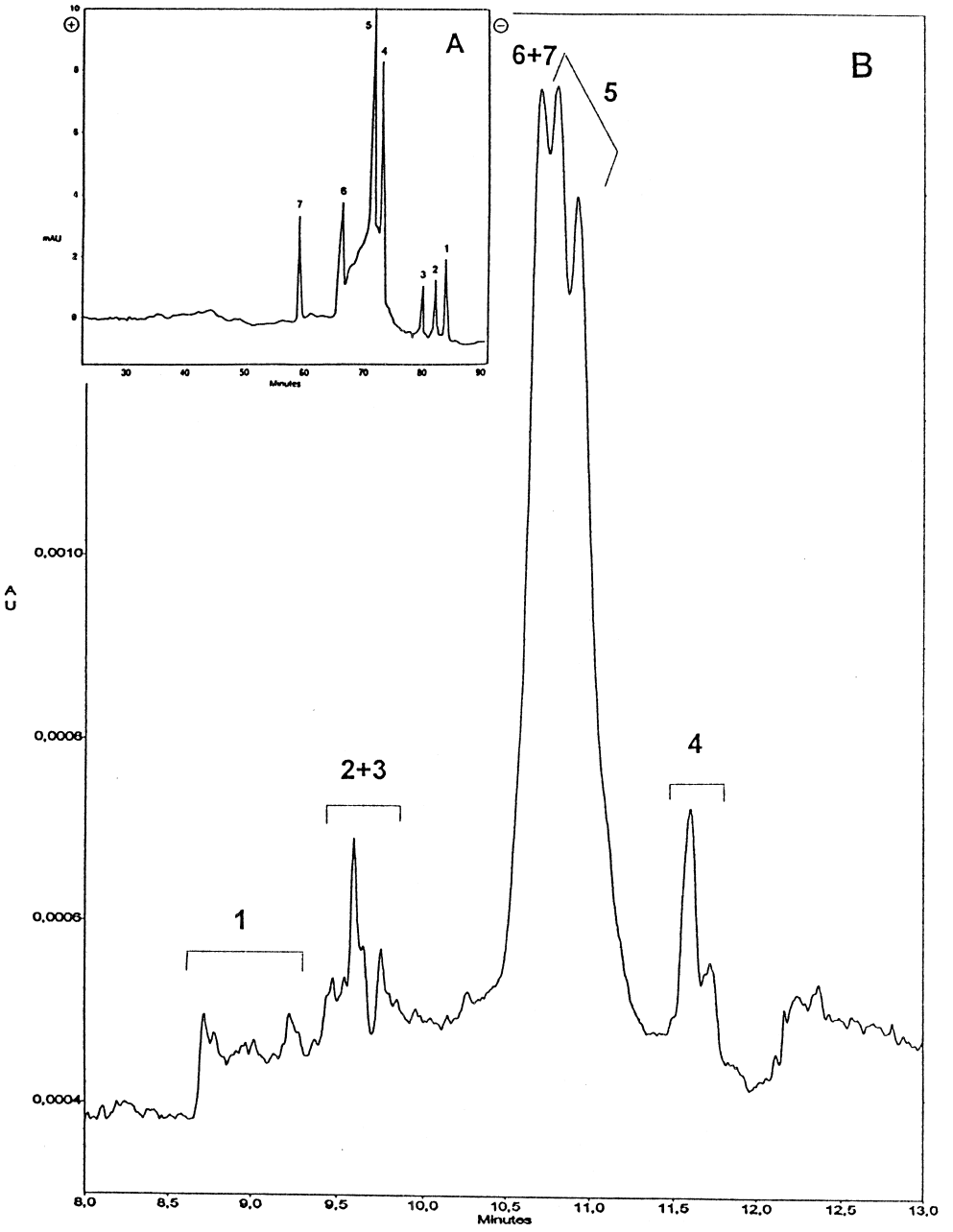


Fig. 18. Capillary electrophoretic separation of rat tail tendon collagen CNBr peptides in the presence of 50 mmol/l SDS in 50 mmol phosphate buffer (pH 2.5). Insert: the same sample run in submicellar detergent concentration (0.1%≈3.5 mmol/l SDS). Both runs in reversed polarity mode. Note changes in the peak sequence when high surfactant concentration in the background electrolyte is used (insert taken from our previous report – see Ref. [87]). Peak identification: **A**: 1, α_1(I)CB$_2$; 2, α_1(I)CB$_4$; 3, α_1(III)CB$_4$; 4, α_1(I)CB$_6$; 5, α_1(I)CB$_7$+α_1(I)CB$_8$; 6, α_2(I)CB$_4$; 7, α_2(I)CB$_{3,5}$+(α_1(III)CB$_9$)$_3$; **B**: 1, α_1(I)CB$_2$, precise location unknown; 2+3 as ad A; 4 as ad A; 5, contains α_1(I)CB$_7$+α_1(I)CB$_8$+incompletely cleaved products, 6+7 ad A. None of the peaks shown in B represents a pure peptide except No. 4 (α_1(I)CB$_6$). Reprinted from Mikšík and Deyl [88].

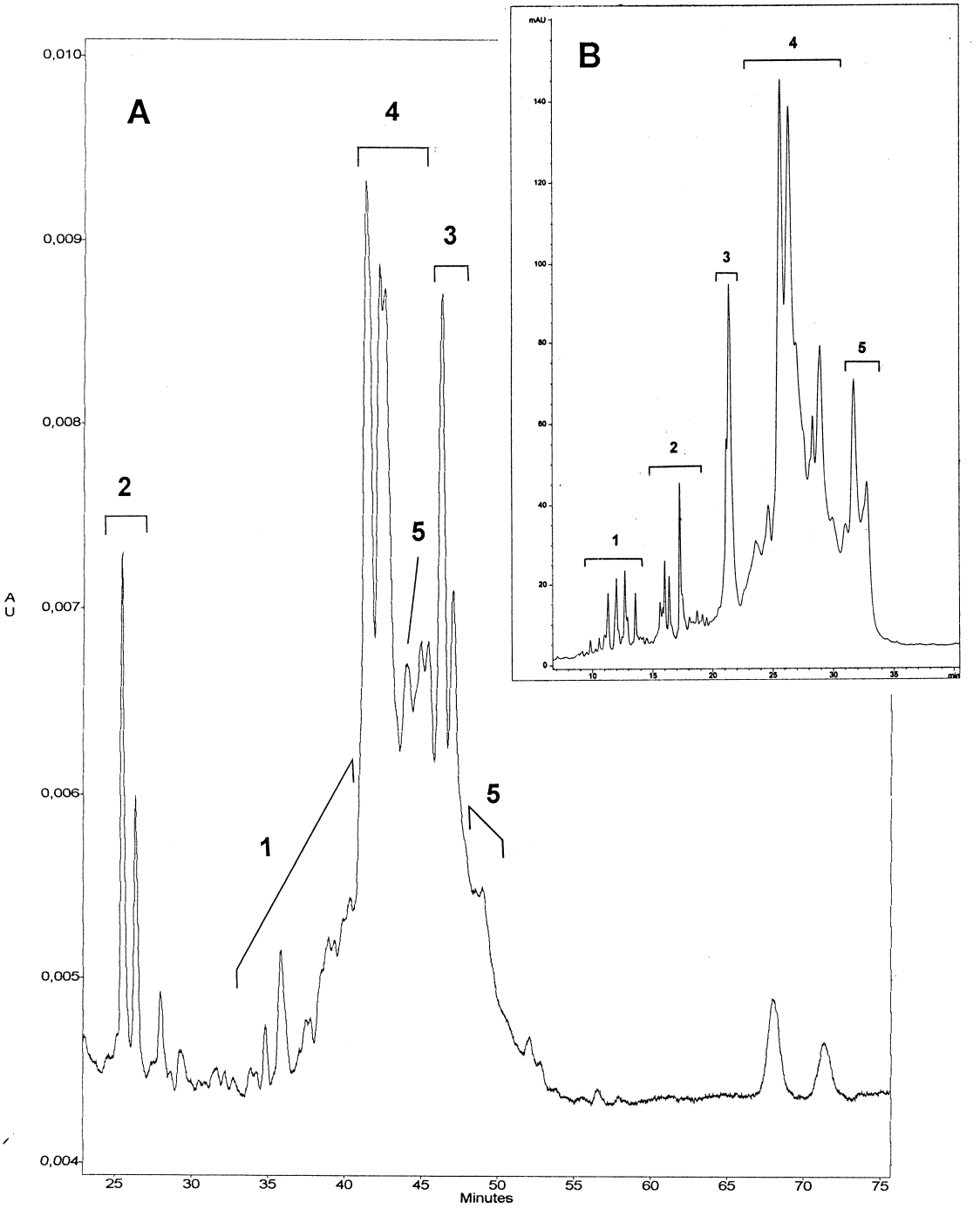


Fig. 19. Separation of rat tail tendon collagen CNBr peptides by (**A**) capillary electrophoresis in the Pluronic copolymer media (10 m*M* Tris and 75 m*M* phosphate buffer, pH 2.5, containing 7.5% Pluronic F127 copolymer) and by (**B**) reversed-phase high-performance liquid chromatography (for details see Ref. [79]). Identification of individual fractions: 1, α_2(I)CB$_2$, α_1(I)CB$_2$, α_1(I)CB$_5$; 2, α_1(I)CB$_4$, α_1(III)CB$_3$, α_1(III)CB$_6$, α_1(I)CB$_3$, α_1(III)CB$_4$; 3, α_1(I)CB$_6$; 4, α_1(III)CB$_5$, α_1(I)CB$_7$, α_1(I)CB$_8$, α_2(I)CB$_4$ and incomplete cleavage products; 5, α_2(I)CB$_{3,5}$, (α_1(III)CB$_9$)$_3$. Reprinted from Mikšík and Deyl [79].

polymers is possible as mentioned in the preceding section of this review.

In analogy to the separation of complete parent α-chains and chain polymers the separation mechanism apparently involves interaction of the solutes with the surfactant decreasing thereby their sticking to the capillary wall.

The third possibility of separating fibre forming collagen (type I) CNBr peptides is to run the separation in very acid buffers (pH 2.5) at a high surfactant concentration. The separations are conveniently materialised in 50 m*M* phosphate buffer made 50 mmol/l with respect to SDS. With this system at least for some peptides plate counts of 100 000 can be achieved. The main feature of this system is that the peptide-micelle associates move rapidly to the anode. Owing to the practically negligible endoosmotic flow at this pH the system has to be run in the negative polarity mode. At the supramicellar concentration of the detergent the partition mechanism apparently reflects interaction (sticking) of the individual peptides to the capillary wall and their elution by the passing by buffer. Supporting evidence can be drawn from an experiment with surface treated capillary in which the separation is virtually lost. If the separation is carried out at low (submicellar) concentration of the surfactant [87], the solutes (peptides) associate with the detergent molecules by which the selectivity of the system is increased and, perhaps, the peptide-detergent associates are at least partly prevented from the interaction with the capillary wall. At supramicellar concentration of the detergent association of the adsorbed peptides occurs with the surfactant clusters (micelles) which in turn results in more easy detachment and faster anodic movement, however, the selectivity is worse than in the previous case. In systems using either sub or supramicellar concentration of the detergent (SDS), the separation is more rugged and less dependent on the amount of sample injected (the capillary is less prone to clogging as seen in standard CZE without detergents). As the anodic movement of individual peptides in systems exploiting SDS in the background electrolyte is quite fast, electrophoretic separations must be run with the reversed polarity.

However, it has to be stressed that in the procedure in which supramicellar concentration of the detergent is used, association of the peptides may not follow strictly the number of hydrophobic domains in the molecule (e.g. for sterical reasons). Another reason could be that the peptide may be simply too small to contain distinct hydrophobic domains (this is the case of small CNBr peptides as discussed in connection with quantitation of different collagen types in tissues using marker peptides α_1(III)CB_2 and α_1(V)CB_1). Consequently the sequence of emerging peaks need not necessarily follow the number of such domains and the general patterns cannot be interpreted in terms of the molecular mass of the analyte as some of the peaks will be displaced.

As long as a considerable amount of evidence is available about the involvement of both ionic and hydrophobic interactions in the separation of collagen derived CNBr peptides (and intact parent α-chains as well) it may be plausible to investigate the possibility of exploiting gel matrices which are capable of introducing still another separation principle in the system, namely molecular sieving. Random polymers of the poly(acrylamide) type favour the molecular sieving mechanism as discussed already. Therefore other sieving media should be looked for: with Pluronic, poly(ethylene oxide)-poly-(propylene oxide) liquid crystals such sieving media have become available; they exhibit the molecular sieving effect, they exert hydrophobic domains capable of hydrophobic interactions (they act in a way similar to surfactants) and they are capable of hydrophilic (ionic) interactions as well. Indeed CNBr fragments can be separated in these matrices using Tris–phosphate (10 and 75 m*M*, respective) buffer pH 2.5 containing 7.5% Pluronic copolymer as shown in Fig. 19. The separation mechanism here is a typical multimode interaction and therefore it is not surprising that the order of emerging peaks is not strictly that of following the increase in rel. mol. mass. This means that the individual separation mechanisms are not completely synergistic, though generally good separations can be obtained.

6. Conclusions

A broad armamentum of techniques has developed over the years in parallel to the broadening of the family of collagen proteins. Generally these tech-

niques involve ion-exchange chromatography, gel permeation, reversed-phase separations, gel electrophoresis (one- and two-dimensional) and recently also capillary electromigration methods. As far as high-performance liquid chromatography is concerned, a rapid fractionation strategy has been developed about 13 years ago, which, though a little outdated today can serve as a guidance for the more recently discovered collagen species as well [89]. Some of the methods like gel electrophoresis of parent α-chains and their CNBr fragments are routinely used, though with minor variations. Still the long time known fact that e.g. α_1(I) and α_2(I) chains possessing large internal homogeneity and very close in their aminoacid sequence can be separated by conventional polyacrylamide gel electrophoresis has not yet been satisfactorily explained. Some light on the separational problems was shed by using capillary electromigration techniques. Apparently all separations of parent collagen α-chains and their fragments are based on multiple (multimodal) interactions, apparently ionic and hydrophobic in nature; smart combinations of appropriate conditions, involving molecular sieving represent a challenge for more perfect and possibly also validated quantitative determinations of different collagen α-chains and their fragments. Gel filled capillaries prepared in situ with compounds that (after polymerisation) aside to molecular sieving exhibit also e.g. surfactant properties may open a new separation pathway in which the strictly electrokinetic mechanisms merge with those of micellar electrokinetic chromatography (surface active agents) and capillary electrochromatography (gel filled capillaries).

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