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Human Collagen 'Fingerprints' Produced by Clostridiopeptidase A Digestion and High-Pressure Liquid Chromatography

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Samples (1–2mg) of purified human type I, II and III collagens and α1(I) and α2 chains were digested with clostridiopeptidase A and the released peptides analysed by ion-exchange high-pressure liquid chromatography. Specific 'fingerprints' were produced for each type of collagen. The reproducible nature of these 'fingerprints' and the reconstitution of the type I 'fingerprint' from the 'fingerprints' of the component α1(I) and α2 chains showed that the specificity of these 'fingerprints' was related to the primary structure of each type of collagen. In addition, some of the differences observed between the 'fingerprints' of the α1(I) and α2 chains of type I collagen were shown to be suitable for the quantitative analysis of these chains.

Recent studies have revealed the existence of at least four structurally distinct types of collagen (see reviews by Gross, 1974; Gallop & Paz, 1975). Type I tropocollagen, consisting of two identical α1(I) chains and one α2 chain, is the predominant form in bone, dentin, tendon and adult dermis, whereas type II, composed of three α1(II) chains, is found in cartilage and intervertebral disc. Type III, made of three α1(III) chains, is present in dermis, blood vessels and synovium, and type IV, the basement-membrane collagen, contains three α1(IV) chains.

It is apparent from the distribution of these collagens that some tissues contain more than one type of collagen (Bradley *et al.*, 1974; Epstein, 1974; Seyer *et al.*, 1974*a*,*b*; Trelstad, 1974; Eyre & Muir, 1975*b*,*c*). This heterogeneity, together with variations in the post-translational modifications of the collagen molecule and the insolubility of the collagen fibres (Henneman, 1972), complicates the study of the primary structure of tissue collagen.

With a view to facilitating the study of human genetic connective-tissue diseases with suspected primary-structure anomalies of collagen (McKusick, 1972), we have developed a technique of analysing the primary structure of collagen by using high-pressure liquid-chromatographic separation of the peptides released from collagen by clostridial collagenase.

We describe the specific 'fingerprints' obtained by high-pressure liquid chromatography of the peptides released from human type I, II and III collagen by clostridiopeptidase A. We also show that this technique is suitable for the analysis of minute amounts of collagen.

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Materials and Methods

Reagents

Pepsin (EC 3.4.23.1) (twice crystallized), azocasein, clostridiopeptidase A (EC 3.4.24.3) (described as collagenase type III from *Clostridium histolyticum*), 4-hydroxy-L-proline and δ-hydroxylysine hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The tripeptides Gly-Pro-Hyp, Gly-Ala-Ala, Gly-Pro-Ala and Gly-Pro-Pro came from Fox Chemical Co. (Los Angeles, CA, U.S.A.). DEAE-cellulose (DE-52) and CM-cellulose (CM-52) were obtained from Whatman (Maidstone, Kent, U.K.). Pierce (Rockford, IL, U.S.A.) supplied ninhydrin reagent (Nin-sol) and amino acid standard (hydrolysate, in sodium citrate buffer) which was completed with weighed hydroxyproline and hydroxylysine. Reagents for gel electrophoresis were from Eastman Kodak Co. (Rochester, NY, U.S.A.), except for sodium dodecyl sulphate (specially pure), which was obtained from BDH Chemicals (Montreal, P.Q., Canada). All the other reagents were from Fisher Scientific (Montreal, P.Q., Canada). The insoluble material found in guanidine hydrochloride (purified) was eliminated by filtration before use.

General procedures

Tissue specimens were milled for 4min, at liquid-N_2 temperature in a freezer-mill (Spex Industries, Metuchen, NJ, U.S.A.). The centrifugations to collect precipitated material were for 30min at 4°C and 37000*g* ($r_{av.}$ 8.25cm) in the SS-34 rotor of the Sorvall RC-2B centrifuge. Tris/HCl buffers were prepared by weighing the required amount of Tris,

dissolving it in half of the final volume and adjusting the pH with 1M-HCl. The solution was then adjusted to final volume. Dialyses were done with stirring at 4°C in Spectrapor no. 2 membranes (specified mol.wt. cut-off 12000–14000) (Spectrum Medical Industries, Los Angeles, CA, U.S.A.).

Purification of human type I, II and III collagens

Skin samples were collected at autopsy from patients and foetuses who had died from conditions not likely to have been associated with collagen abnormalities. Foetal dermis was used as the source of type III collagen, and both foetal and adult dermis were used as the sources of type I collagen. These collagens were isolated and purified by the techniques described by Epstein (1974). The type III collagen obtained was found to contain a little type I collagen. When chromatographed on CM-cellulose, the type III collagen was co-eluted with the $\beta 12$ chains of type I collagen. Pure type III collagen was precipitated from this co-eluted material by dialysis against 0.05M-Tris/HCl buffer, pH7.5, containing 1.71M-NaCl.

Hyaline costal cartilage was obtained at autopsy from a 3-year-old child who had died of cardiac disease. After excision of the perichondrium and milling of the cartilage, the proteoglycans were extracted at 4°C with 5.5M-guanidine hydrochloride/0.15M-potassium acetate, pH6.3 (10ml/g wet wt), changed daily for 5 days (Rosenberg, 1975). Type II collagen was obtained by limited pepsin digestion of the residue by using the techniques of Miller (1972) and of Eyre & Muir (1975*a*). The type II collagen obtained was chromatographed on both CM- and DEAE-cellulose.

CM- and DEAE-cellulose chromatography

CM-cellulose chromatography was performed as described by Lichtenstein *et al.* (1975). The bed size was 1.5cm × 20cm and the column was eluted at 45°C with a 400ml linear gradient from 0 to 0.1M-NaCl in 0.03M-sodium acetate buffer, pH4.8, containing 4M-urea. The A_{230} of eluted material was continuously measured with a Schoeffel 440 Spectroflow monitor equipped with a 8μl cell.

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

Gel electrophoresis

Urea/sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed by the technique of Goldberg *et al.* (1972) in a Büchler Polyanalyst instrument. The gels were stained with 1% Amido Black in 7% (v/v) acetic acid for 30min. Destaining was performed in a diffusion destainer (model 172, Bio-Rad, Richmond, CA, U.S.A.) by using a solution of acetic acid (50ml), methanol (75ml) and water to 1 litre. Gels were scanned with a scanning microdensitometer (model 445-50 Densicomp; Clifford Instrumentation, Natick, MA, U.S.A.) equipped with an interferential filter centred at 550nm.

Clostridiopeptidase A digestion

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

Samples (1–2mg) of collagen were suspended in 0.5ml of 0.02M-Tris/HCl buffer, pH7.5, containing 5mM-$CaCl_2$. Azocasein (0.01%, w/v) was added to this mixture as an indicator of non-specific proteinase activity. Clostridiopeptidase A solution (10μl; 2000units/ml, where 1 unit liberates amino acids from collagen equivalent in ninhydrin colour to 1μmol of L-leucine at 37°C, pH7.4 in 18h) in the same buffer was added and the mixture was maintained at 37°C for 15h with mild agitation. Then 0.5ml of 10% (w/v) trichloroacetic acid was added and the mixture left for 30min at 4°C. The suspension was centrifuged for 10min at 4°C and 9200*g* (r_{av}. 8.25cm). The pellet was discarded and the supernatant extracted three times with 3ml of ether to remove trichloroacetic acid. The residual solution was freeze-dried.

High-pressure liquid chromatography of the clostridiopeptidase A digestion product

The automatic analysis of the small peptides released by the clostridiopeptidase A digestion was performed on the Durrum D-500 amino acid analyser (Durrum Instruments Co., Palo Alto, CA, U.S.A.). Each freeze-dried sample was dissolved in the sodium citrate sample buffer, pH2.2 (200μl/mg), and a portion (40μl) transferred to a sample-holder unit. The chromatography was performed with a constant flow rate of 8.25ml/h at a pressure of 13.5×10^6Pa on the standard DC-4A-resin column (1.75mm bore, 48cm long) by using a modified Durrum sodium citrate buffer system to produce a discontinuous pH and ionic-strength gradient. The elution system was as follows (zero time refers to sample injection): 0–44.5min, pH3.25, Na^+, 0.2M; 44.5–52min, pH3.75, Na^+, 0.2M (prepared as a 1:1 mixture of the 0.2M-Na^+ standard buffers, pH3.25 and 4.25); 52–80min, pH4.25, Na^+, 0.2M; 80–130min, pH4.50, Na^+ 0.38M (prepared by addition of 6M-HCl to the 0.38M-Na^+ standard buffer, pH5.02); 130–165min, pH7.9, Na^+ 1.1M. The column temperature was 50°C and was raised to 65°C at 10min. The elution times of the tripeptides Gly-Pro-Hyp,

Gly-Ala-Ala, Gly-Pro-Ala and Gly-Pro-Pro were determined with synthetic standards.

Reaction with ninhydrin was performed at 126°C under 7.43×10^5 Pa back-pressure and the absorbance of the reaction products read with a 590nm photometer.

Amino acid analysis

Samples were hydrolysed under N_2 in constant-boiling 6M-HCl for 20h at 110°C and analysed with the Durrum D-500 amino acid analyser by using the standard four-buffer system recommended for collagen analysis by the Durrum Instrument Co. Hydroxyproline and proline reaction products were detected with a 440nm photometer.

Results

Characteristics of the isolated collagen

The behaviour of our type I collagen preparation on CM-cellulose chromatography (Fig. 1) and polyacrylamide-gel electrophoresis (Fig. 2) conformed to the characteristics of type I collagen reported by Bornstein & Piez (1964). Characteristic electrophoretic mobilities and amino acid contents were also observed for the α1(I)/β11 mixture, β12 and α2 chains eluted from CM-cellulose. The slight heterogeneity observed in the electrophoretic pattern of type I collagen and the α1(I) chains has also been observed by others (Chung & Miller, 1974; Eyre & Muir, 1975*b*) and attributed to the production of some shorter chains by the action of pepsin.

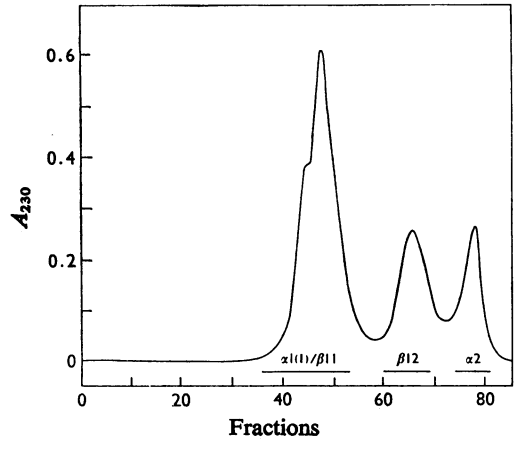

Fig. 1. *CM-cellulose chromatogram of type I collagen*
A sample (30mg) of purified type I collagen in 40ml of starting buffer was placed on the column and chromatographed as described in the Materials and Methods section. The flow rate was 70ml/h and the volume of the fractions 4.5ml. Fractions 36–53 constituted purified α1(I) and dimeric β11 chains, fractions 60–69 purified β12 chains and fractions 74–81 purified α2 chains.

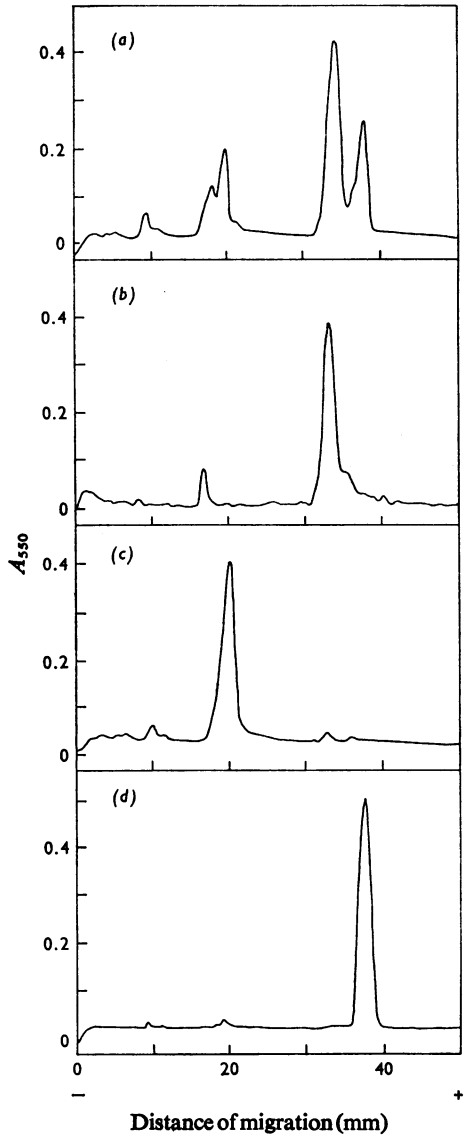

Fig. 2. *Electrophoretograms of type I collagen and its component chains*
Samples (25–50μg) of collagen in 100μl of sample buffer were applied to urea/sodium dodecyl sulphate/polyacrylamide gels and electrophoresis was performed as described by Goldberg *et al.* (1972). The samples consisted of purified type I collagen (*a*) and fractions eluted from CM-cellulose (Fig. 1) α1(I)/β11 mixture (*b*), β12 chains (*c*) and α2 chains (*d*).

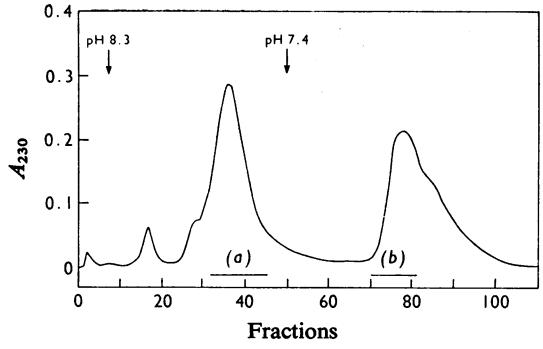

Fig. 3. *DEAE-cellulose chromatogram of cartilage collagen* A sample (40mg) of cartilage collagen in 40ml of starting buffer was applied to the column and eluted as described by Seyer *et al.* (1974*a*). The flow rate was 70ml/h and the volume of the fractions 8ml. Fractions 32–45, constituted component (*a*) of cartilage collagen, and fractions 70–81, component (*b*).

Table 1. *Amino acid composition of cartilage collagen* Cartilage collagen was chromatographed on CM-cellulose and yielded a single fraction referred to as CM-cellulose. When chromatographed on DEAE-cellulose two major fractions, (*a*) and (*b*), were obtained.

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

The cartilage collagen produced a single peak when chromatographed on CM-cellulose, and when chromatographed on DEAE-cellulose, this peak was resolved into two main fractions (Fig. 3). These fractions had identical amino acid compositions (Table 1) and hexose contents as determined by the

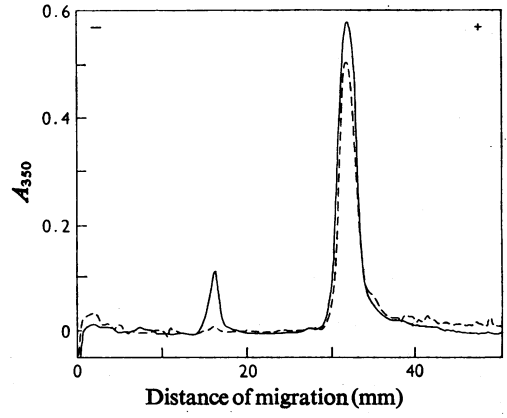

Fig. 4. *Gel electrophoresis of cartilage collagen components* Samples (25–50μg in 100μl of sample buffer) of cartilage collagen obtained by DEAE-cellulose as fraction (*a*) (----) and fraction (*b*) (——) shown in Fig. 3 were applied to urea/sodium dodecyl sulphate/polyacrylamide gels. Electrophoresis was performed as described by Goldberg *et al.* (1972).

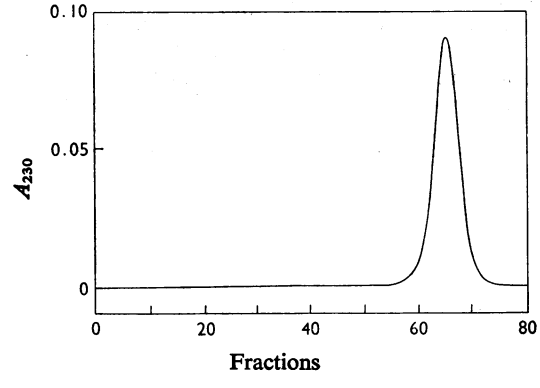

Fig. 5. *CM-cellulose chromatography of type III collagen* A sample (10mg) of purified type III collagen in 50ml of starting buffer was chromatographed as described in Fig. 1.

method of Robertson & Harvey (1972). Apart from higher hydroxylysine contents, their amino acid compositions were similar to the composition of human type II collagen calculated from its CNBr fragments by Miller & Lunde (1973). As a result the major peaks obtained from CM- and DEAE-cellulose chromatography were considered to contain type II collagen. The presence, shown by electrophoresis, of some β chains in the second DEAE-cellulose fraction (Fig. 4) suggests that the type II collagens in these fractions differed in their state of aggregation. In

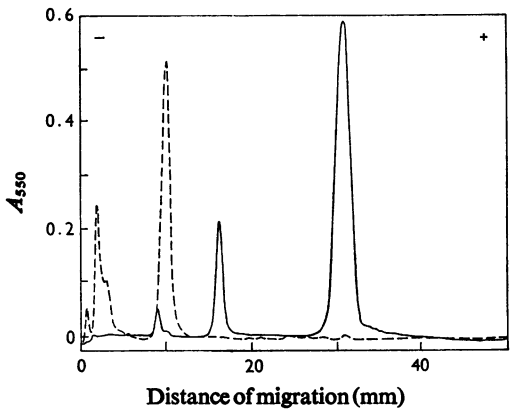

Fig. 6. *Electrophoretograms of type III collagen*
Samples (50 μg) of purified type III collagen in 100 μl of sample buffer were applied to urea/sodium dodecyl sulphate/polyacrylamide gels as described in Fig. 2. Samples consisted of unreduced type III collagen (-----) and type III collagen reduced with 1% β-mercaptoethanol (——).

contrast with Seyer *et al.* (1974*a*,*b*), who found both type I and II collagens in bovine cartilage, we only found type II collagen in our isolated human material.

A characteristic single symmetrical peak was obtained when our unreduced type III collagen preparation was chromatographed on CM-cellulose (Fig. 5). On gel electrophoresis the same preparation showed a major γ band and some higher-molecular-weight material (Fig. 6). After reduction of the disulphide bonds with β-mercaptoethanol, the characteristic conversion of the slowly migrating γ chains into the faster migrating α1 chains was observed (Eyre & Muir, 1975*a*). The presence of some dimeric (β) and trimeric (γ) material was probably due to the presence of some non-disulphide cross-links. The amino acid composition of this type III collagen preparation was also found to be similar to the compositions reported by Epstein (1974) and Chung & Miller (1974).

Analysis of the peptides released by clostridiopeptidase

After digestion with clostridiopeptidase A, each type of collagen showed a specific elution profile on high-pressure ion-exchange liquid chromatography (Fig. 7). More than 40 peaks were resolved. Specific peaks were identified for each type of collagen. The specific peaks for type I collagen were eluted after 88min 58s and 139min 9s, for type II collagen after 100min 40s and 105min 17s, and for type III collagen after 95min 37s, 97min 10s, 98min 73s and 122min 55s. Peaks corresponding to the elution times of the tripeptide standards were identified. Partial resolution between the peaks Gly-Pro-Hyp and Gly-Ala-Ala and between Gly-Pro-Ala and Gly-Pro-Pro was obtained.

Three peaks were produced by non-collagenous materials. A urea peak eluted after 9 min was found in the digests of chromatographically purified chains. The peak eluted after 78min was an artifact also present in blanks, and the peak eluted after 111min was due to NH_3.

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

The elution patterns obtained from different runs of the same peptide preparation were almost identical. The elution pattern obtained from separate digests of the same substrate were also almost identical provided that the same conditions, in particular the same enzyme preparation, were used. We observed several differences when collagenase form III from Advance Biofacture Corp. (Lynnbrook, NY, U.S.A.) was used instead of the Sigma type III collagenase. These differences were probably related to the known heterogeneities of this enzyme (Miyoshi & Rosenbloom, 1974).

An assessment of the suitability of this technique for the quantitative analysis of collagen was made. Purified type I collagen, α1(I), α2 and β12 chains were separately digested with clostridiopeptidase A and the resulting peptides chromatographed. Two peaks were selected; the one eluted at 88min 58s (peak 88/58) was considered specific for the α1(I) chain and the one eluted at 150min 58s (peak 150/58) specific for α2 chain (Fig. 8*a*). Using these peaks as markers, we recalculated the chain composition of type I collagen and β12 chains. The results given in Table 2 show close agreement between the experimental and theoretical chain compositions.

Using the quantitative information obtained from this experiment, we mixed the peptides produced by the separate digestions of α1(I) and α2 chains in a 2:1 molar ratio, simulating type I collagen. The resulting elution profile was almost completely superimposable on that obtained for type I collagen (Fig. 8*b*).

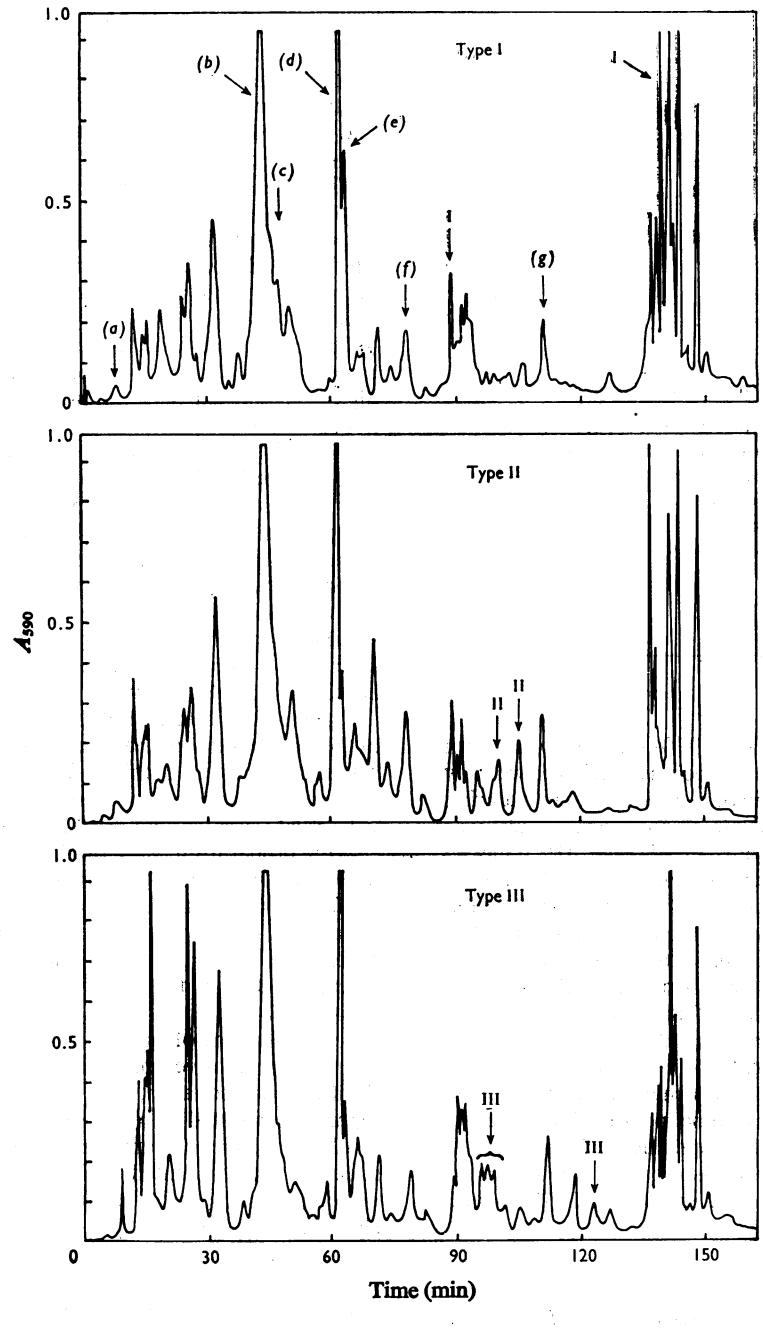

Fig. 7. *High-pressure liquid chromatography of the various collagens after collagenase digestion*
The peptides released by clostridiopeptidase A digestion of 200 μg of type I, type II and type III collagens were dissolved in 40 μl of sample buffer and chromatographed on the Durrum D-500 amino acid analyser as described in the Materials and Methods section. Specific peaks for type I, II and III collagens are indicated by the numbers I, II and III respectively. Peaks indicated by letters include: urea, 9 min (*a*); Gly-Pro-Hyp, 43 min (*b*); Gly-Ala-Ala, 46 min (*c*); Gly-Pro-Ala, 61 min 15 s (*d*); Gly-Pro-Pro, 62 min 20 s (*e*); artifact, 78 min (*f*); NH_3, 111 min (*g*).

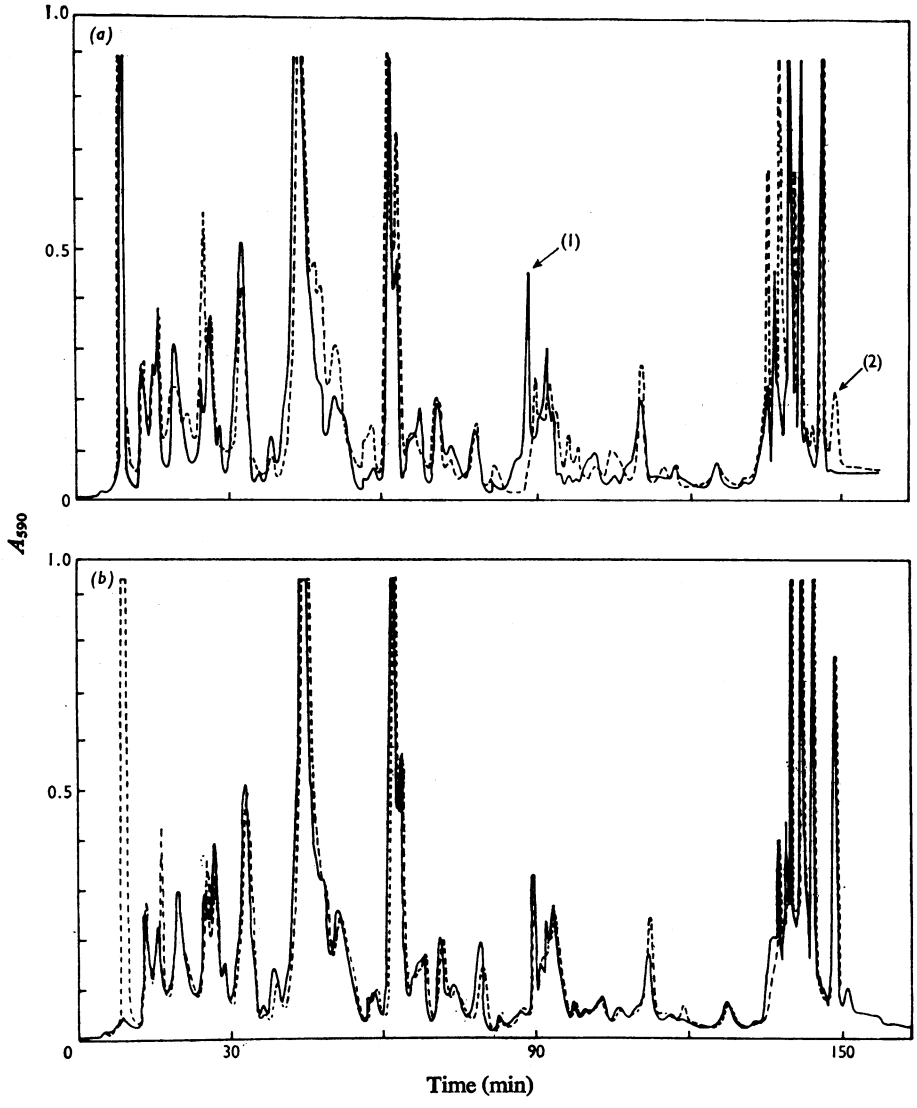

Fig. 8. *Reconstitution of type I collagen 'fingerprint' from digested α1(I) and α2 chains*
(*a*) The peptides released by clostridiopeptidase A digestion of α1(I) (——) and α2 (----) chains were chromatographed as described in Fig. 7. Quantification of α1(I) and α2 chains in type I collagen was made by using peak (1) [specific for α1(I) chain] and peak (2) (specific for α2 chain). (*b*) Chromatograms of digested type I collagen (——) and of separately digested α1(I) and α2 chains mixed in a 2:1 molar ratio (----).

Discussion

Clostridiopeptidase A has been shown to contain two main fractions termed fractions A and B (Miyoshi & Rosenbloom, 1974). Fraction A cleaves the collagen molecule at the bond between X and Gly in the sequence -P-X-Gly-P-X-, where P is Pro or Hyp and X is any amino acid (Harper & Kang, 1970). In addition to this site of cleavage, fraction B is able to cleave at the amino end of glycine in some sequences where P is replaced by other amino acids (Bornstein, 1967; Harper & Kang, 1970).

In our study we observed that specific 'fingerprints' of 1–2mg samples of human type I, II and III collagens and α1(I) and α2 chains were produced by the clostridiopeptidase A-released peptides after their separation by high-pressure liquid chromatography and reaction with ninhydrin. The differences in these 'fingerprints' were considered to reflect differences in

Table 2. *Quantitative analysis of type I collagen*
By using the described high-pressure liquid-chromatography analysis of the collagenase-digested collagens, the proportions of chains α1(I) and α2 were evaluated from the relative area of the peak eluted at 88min 58s [considered as specific for the α1(I) chain] and of the peak eluted at 150min 58s (considered as specific for the α2 chain). These peaks are indicated by arrows on Fig. 8. The experimental values are expressed as the ratio ($\times 10^3$) between the area of the analysed peak and the summated areas of all the eluted peaks (excluding artifacts).

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

the primary structure of each of these collagens. This view is supported by our observation that the type I collagen 'fingerprint' could be reconstituted from the 'fingerprints' of its component α1(I) and α2 chains. In addition, some of those differences between the 'fingerprints' for α1(I) and α2 chain were shown to be suitable for the quantitative analysis of the relative amounts of these chains.

Four tripeptide peaks were identified by comparison of their elution times with standards. Two of those, containing Gly-Pro-Pro and Gly-Pro-Hyp, may be used to estimate the completeness of the post-translational step of proline hydroxylation as shown by Paz & Gallop (1975).

We were concerned about the effects of non-specific proteinases on the peptides released by clostridial collagenase. However, the reproducibility of the 'fingerprints', the lack of coincident peaks when compared with amino acid profiles and the minimal release of trichloroacetic acid-soluble fragments from azocasein suggested that our digestion system contained relatively low non-specific proteinase activity. The activity of these non-specific proteinases may vary in different clostridial collagenase preparations. This activity can be assessed by using azocasein or [^{3}H]tryptophan-labelled chick proteins (Peterkofsky & Diegelmann, 1971) and when found to be significant its activity can be decreased by further purification of the enzyme preparation and the addition of non-specific proteinase inhibitors.

This technique of primary-structure analysis has several features that make it of potential value for the study of tissue collagen in patients with genetic connective-tissue diseases. These features include the ability to produce 'fingerprints' from 1–2mg of collagen, the possibility of assessing the completeness of proline hydroxylation and of determining the relative amounts of different collagen types within a tissue.

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References

Bornstein, P. (1967) *Biochemistry* **6**, 3082–3093
Bornstein, P. & Piez, K. A. (1964) *J. Clin. Invest.* **43**, 1813–1823
Bradley, K., McConnell-Breul, S. & Crystal, R. G. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 2828–2832
Chung, E. & Miller, E. J. (1974) *Science* **183**, 1200–1201
Epstein, E. H. (1974) *J. Biol. Chem.* **249**, 3225–3231
Eyre, D. R. & Muir, H. (1975*a*) *Connect. Tissue Res.* **3**, 165–170
Eyre, D. R. & Muir, H. (1975*b*) *Connect. Tissue Res.* **4**, 11–16
Eyre, D. R. & Muir, H. (1975*c*) *Biochem. J.* **151**, 595–602
Gallop, P. M. & Paz, M. A. (1975) *Physiol. Rev.* **55**, 418–487
Goldberg, B., Epstein, E. H. & Sherr, C. J. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 3655–3659
Gross, J. (1974) *Harvey Lect.* **68**, 351–432
Harper, E. & Kang, A. H. (1970) *Biochem. Biophys. Res. Commun.* **41**, 482–487
Henneman, D. H. (1972) *J. Med. Primatol.* **1**, 58–67
Lichtenstein, J. R., Byers, P. H., Smith, D. S. & Martin, G. R. (1975) *Biochemistry* **14**, 1929–1933
McKusick, V. A. (1972) *Heritable Disorders of Connective Tissue*, 4th edn., p. 52, C. V. Mosby, St. Louis
Miller, E. J. (1972) *Biochemistry* **11**, 4903–4909
Miller, E. J. & Lunde, L. G. (1973) *Biochemistry* **12**, 3153–3159
Miyoshi, M. & Rosenbloom, J. (1974) *Connect. Tissue Res.* **2**, 77–84
Paz, M. A. & Gallop, P. M. (1975) *In Vitro* **11**, 302–311
Peterkofsky, B. & Diegelmann, R. (1971) *Biochemistry* **10**, 988–994
Robertson, W. V. B. & Harvey, J. (1972) *Biochem. Med.* **6**, 246–256
Rosenberg, L. (1975) in *Dynamics of Connective Tissue Macromolecules* (Burleigh, P. M. C. & Poole, A. R., eds.), pp. 105–124, North-Holland Publishing Co., Amsterdam and Oxford, and American Elsevier Publishing Co., New York
Seyer, J. M., Brickley, D. M. & Glimcher, M. J. (1974*a*) *Calcif. Tissue Res.* **17**, 25–41
Seyer, J. M., Brickley, D. M. & Glimcher, M. J. (1974*b*) *Calcif. Tissue Res.* **17**, 43–55
Trelstad, R. L. (1974) *Biochem. Biophys. Res. Commun.* **57**, 717–725
Trelstad, R. L., Kang, A. H., Toole, B. P. & Gross, J. (1972) *J. Biol. Chem.* **247**, 6469–6473