Characterization of a Collagen from Codfish Skin Containing Three Chromatographically Different α Chains*

Karl A. Piez

ABSTRACT: Collagen from codfish skin was fractionated in the denatured form into single (α), double (β), and triple chain (γ) components by recycling on columns of polyacrylamide or cross-linked dextran gels. Chromatography of these fractions on carboxymethylcellulose showed the presence of three different α chains and the predicted β and γ components arising from intramolecular cross-links.

The α chains, designated $\alpha1$, $\alpha2$, and $\alpha3$, have molecular weights of about 95,000 but different amino acid compositions. It is suggested on the basis of these results that the $\alpha1$ fraction of other collagens may contain two different chains which are not resolvable by the procedures used.

Studies in this laboratory (Piez *et al.*, 1961, 1963; Bornstein *et al.*, 1964; Bornstein and Piez, 1964; Lewis and Piez, 1964a,b) on a variety of collagens from vertebrate species have shown that the collagen molecule is assembled from three chains of which one, the $\alpha2$ chain, is clearly different in amino acid composition and chromatographic behavior from the other two, the $\alpha1$ chains. In addition to these single chains, denatured collagen samples contain varying amounts of covalently linked dimers of α chains consisting of all possible pairs (β_{11}, β_{12}, and β_{22}) which arise from intra- and intermolecular cross-links. The trimer, γ_{112}, may also be present.

As part of a continuing program to obtain additional comparative data, collagen from codfish skin was studied by chromatography in the denatured form on carboxymethylcellulose. The chromatograms obtained were more complex than observed for any other collagen. α and β components were each present in at least three parts of the chromatogram. Prior separation into molecular weight classes by molecular sieve chromatography permitted interpretation of the carboxymethylcellulose chromatograms in terms of three different α chains and the predicted β components. These experiments together with a further characterization of the α chains are reported here.

Codfish skin collagen has been studied most extensively by Gustavson (1956) and Young and Lorimer (1960, 1961). The native protein is similar to other vertebrate collagens. It is characterized by a denaturation temperature in solution of about 13° and a low content of hydroxyproline and proline.

Methods

Codfish Skin Collagen. Codfish skin was obtained either frozen (through the courtesy of Dr. Joseph Nichols, Ethicon, Inc., Somerville, N. J.) or from fresh fish bought commercially in Boston (through the courtesy of Dr. Jerome Gross). Identical results were obtained with collagens from both sources. All operations were conducted below 5°. The skins were cut into small pieces and extracted one to three times with approximately five volumes of 3% acetic acid. The extracts were clarified by filtration through cheesecloth followed by centrifugation. The collagen was precipitated by the addition of sodium chloride to give a 5% solution and then separated by centrifugation. The residue was suspended in water to give a volume of about one-fifth the original volume and dissolved by dialysis against 1% acetic acid. After clarification by centrifugation, the pH of the solution was slowly raised to 7.0 by the addition of 1 N sodium hydroxide with cooling and stirring. If a precipitate appeared at this point and did not redissolve after continued stirring, it was removed by centrifugation. Collagen was precipitated from the neutral solution by addition of sodium chloride to give a 20% solution. The residue was redissolved by dialysis against 1% acetic acid and precipitated again from neutral solution. The residue was redissolved, dialyzed extensively against 1% acetic acid, clarified by centrifugation, and lyophilized. The purified collagen was stored over calcium chloride at 5°.

Molecular Sieve Chromatography. Two columns were used with volumes of 450 (2.4 × 100 cm) and 1100 ml (3.8 × 100 cm). The columns were constructed from glass or Lucite tubing with piston-like fittings at the ends of the column. The fittings were machined from Teflon and had O-ring seals. Porous polyethylene disks were placed over the fittings to retain the packing. These are similar to columns available commercially (LKB Instruments) for recycling chromatography. Two solvents were used, 0.15 M, pH 4.8, potassium acetate at

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room temperature or 1 M calcium chloride adjusted to pH 7.0 at 10°. Sephadex G-200 (Pharmacia) or Bio-Gel P-200 (Bio-Rad Laboratories) was allowed to swell in solvent for several days and then mixed with about a one-tenth volume (settled) of cellulose (Whatman powder CF 11) in the same solvent. This allowed a flow rate approximately twice the rate possible without the cellulose. The mixture was poured into the column with the bottom fitting and porous disk in place. The flow rate was restricted with a clamp to about double the final value expected. The restriction was removed when the column was approximately half packed. When fully packed, the column was washed under gravity flow overnight using a liquid head of about 6 ft. At this time the flow rates were 60–100 ml/hr for the large column and 30–50 ml/hr for the small column. The flow was stopped and the top fitting was put in place. Connections were made from the bottom of the column to a pump (Sigmamotor), from the pump to a flow cell (1-cm light path) in a spectrophotometer (Beckman DB) with recorder (Sargent SRL), and from the flow cell to the top of the column in the manner described by Porath and Bennich (1962) for recycling chromatography using reverse flow.

It was found advisable to include an upside down glass "T" filled with solvent (1-mm i.d. inlet and outlet, 8-mm i.d. upright closed at the top with a piece of tubing and a clamp) between the top of the column and the spectrophotometer to act as a bubble trap. This helped to prevent the collection of bubbles in the flow cell. The sample compartment of the spectrophotometer was cooled. If the column were operated at room temperature, it was also advisable to deaerate the solvents by warming under reduced pressure.

The system was washed for 24 hr with fresh solvent with the pump set to give a flow rate approximately half the rate obtainable under gravity flow. If accidents were avoided, a column could be operated in this manner for several weeks or longer. The sample (a maximum of 50 mg in 10 ml for the small column or 150 mg in 30 ml for the large column) was pumped onto the column and the system was closed for recycling. At this point the bubble trap was opened and then raised several feet and closed to put a slight positive pressure on the system. This prevented the possibility that air bubbles might be drawn into the system at the connections. The spectrophotometer was set at a wavelength between 230 and 236 mμ, depending on the sensitivity desired. At any time cycling could be momentarily stopped, the system opened between pump and spectrophotometer, and solvent pumped in while fractions were collected without interrupting the recording of concentration. If desired, cycling could be re-established to obtain further resolution. Molecular sieve chromatography has also been used by Francois and Glimcher (1965) to fractionate denatured calf skin collagen.

Carboxymethylcellulose Chromatography. The procedure previously described (Piez *et al.*, 1963) was employed except the column (18 × 150 mm) was eluted at 200 ml/hr (twice the previous rate) and was operated at room temperature since codfish skin collagen is fully denatured above 20°. The eluting buffer was 0.08 ionic strength sodium acetate buffer, pH 4.8, on which was superimposed a linear gradient from 0.0 to 0.1 M sodium chloride over a total volume of 800 ml. The adsorbent[1] was Schleicher and Schuell CM-cellulose, standard grade, lot 1310, 0.8-mequiv/g capacity. A column can be used nearly indefinitely if the adsorbent at the top of the column is replaced as it becomes colored. In fact, the resolving capacity of a column improves with use for a time. The ionic strength of the eluting buffer may have to be altered by 0.01 or 0.02 depending on the properties of the adsorbent.

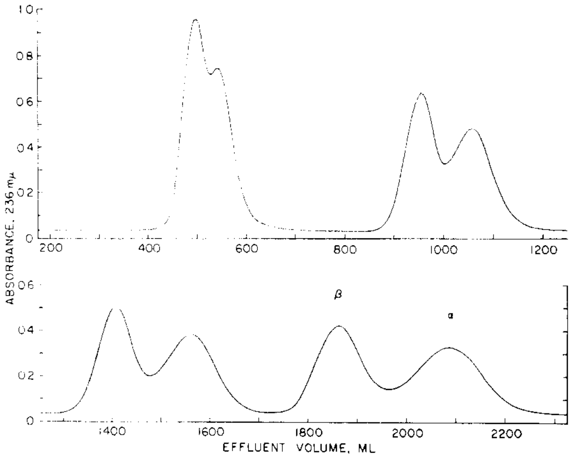

FIGURE 1: Recycling chromatography of approximately 150 mg of denatured codfish skin collagen on a column (3.8 × 100 cm) of P-200 acrylamide gel containing 10% cellulose. Conditions: pH 4.8, 0.15 M potassium acetate, room temperature, 30 ml/hr.

Air bubbles can be prevented from forming in the flow cell in the spectrophotometer by deaerating the buffer and mounting the gradient flasks on a magnetic stirrer with a hot plate set to keep the buffer at about 40°. It is also useful to use a sealed column of the type used for recycling without air or free liquid space and upward flow of buffer. The sample is then pumped onto the column, followed by eluting buffer. Protein was isolated by desalting selected chromatographic fractions on Sephadex G-25 using a pyridine acetate buffer, followed by lyophilization (Piez *et al.*, 1963).

Amino Acid Analysis. Proteins were hydrolyzed under a nitrogen atmosphere for 24 hr in 6 N HCl at 106°. Hydrolysates were analyzed on an automatic instrument (Piez and Morris, 1960) modified for high-speed analysis. Corrections were made for destruction of threonine, serine, methionine, and tyrosine and incomplete release of valine as previously described (Piez *et al.*, 1960).

Tyrosine and tryptophan contents were calculated from the ultraviolet spectra as described by Beavan and

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[1] A reliable commercial source of carboxymethylcellulose has not been found. Most lots have given poor chromatograms.

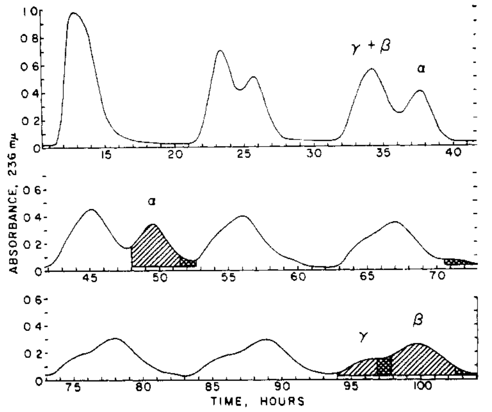

FIGURE 2: Recycling chromatography of approximately 50 mg of codfish skin collagen on a column (2.4 × 100 cm) of G-200 cross-linked dextran containing 10% cellulose. Conditions: pH 4.8, 0.15 M potassium acetate, room temperature, 15 ml/hr. The parts of the chromatogram which are shaded indicate times when the effluent was collected and replaced with buffer. The cross-hatched portions were discarded; the others were chromatographed on carboxymethylcellulose (Figure 3).

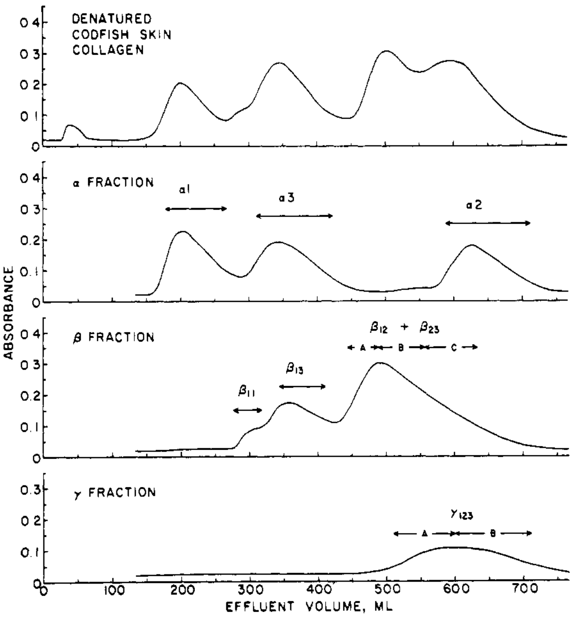

FIGURE 3: Chromatography of denatured codfish skin collagen on carboxymethylcellulose. Top chromatogram: approximately 50 mg of unfractionated collagen. Bottom three chromatograms: the α, β, and γ fractions obtained by molecular sieve chromatography of approximately 150 mg of collagen (Figure 2). The arrows indicate fractions taken for amino acid analysis (Tables I and III); see text for conditions.

Holiday (1952). Solutions were prepared in pH 4.8, 0.15 M, potassium acetate and concentrations were determined by polarimetry at 313 mμ. The specific rotation, measured on solutions of codfish skin collagen whose concentrations had been determined by micro-Kjeldahl, was found to be −750°. A measured proportion of 1 N sodium hydroxide, calculated to neutralize the buffer and make the mixture 0.1 N in base, was added. Spectra were measured in a Model 14 Cary spectrophotometer, using cells with a 1-cm light path.

Molecular Weight. Molecular weights of α chains were determined by high-speed sedimentation equilibrium in the Spinco Model E ultracentrifuge as described by Yphantis (1964). A six-hole cell was used for the simultaneous examination of three samples in an An-D rotor at 10°. The solvent was 1 M calcium chloride adjusted to pH 7.0. Samples were either dissolved in the solvent to give concentrations between 0.05 and 0.25 mg/ml and dialyzed against solvent, or chromatographic fractions with absorbances between 0.1 and 0.5 at 230 mμ were dialyzed against several changes of solvent. Speeds between 19,160 and 24,630 rpm were used. Measurement of Rayleigh patterns at various times demonstrated that equilibrium was achieved in less than 16 hr and that the patterns were unchanged for at least an additional 24 hr.

Measurements were made along the radius (r) at half fringes if the concentration gradient was sufficient to give five or more fringes. With lower gradients, the concentration (y displacement) was measured at 100-μ intervals along r. The weight-average molecular weight (M_w) was calculated for the whole cell from the slope of a least-square line through values of ln y plotted against r^2. Values of M_w were also calculated across the gradient taking slopes from successive overlapping sets of three points. M_w was then plotted against y, and M_w at $y = 0$ was calculated from a least-square line (Figure 5). M_z was obtained by extrapolating M_w to the cell bottom (6) employing a plot of M_w against $b^2 - r^2$. Calculations were performed by computer using a program written by Drs. Parker Small and Jerome Resnick.

The apparent partial specific volume of denatured codfish skin collagen was measured in 1 M calcium chloride, pH 7.0, at 20°. Densities were determined both with a 25-ml pycnometer and a Linderstrøm-Lang density gradient column using approximately 1% solutions of protein dialyzed against solvent. Concentrations were determined after dialysis by a carefully calibrated micro-Kjeldahl procedure with a reproducibility better than 1%. The nitrogen content of codfish skin collagen was assumed to be 18.6%, as measured by micro-Kjeldahl. The value calculated from the amino acid composition is 19.0%, suggesting that a small amount of nitrogen is not converted to ammonia during digestion or that collagen is difficult to dry completely. The density gradient column gave a value of 0.693 and the pycnometer a value of 0.695 for the apparent partial specific volume, with an estimated error of 1%. The latter value was used in the calculations.

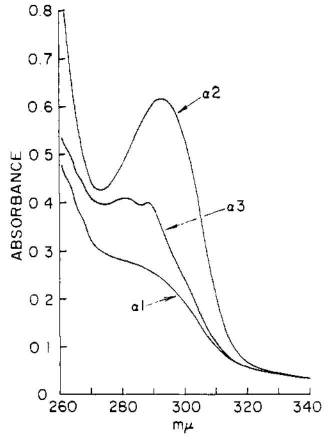

FIGURE 4: Absorption spectra of α chains from codfish skin collagen. Concentrations were 3.4 mg/ml in 0.1 N sodium hydroxide.

Results

Molecular Sieve Chromatography. Good resolution of α and β components could be achieved in two to four cycles through columns of either Bio-Gel P-200 (polyacrylamide gel) or Sephadex G-200 (cross-linked dextran). The examples in Figures 1 and 2 are representative of some of the better experiments. Some difficulty was experienced in preparing columns which gave reproducible separations. The Bio-Gel P-200 excluded at a slightly lower molecular weight than the Sephadex G-200 and thus was not effective in separating β and γ components. This separation was marginal on G-200 requiring many cycles (Figure 2). A practical procedure will require a gel that excludes at a higher molecular weight.

Experiments in pH 4.8 acetate buffer at room temperature which lasted 2–3 days yielded samples of α component that showed evidence of heterogeneity when examined by sedimentation equilibrium. For this reason experiments were also performed in 1 M calcium chloride, pH 7.0, at 10°, conditions under which denatured collagen is more stable. The lower temperature is possible since 1 M calcium chloride lowers the melting point of collagen about 15° (von Hippel and Wong, 1963). This procedure gave a better product but, for reasons which are not clear, resolution was not as good as in acetate buffer.

Carboxymethylcellulose Chromatography and Amino Acid Analysis. An example of the complex chromatograms obtained from denatured codfish skin collagen before molecular sieve chromatography is shown in Figure 3, top. After separation into α, β, and γ fractions simpler chromatograms were obtained which could be interpreted on the basis of the amino acid composition of protein in the peaks.

The α fraction showed three major chromatographic peaks of equal size (Figure 3). Since protein isolated from the peaks all had the same extinction coefficient at 230 mμ ($E_{1\text{cm}}^{1\%}$ 22 ± 1), approximately equal amounts

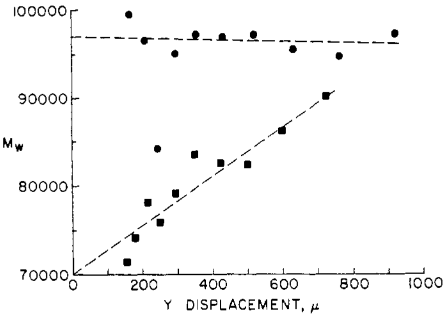

FIGURE 5: Molecular weight data from two typical high-speed sedimentation equilibrium experiments on samples of α chains from codfish skin collagen. One sample (●) was a portion of the effluent (α1 chain) from a carboxymethylcellulose column (Figure 3) dialyzed against solvent (1 M calcium chloride); the other sample (■) was protein (α2 chain) from a similar chromatogram which was isolated by desalting and drying and then redissolved in solvent. The heterogeneity of the latter, presumably resulting from the isolation procedure, is evident. Speed, 24,630 rpm; initial concentration, about 0.1 mg/ml; see text for additional details.

TABLE I: Amino Acid Composition of Codfish Skin Collagen and Its Constituent α Chains.

	Residues/1000 Total Residues[b]			
Amino Acid[a]	Collagen	α1	α2	α3
4-Hydroxyproline	56	55	52	58
Aspartic acid	52	50	54	51
Threonine	24.5	23.4	26.9	24.5
Serine	73	70	73	73
Glutamic acid	73	76	62	78
Proline	97	98	97	96
Glycine	339	339	348	347
Alanine	109	119	107	101
Valine	17.4	15.5	19.5	20.1
Methionine	18.2	16.7	18.3	16.8
Isoleucine	9.3	10.8	9.2	8.9
Leucine	20.9	18.8	24.4	17.5
Tyrosine	4.2	1.8	4.7	2.6
Phenylalanine	10.8	13.2	9.1	11.0
Hydroxylysine	6.5	5.5	9.5	5.3
Lysine	27.9	31.3	20.6	30.3
Histidine	8.5	5.2	11.5	7.0
Arginine	53	51	54	51
Tryptophan[c]	. . .	0	0	1.0
Amide nitrogen	(46)	(43)	(46)	(54)

[a] No chromatographic evidence was seen (<1 residue/1000) for 3-hydroxyproline or cystine. [b] Assuming a molecular weight of 95,000 and applying the calculated residue weight of 91, the unit residues/1000 is approximately equal to residues/chain. [c] Suggested values, determined by measurements of ultraviolet spectra (Table II and text).

TABLE II: Tyrosine and Tryptophan Content of Codfish Skin α Chains Determined from the Ultraviolet Spectra.

	Residues/1000 Total Residues		
	Expt 1	Expt 2	Average
Tyrosine			
$\alpha 1$	1.0	1.0	1.0
$\alpha 2$	5.5	5.5	5.5
$\alpha 3$	1.6	1.4	1.5
Tryptophan			
$\alpha 1$	0.3	0.6	0.5
$\alpha 2$	0.1	0.3	0.2
$\alpha 3$	0.8	1.2	1.0

of each were present. Amino acid analysis (Table I) indicated that they are distinct but closely related proteins. Nearly every amino acid is present in a different amount in at least one of the α chains. Some of the differences, particularly in the contents of alanine, tyrosine, hydroxylysine, lysine, and histidine, are large.

Although tryptophan has not previously been found in purified collagen, the ultraviolet spectrum of the $\alpha 3$ chain showed a form typical of a mixture of tyrosine and tryptophan (Figure 4). Calculations made from the spectra are shown in Table II. Good agreement was obtained on two different preparations of α chains. The tyrosine contents of $\alpha 1$ and $\alpha 3$ were lower by this method than by chromatography (Table I). The latter values are probably better since at these very low levels, near 2 residues/1000 or about 0.4%, measurements of the spectra are somewhat uncertain. The high protein concentrations necessary to give reasonably good spectra result in a significant absorption of light by scattering. Correction by subtraction of the baseline obtained by extrapolation through the absorbances at 360 and 320 mμ is only approximate. This difficulty applies also to measurement of the tryptophan content. However, the measured amount in $\alpha 3$, 1.0 residue/1000, together with the typical form of the spectrum (Figure 4), is suggestive evidence for the presence of one tryptophan residue/chain. The small values obtained for $\alpha 1$ and $\alpha 2$ are probably not significant. Until additional evidence is available, these results must be considered tentative.

Chromatography of the β fraction on carboxymethyl cellulose (Figure 3) showed one large tailing peak preceded by two smaller peaks. Protein was isolated from the peaks as indicated in Figure 3. The first peak represented about 5% of the total β fraction as measured by the area under the curve. Amino acid analysis (Table III) showed it to be identical in composition with $\alpha 1$, and it was therefore identified as β_{11}. The second peak, representing about 25% of the β fraction, had the composition predicted for β_{13}. The large peak showed small but reproducible differences in the composition of fractions from the tailing and leading sides of the peak with intermediate values for the fraction from the middle of the peak. These differences were of the same size and in the same direction as would be predicted if the peak were a mixture of β_{12} and β_{23} with the leading edge enriched in β_{12} and the trailing edge enriched in β_{23} (Table III). Although the differences are not large, they involve about half the amino acids, and in every case the agreement between the values found and

TABLE III: Partial Amino Acid Composition of Cross-Linked Components from Codfish Skin Collagen.

	Residues/1000 Total Residues											
	Calculated[a]						Found					
Amino Acid	β_{11}	β_{13}	β_{12}	β_{23}	γ_{123}	Collagen	β_{11}	β_{13}	β_{12}[b]	β_{23}[b]	γ_{123}[c]	Collagen
4-Hydroxyproline	56	57	54	55	55	55	58	57	54	55	53	56
Aspartic acid	50	51	52	53	52	52	50	50	51	53	52	52
Glutamic acid	76	77	69	70	72	72	75	77	71	71	74	73
Alanine	119	110	113	104	109	109	122	111	114	109	109	109
Valine	15.5	17.8	17.5	19.8	18.4	18.4	15.7	17.7	19.4	19.0	19.3	17.4
Isoleucine	10.8	9.9	10.0	9.1	9.6	9.6	9.7	9.9	11.0	8.9	9.8	9.3
Leucine	18.8	18.2	21.6	21.0	20.2	20.2	17.3	18.1	20.2	20.5	19.7	20.9
Tyrosine	1.8	2.2	3.2	3.8	3.0	3.0	1.2	1.8	3.3	3.3	3.1	4.2
Phenylalanine	13.2	12.1	11.2	10.1	11.1	11.1	13.0	12.4	11.1	10.2	10.6	10.8
Hydroxylysine	5.5	5.4	7.5	7.4	6.8	6.8	5.1	4.4	6.3	6.5	5.2	6.5
Lysine	31.3	30.8	26.0	25.5	27.4	27.4	27.7	28.7	26.0	24.7	25.2	27.9
Histidine	5.2	6.1	8.4	9.3	7.9	7.9	5.1	5.7	7.8	9.0	7.8	8.5

[a] Calculated from the amino acid composition of the constituent α chains (Table I). [b] β_{12} and β_{23} represent protein from β fractions A and C, respectively (Figure 3). [c] Composition of γ fractions A and B (Figure 3) did not differ significantly.

predicted is within experimental error. Additional confidence can be placed in these identifications by the fact that the chromatographic positions of the various β components are the same as would be predicted on the basis of their constituent α chains, taking into account the retarding effect of the doubled molecular weight.

The γ fraction chromatographed as a broad peak in the position expected for γ_{123}. Amino acid analysis showed it to be the same as predicted for this component and the same as found for whole unfractionated collagen (Table III).

It is clear from the chromatograms in Figure 3 that the chromatographic behavior of the unfractionated collagen can be fully accounted for by the sum of the separated α, β, and γ fractions.

Molecular Weight. The molecular weight and evidence of homogeneity of the α chains was obtained by high-speed sedimentation equilibrium (Yphantis, 1964). Samples prepared by recycling chromatography, chromatography on carboxymethylcellulose, desalting, and redissolution in solvent routinely gave weight average molecular weights in the range 70,000–80,000 and showed evidence of marked heterogeneity (Figure 5). It was further found that at pH 4.8 and room temperature the molecular weight fell as the sample was centrifuged. This was unexpected since previous experiments with α chains from rat skin collagen (Lewis and Piez, 1964a) indicated reasonably good stability at pH 4.8 and 40°. To obtain better values, measurements were made in 1 M calcium chloride, pH 7.0, at 10°. The best results were obtained when samples were taken directly from the chromatographic effluent without desalting and dialyzed against the calcium chloride solvent. Under these conditions higher molecular weights and evidence for reasonably good homogeneity were obtained (Figure 5).

Samples of $\alpha 1$, $\alpha 2$, and $\alpha 3$ gave similar results. Observed weight-average molecular weights ranged from approximately 90,000 to 100,000 and were independent of concentration and speed of rotation within experimental error. Typical results are shown in Table IV. M_z averaged about 10% higher than M_w. This can probably be attributed to the presence of some β component since the effect is greatest in $\alpha 2$ and $\alpha 3$ which would be contaminated with any β component remaining after molecular sieve chromatography. M_w at zero concentration in the cell ($y = 0$) was usually somewhat lower than M_w for the whole cell, indicating the presence of some low molecular weight material. This might be expected since the α chains are not very stable under the conditions used for chromatography on carboxymethylcellulose. Since there is probably some contamination with both high and low molecular weight material, M_w calculated for the whole cell probably gives the best value of the actual molecular weight of the α chains. This value, 95,000, is in good agreement with the value of 98,000 obtained for α chains from rat skin collagen (Lewis and Piez, 1964a).

TABLE IV: Molecular Weight of α Chains from Codfish Skin Collagen.[a]

	M_w		
	Whole Cell	At $y = 0$	M_z $(r = b)$
19,160 rpm			
$\alpha 1$	87,000	79,400	97,600
$\alpha 2$	95,100	82,600	109,500
$\alpha 3$	97,300	86,000	113,300
24,630 rpm			
$\alpha 1$	95,100	94,400	96,600
$\alpha 2$	96,300	81,700	126,900
$\alpha 3$	99,300	103,800	89,800
Average	95,000	88,000	105,500

[a] Initial protein concentrations were approximately 0.1 mg/ml in 1 M calcium chloride, pH 7.0.

Discussion

The finding of three different α chains in denatured collagen from codfish skin is most readily explained by assuming that the native collagen consists of a single molecular species in which each molecule contains three different α chains. The possibility that two or more collagens are present or that the α chains are randomly distributed among the molecules seems unlikely for two reasons. First, the presence of equal amounts of the three α chains indicates a stoichiometric relationship. Second, the finding that the cross-linked components, which in this kind of sample would arise largely from intramolecular cross-links (Bornstein *et al.*, 1964), consist largely of β_{13}, β_{12}, β_{23}, and γ_{123}, while cross-linked components containing identical α chains are largely absent, suggests strongly that two identical α chains do not occur together in the same molecule. The small amount of β_{11} found is consistent with a small degree of intermolecular cross-linking. β_{22} and β_{33} may also be present in small amounts but were not identified. The possibility that one of the α chains might be an artifact arising from another α chain by some minor modification does not seem possible in view of the very different amino acid compositions of the three α chains.

All collagens which have been previously studied by chromatographic methods have been shown to contain only two chromatographically separable α chains. Therefore, codfish skin collagen must be considered unique or unusual in this regard. However, the question is raised whether the difference lies in the fact that the two chains in the $\alpha 1$ fraction of other collagens are actually identical or are different but chromatograph together owing to a similar molecular weight and net charge. Evolutionary considerations suggest that a property as fundamental as the nonidentity of the constituent chains of a protein molecule would be common to all species, certainly to those that are closely related.

Chemical cleavage with cyanogen bromide at the methionine residues of $\alpha 1$ and $\alpha 2$ from rat skin collagen supports the idea that the $\alpha1$ fraction contains two different chains. Preliminary studies (Bornstein and Piez, 1965) found approximately twice as many peptides from $\alpha1$ as would be predicted from the methionine content while $\alpha2$ yielded approximately the predicted number. Although strongly suggestive, additional studies are necessary to further characterize the peptides before this can be considered conclusive evidence of the nonidentity of all three α chains in collagens other than codfish skin collagen.

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