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ANOMALIES IN THE ELECTROPHORETIC MOLECULAR-WEIGHT DETERMINATION OF COLLAGEN DENATURATION PRODUCTS

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SUMMARY

It has been demonstrated that the α_1-and α_2-chains of collagen give parallel lines in Ferguson plots and therefore have identical retardation coefficients during polyacrylamide gel electrophoresis. Hence it is proposed that both of these polypeptide chains are of identical molecular weight. The electrophoretic separation reflects the charge difference between these two chains. The optimal separation conditions are therefore obtained at zero gel concentration; it has been proved that the best separations are obtained in practice in 3.0% gels. The charge of a higher polymer is the sum of charge contributions of individual α-chains constituting such a polymer. Hence, if the Ferguson plot of a given polymer is known, it is possible to determine the composition of another polymer in terms of the α-chains, provided that the Ferguson plot of the latter is parallel. The electrophoretic mobilities of collagen polypeptide chains, as well as fragments and polymers in SDS gels, differ substantially from a standard calibration series of globular proteins, which therefore cannot be used for molecular-weight determinations. The reason why the charge difference between α_1- and α_2-chains cannot be eliminated by using SDS remains to be elucidated.

INTRODUCTION

Polyacrylamide gel electrophoresis appears to be the most reliable and simple procedure for the determination of the molecular weight of proteins. Since the publication of the classical paper of Osborn and Weber[1], it has been well established that there is a linear relationship between the molecular weight and electrophoretic mobility. This relationship holds with an adequate degree of accuracy for substances with molecular weights of up to 100,000 daltons. For large molecules, separation on diluted gels is necessary because otherwise these molecules are easily retained at the start. In the particular case of collagen denaturation products, sodium dodecyl-

sulphate (SDS) polyacrylamide gel electrophoresis has been used by Furthmayr and Timpl[2].

The collagen molecule is composed of two types of polypeptide chains which do not differ appreciably in their amino acid composition and have a common over-all building scheme. Therefore, the chain polymers assayed are of high internal homogeneity. In such a situation, the presence of SDS should be unimportant for the final resolution. Fundamental data about the molecular weights of individual α-chains of collagen were derived by Sykes and Bailey[3]; these workers proved that collagen extracted from lathyritic porcine aorta was clearly separated into two α-components during SDS-polyacrylamide gel electrophoresis. The α_1- and α_2-chains, being so similar chemically, are unlikely to bind different amounts of SDS. It is equally unlikely that SDS–collagen complexes would adopt different conformations. If, then, the difference in mobilities of the α-components is not due to conformational anomalies, it should necessarily reflect a true difference in molecular weight between α_1- and α_2-chains. This difference was established by Sykes and Bailey[3] to be 6000 daltons and the corresponding molecular weights of α_1- and α_2-chains were determined to be 100,000 and 94,000 daltons, respectively.

Although this evidence is only indirect, its validity can be substantiated by the findings of Reynolds and Tanford[4] that all proteins, regardless of their compositions, bind identical amounts of SDS and adopt similar conformations.

The procedure of Sykes and Bailey[3] enables one also to differentiate between β_{11}- and β_{12}-fractions, which means that this technique may reveal a difference of 6000 daltons in proteins of molecular weight 200,000 daltons; however, it is not possible to separate higher polymers of α-chains as these migrate as a single band with a low electrophoretic mobility.

It seemed attractive, therefore, to establish whether or not it is possible (1) to verify that there is a difference in molecular size between α_1- and α_2-chains which can be reflected by different retardation coefficients; (2) to make a suitable choice of conditions under which even the higher polymers of α-chains would be separated; (3) to establish the composition of these polymers in terms of α-chains according to their electrophoretic mobility; and (4) to obtain identical results with proteins of high internal homogeneity on polyacrylamide gel electrophoresis both with and without SDS.

EXPERIMENTAL

For estimating the molecular properties of collagen polymers, two kinds of polyacrylamide gel electrophoresis were used, in acidic and alkaline media.

In acidic media (without SDS)

These separations were carried out in tubes of dimensions 0.5×8 cm or 0.5×15 cm at 4 mA per gel. β-Alanine–acetic acid buffer (31.2 g of β-alanine and 9.0 ml of acetic acid per litre; pH 4.5) was used in the electrode vessels. Polyacrylamide gels were prepared as described by Reisfeld[5]. Lower gel preparations were made according to the desired gel concentration (Table I). The gels were stained with 1% amido black in 7.5% acetic acid. The staining solution was used in the cathode vessel and after the dye front had passed the gel the dye solution was replaced by

TABLE I

SCHEME FOR MIXING OF STOCK SOLUTIONS FOR GELS OF VARIOUS CONCENTRATIONS

Gel concentration (%)	*Stock solution**			
	A (ml)	*C (ml)*	*F (ml)*	*H (ml)*
3	2.0	2.4 + 1.6 water	6.0	4.0
4	2.0	3.2 + 0.8 water	6.0	4.0
5	2.0	4.0	6.0	4.0
6	1.84	4.8	5.52	3.84
7	1.33	6.0	5.0	3.68

* (A) 24 ml of 1 *N* KOH + 8.6 ml of glacial acetic acid + 2 ml of N,N,N',N'-tetramethylenediamine + water to 50 ml.
(C) 10 g of acrylamide + 0.4 g of N,N'-methylenebisacrylamide + water to 50 ml.
(F) 180 mg of ammonium persulphate + water to 100 ml.
(H) 48 mg of of urea + water to 100 ml.

7.5% acetic acid. It should be noted that before the dyeing operation and during the destaining of gels, it is necessary to reverse the polarity of the electrodes. During separations, the upper electrode vessel is connected to the positive terminal.

In alkaline media (with SDS)

This procedure was carried out according to Furthmayr and Timpl[2]. A 14-g amount of acrylamide and 0.37 g of methylenebisacrylamide were dissolved in 50.0 ml of 0.2 *M* phosphate (pH 7.2) and 10.0 ml of 2% SDS were added. After adding 0.1 ml of N,N,N',N'-tetramethylenediamine, the mixture was diluted to 100 ml with distilled water. Equal volumes of this solution and of ammonium persulphate solution (150 mg of ammonium persulphate in 100 ml of 0.1 *M* phosphate; pH 7.2) were used for the preparation of a 7.0% gel. Correspondingly lower amounts of acrylamide and the bisacrylamide were used for lower gel concentrations. Tubes of 0.9 × 10 cm were used at 6 mA per gel. The upper electrode vessel was connected to the negative terminal.

Gels were stained with Coomasie brilliant blue (1.25 g per 46 ml of glacial acetic acid and made up to 500 ml with 50% methanol). For destaining, a solution of 75 ml of glacial acetic acid plus 50 ml of methanol made up to 1000 ml with distilled water was used. The staining procedure followed that described by Osborn and Weber[1] and appropriate corrections were introduced so as to eliminate changes in gel length and band displacement during the staining procedure.

The collagen used was prepared from calf skin as described by Rubin *et al.*[6]. Individual α-chains were isolated by combined chromatography on Sepharose 4B and carboxymethylcellulose (Piez *et al.*[7]). α-Chains (II) from chicken sternal cartilage were prepared according to Miller and Matukas[8]. Individual α-chains were subjected to cyanogen bromide cleavage[9] and some of the resulting peptides were isolated on a carboxymethylcellulose column[10]. Polymers of individual α-chains were obtained

by adding a 1% solution of formaldehyde dropwise to the samples as described by Veis and Drake[11].

RESULTS AND DISCUSSION

In the early stage of this work it was observed that the plot of the logarithm of the molecular weight of collagen polypeptide chains *versus* the electrophoretic mobility is not exactly a straight line, assuming molecular weights for the α_1- and α_2-chains of 100,000 and 94,000 daltons, respectively. These deviations occur both in the presence and in the absence of SDS and become more distinct in more diluted gels, as shown in Fig. 1. It is also obvious from Fig. 2 that the α_1-chain, as well as both its polymers and fragments, shows a straight-line dependence of the logarithm of the molecular weight on the electrophoretic mobility, which differs from a similar relationship for polymers and fragments derived from the α_2-chain. Neither of these straight lines fits the calibration of polyacrylamide gel with standard protein series of known molecular weight. Similar differences in electrophoretic behaviour have already been observed with cyanogen bromide fragments of α_1- and α_2-chains by Furthmayr and Timpl[2]. These workers stated that the differences between standard calibration series and collagen fragments reflect steric hindrances rather than changes in the ability to bind SDS. This may be true when comparing the standard calibration series with collagen polymers or fragments, but it is unlikely that these hindrances affect the different behaviour of α_1- and α_2-chains because the distributions of amino acids of both chains are very similar (Bornstein and Piez[12]).

The Ferguson plots shown in Fig. 2 indicate that straight lines corresponding to α_1- and α_2-chains are parallel; it can therefore be concluded that the retardation

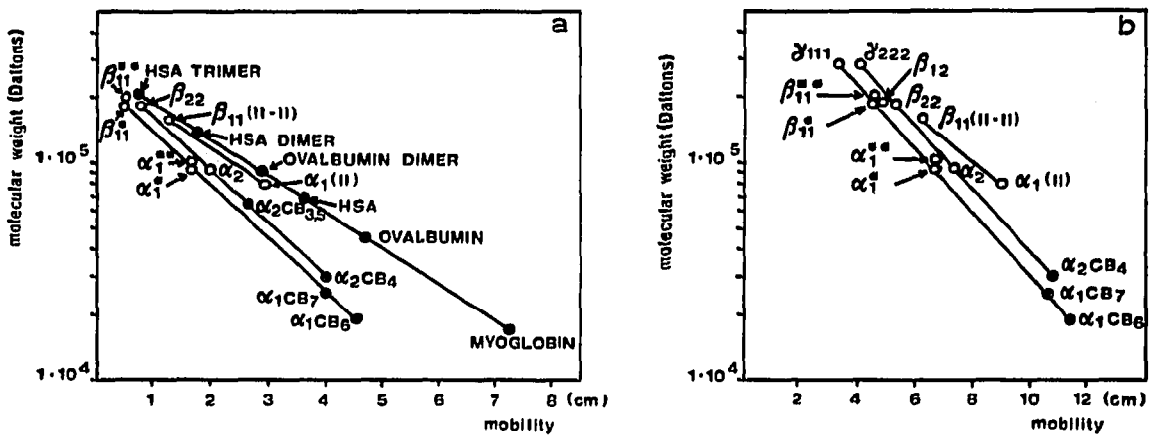
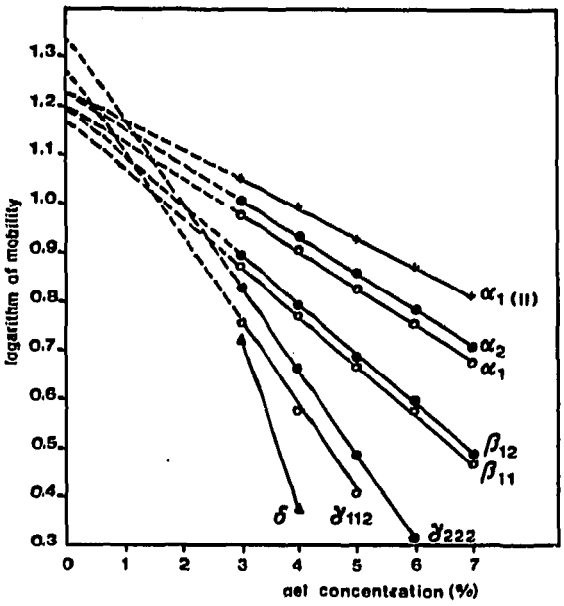


Fig. 1. Semilogarithmic plot of migration distance *versus* molecular weight for various cyanogen bromide peptide denaturation products of collagen and α-chain polymers: (a) in the presence of SDS, 5% gels, and (b) in the absence of SDS, 5% gel. ●, Standards of known molecular weights; ○, chemical entities, the molecular weights of which are estimated by polyacrylamide gel electrophoresis; *, molecular weights of α_1- and β_{11}-chains considered to be 94,000 and 188,000 daltons, respectively; **, molecular weights of α_1- and β_{11}-chains considered to be 100,000 and 200,000 daltons, respectively. HSA = Horse serum albumin.



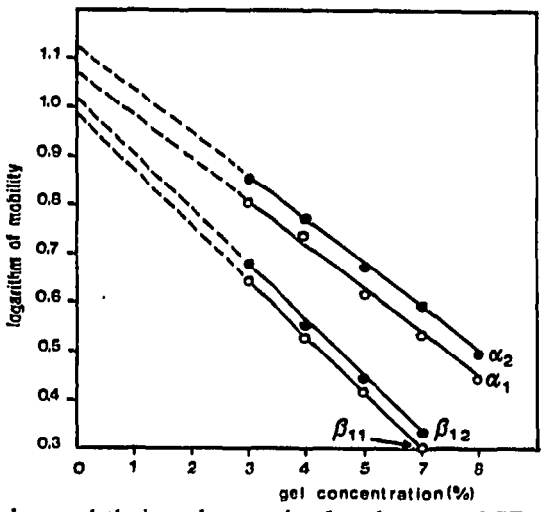


Fig. 2. Ferguson plots of individual collagen α-chains and their polymers in the absence of SDS.

Fig. 3. Ferguson plots of individual collagen α-chains and their polymers in the presence of SDS.

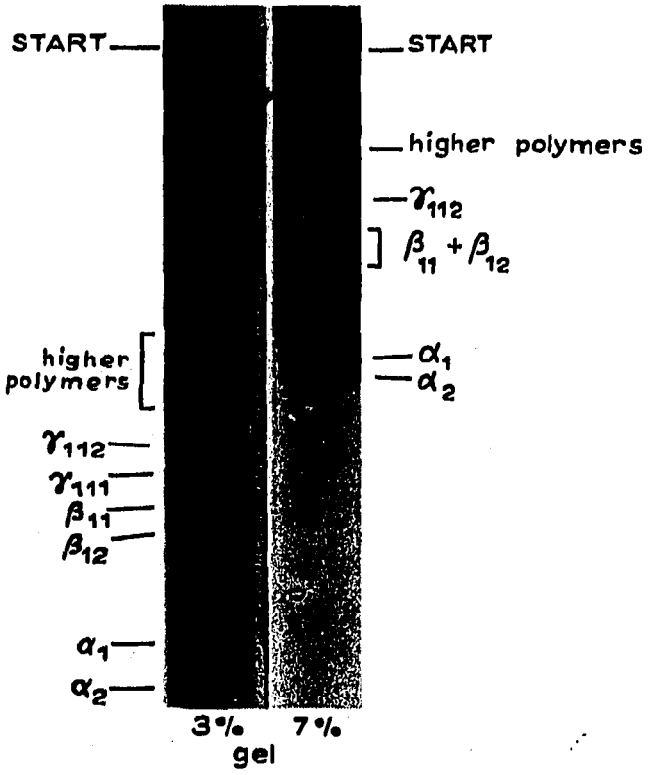


Fig. 4. Polyacrylamide gel electrophoresis patterns of chain polymers containing collagen samples.

coefficients (the slopes of these lines) are identical and therefore both of these polypeptide chains should be equal in molecular size. The same applies to the Ferguson plots obtained in the presence of SDS, as indicated in Fig. 3. It appears that the difference in electrophoretic mobility is the result of a charge difference represented by the antilogarithm of the difference in the y-intercepts of these two lines. It has been demonstrated by Chrambach and Rodbard[13] that for the separation of substances with parallel lines on a Ferguson plot, the optimal separation is obtained at zero gel concentration, which in practice is approximated by a 3% gel with 20% cross-linkages. This is demonstrated in Fig. 4.

For polymers composed of α_1- and α_2-chains, the dependence of the electrophoretic mobility on the logarithm of the molecular weight falls between the two extreme lines given by polymers consisting of α_1- or α_2-chains only, *e.g.*, the point for the β_{12}-fraction lies mid-way between these extreme lines.

TABLE II

DIFFERENCES IN ELECTROPHORETIC MOBILITIES BETWEEN VARIOUS COLLAGEN FRACTIONS AT ZERO GEL CONCENTRATION

Extrapolated values from Ferguson plots.

Collagen fractions	*Difference in mobility (cm)*		*Theoretical assumption in multiples of the differences of mobility, α_2 versus α_1*
	Gel with SDS	*Gel without SDS*	
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

The difference between the above antilogarithms for α_1- and α_2-chains at zero gel concentration equals the difference between the antilogarithms for β_{11}- and β_{12}-chains, and similar relationships hold for other types of α-chain polymers, as indicated in Table II. Also, there is no difference whether the separation is carried out in the presence or in the absence of SDS, which means that the separation of α_1- and α_2-chains is the result of the charge difference which, however, cannot be eliminated by using SDS. Identical values of the retardation coefficients indicate that the two types of α-chain are of identical molecular size. The charge difference being constant for single α_1- and α_2-chains, as well as for different combinations of these chains in polymers, enables the composition of an unknown zone to be determined in terms of α_1- and α_2-chains, provided that the Ferguson plot for one polymer of known composition can be determined. Thus, for example, a line parallel to the γ_{112} line which differs by two increments of the $\alpha_1 - \alpha_2$ difference in free mobility must necessarily be a trimer composed of three α_2-chains. As there is no marked influence of SDS upon the final separation pattern, it might seem appropriate to

suggest that all separations could be carried out in acidic media, in which the runs are faster and also staining and destaining can be achieved more rapidly. However, according to the recent results of Davison *et al.*[14] on the redistribution of labile cross-links in acidic media, this procedure cannot be generally recommended unless the labile cross-links are stabilized before electrophoresis by borohydride reduction.

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