CHROM. 5907

## A convenient gel chromatography method for determining the molecular weight distributions and averages of synthetic polypeptide collagen models

Polydisperse synthetic sequential polypeptides such as poly-(L-alanylglycyl-L-proline) have been and are involved in diverse studies as simplified models of collagen, the component chains of which have a high content of glycine and imino acids, with glycine situated with practically uninterrupted regularity at every third position. The topics in collagen chemistry and biology which have been approached through investigations of sequential polypeptide models include conformational studies in solution (*e.g.* refs. 1 and 2) and in the solid state (*e.g.* refs. 3–5), the action of enzymes for which collagen is the natural substrate (*e.g.* refs. 6 and 7), and the immunology of collagen[8].

It is clearly essential that the characterization of such materials must be thorough and reliable if the conclusions drawn from conformational or biological studies on them are to be accepted with confidence*. Among the items of characterization which are crucial in the case of collagen models are the molecular weight distributions and averages, since both conformational and biological properties are molecular weight dependent[1–6,8,10]. Unfortunately, determination of the molecular weight averages of polydisperse synthetic polypeptides by conventional means (osmometry, ultracentrifugation, etc.) is a tedious and often unreliable undertaking. Gel chromatography has the advantage of simplicity of operation and also the highly attractive feature that it provides a means of determining simultaneously not only the number and weight averages but also the molecular weight distribution[11,12]. Gel chromatography has been applied to synthetic collagen models in several reported instances but generally for fractionation purposes rather than molecular weight determination[1,2,6–8]. This incomplete realization of the potentialities of the technique would appear to be due to the lack of a suitably calibrated system.

We are currently engaged in the synthesis and characterization for immunological studies of an extensive series of collagen models of the general formula H–(Ala-Gly-X)$_n$–OH where X is an imino acid. We required a means of determining the molecular weight distributions and averages of these materials routinely, and have therefore devised a generally applicable calibrated gel chromatography system for this purpose.

It is now well established that the determination of molecular weights by gel chromatography is only reliable if the substances employed for calibration are chosen so as to be as closely related as possible in composition and molecular shape to the material under investigation. When the molecular weight of a typical enzymic protein is to be determined, these requirements are met if denaturing media are used in conjunction with globular proteins of known molecular weight as calibrants. Provided all the materials are in "random" conformations, effects due to differences in the shapes of the native proteins are eliminated. Implicit in this method is the assumption that the "random" conformation of one protein is similar to that of any other. This seems a fair assumption only if the amino acid compositions are similar.

* DETAR has given a critical discussion of this matter[9].

Collagen and peptides related to it are, however, highly atypical in their composition. The high content of glycine and proline, both of which have special stereochemical roles, probably invalidates the use of "random" enzymic proteins as calibrants for the determination of the molecular weight of a "random" collagen peptide by gel chromatography. In the related technique of sodium dodecylsulphate–polyacrylamide gel electrophoresis[13] this is certainly the case. PIEZ[14] has described two gel chromatography systems for the determination of molecular weights of collagen peptides which are not open to objections of this kind. These involve columns of Bio-Gel P-150 or A-1.5 calibrated with independently characterized cyanogen bromide fragments of collagen and eluted with 1 *M* calcium chloride. Excellent linear correlations of the logarithm of the molecular weight with elution volume were obtained. These systems would have been ideal for our purpose except that most of our polymers are insoluble in all except mixtures containing high proportions of carboxylic acids. We have therefore modified PIEZ's Bio-Gel P-150 system by changing the solvent to 50% acetic acid, in which almost all our materials dissolve easily.

*Experimental*

A column (79 × 1.2 cm) of Bio-Gel P-150 (100–200 mesh, control number 5592) was made up in 50% acetic acid, stabilized by the passage of several bed volumes of solvent, and calibrated using a series of collagen cyanogen bromide fragments of known molecular weight provided by Dr. K. A. PIEZ and some low molecular weight oligomers of L-alanylglycyl-L-proline synthesized in this laboratory. The column was eluted at a rate of 3 ml/h, fractions of 1.3 ml being collected; the void volume was determined with Blue Dextran 2000 and found to be 22.0 fractions

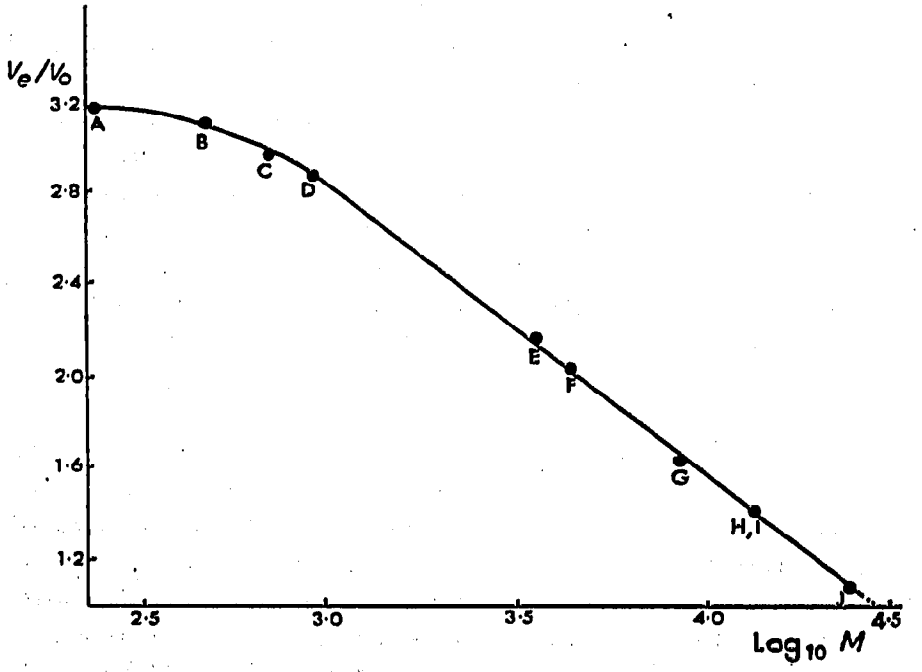


Fig. 1. Calibration of the column of Bio-Gel P-150 in 50% acetic acid. The calibrants were: A–D = H–(L-Ala-Gly-L-Pro)$_n$–OH where $n$ = 1 ($M$ = 243), 2 ($M$ = 468), 3 ($M$ = 693) and 4 ($M$ = 918), respectively; E–H = cyanogen bromide peptides α1-CB5 ($M$ = 3,600), α1-CB4 ($M$ = 4,400), α1-CB6A ($M$ = 8,500), and α1-CB3 ($M$ = 13,500), respectively, of chick bone collagen; I and J = cyanogen bromide peptides α1-CB3 ($M$ = 13,500) and α1-CB7 ($M$ = 24,500), respectively, of rat skin collagen.

(28.6 ml); the elution volume of each calibrant was determined by monitoring the eluant polarimetrically after application of 1.5 mg of the calibrant dissolved in 0.2 ml of 50% acetic acid. The results of this calibration are presented graphically in Fig. 1. Comparison with the results obtained by PIEZ[14] shows that the effect of changing the solvent from 1 *M* calcium chloride to 50% acetic acid on the behaviour of Bio-Gel P-150 towards collagen peptides is small. The linear portion of Fig. 1 is described by the equation

$$\log_{10} M = 5.2 - \left(0.78 \frac{V_e}{V_0}\right),$$

whereas the linear portion of the graph given by PIEZ[14] corresponds to

$$\log_{10} M = 5.2 - \left(0.66 \frac{V_e}{V_0}\right).$$

For the investigation of the molecular weight distribution of a synthetic sequential polypeptide collagen model *ca.* 10 mg of the polymer were dissolved in *ca.* 0.5 ml of 50% acetic acid by warming at 80° for 1 min, and this solution was applied to the calibrated column immediately after rapid cooling. Elution conditions were as for the calibration runs. The relative amounts of polymer in each fraction were determined by ninhydrin estimation after total alkaline hydrolysis or, when it could be demonstrated that the optical rotation was sufficiently high and dependent only on concentration (*i.e.* not molecular weight dependent), by polarimetry. Polarimetry was, when applicable, the more convenient and reproducible technique. A typical elution profile obtained with a preparation of poly-(L-alanylglycyl-L-proline) using polarimetry is shown in Fig. 2. In this case it was shown that the optical rotation of each fraction was directly proportional to the amount of polymer present by ninhydrin determinations on selected fractions and by the determination of the specific rotations in 50% acetic acid of five preparations of the same polymer of weight average molecular weight 2000, 3000, 6000, 11,000 and 15,000. All had

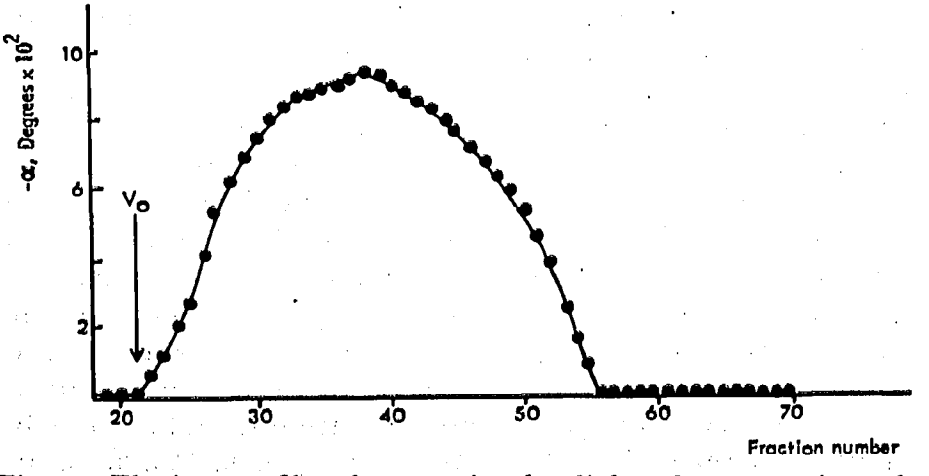


Fig. 2. Elution profile of a previously dialyzed preparation of poly-(L-alanylglycyl-L-proline) on the calibrated column. The sample applied contained 8 mg in 0.5 ml. Optical rotations were measured at 436 nm in a 1-dm cell at 20°.

$[\alpha]_{436}^{20}$ — 500 ± 15° (*c* 0.1, 50% AcOH). Since the amount of polymer in the *i*th eluant fraction is therefore proportional to the optical rotation $\alpha_i$, the proportion of the total weight (weight fraction $w_i$) present was calculated from $w_i = \alpha_i/\Sigma\alpha_i$. The molecular weight $M_i$ corresponding to each fraction was available from Fig. 1,

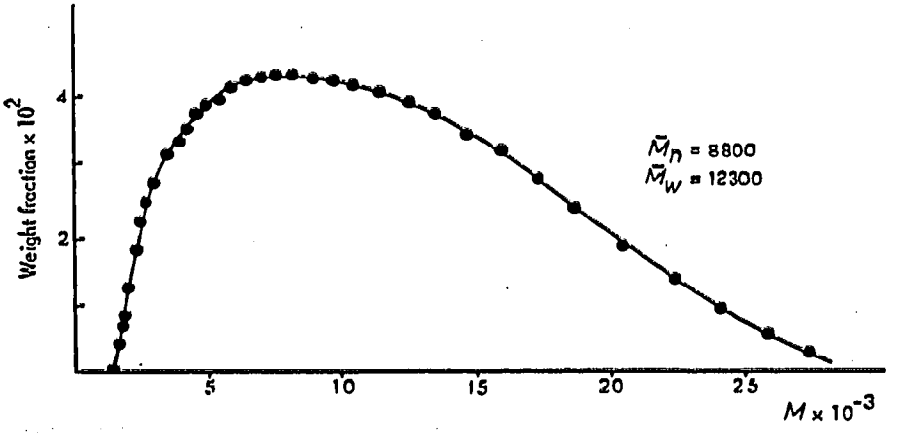


Fig. 3. Differential molecular weight distribution curve derived from Fig. 2.

and it was therefore possible to derive a differential molecular weight distribution curve (Fig. 3) and to calculate the number average $\bar{M}_n$ and weight average molecular weights $\bar{M}_w$ from

$$\bar{M}_n = \frac{\Sigma\alpha_i}{\Sigma(\alpha_i/M_i)}$$

and

$$\bar{M}_w = \frac{\Sigma(\alpha_i M_i)}{\Sigma\alpha_i}.$$

*Results*

Since the calibrants are so similar in composition and sequence to the polymers under investigation, errors due to deviations from the correlation shown in Fig. 1 are probably negligible, but we recognize two other sources of error in this method. The first, which is general in molecular weight distribution analysis by gel chromatography, stems from the fact that the naive treatment we have adopted assumes that all species of a given molecular weight elute in an infinitely sharp band. The result of this assumption is that the apparent molecular weight distribution is slightly broader than the true distribution. Several sophisticated mathematical procedures have been devised to correct for this deficiency, but the errors due to the neglect of such corrections are small except for very narrow distributions[11,12]. The second possible source of error is more peculiar to the sequential polypeptide collagen models in which we are interested. These materials are prone to aggregation in solution[1,2]. The presence of high molecular weight aggregates in the solution applied to the column or the occurrence of aggregation during elution would obviously result in false molecular weight distributions and erroneous averages. When preparations of poly-(L-alanylglycyl-L-proline) of high average molecular weight such as that used in Fig. 2 were dissolved in 50% acetic acid without heating and applied

to the calibrated column, the presence of high molecular weight aggregates was indicated by the appearance of considerable material in the void volume. However, brief heating at 80° followed by rapid cooling to room temperature immediately before application to the column (our routine procedure) sufficed for complete dissociation, as judged by the absence of excluded material from the chromatograms. Gel chromatography and ultracentrifuge criteria indicated that reaggregation after such treatment was slow in dilute solutions, and we are satisfied that the molecular weight distributions obtained by our method are not seriously distorted by aggregation phenomena.

As an independent check on the method, we have compared the weight average molecular weights obtained in this way with those obtained by ultracentrifugation using three different preparations of poly-(L-alanylglycyl-L-proline). The sedimentation equilibrium method was used on 0.3% solutions in 50% acetic acid at 20° which had been heated briefly at 80° to disaggregate them immediately before analysis. This gave results of 6000, 11000 and 14000 on preparations which had weight average molecular weights by gel chromatography of 6000, 11000 and 15000. Such close agreement must be regarded as to some extent fortuitous since the ultracentrifuge results are uncertain to an extent of at least ± 10%, mainly because of uncertainty in the partial specific volume — a value of 0.72 was used in these determinations. Comparison of independently obtained number average molecular weights with those obtained by gel filtration proved impossible because our polymers were insoluble in solvents suitable for use in the osmometer at our disposal and end-group labelling by dinitrophenylation of suspensions gave absurdly exaggerated estimates. However, it does appear from the ultracentrifuge results that the molecular weight distributions and averages of synthetic sequential polypeptide collagen models can be determined by the method exemplified above without gross error. We have found it convenient and reproducible in applications involving many preparations of poly-(L-alanylglycyl-L-proline) and a series of analogues of this sequence, comprising all the possible diastereoisomers and polymers in which proline is replaced by another imino acid. Only in the case of poly-(L-alanylglycyl-L-thiazolidine-4-carboxylic acid) did inadequate solubility in 50% acetic acid render the method inapplicable. In contrast to Sephadex G-150 in 50% acetic acid[15], columns of Bio-Gel P-150 in this solvent remain unchanged after several months at room temperature.

We are grateful to Dr. K. A. PIEZ for providing generous samples of the collagen cyanogen bromide peptides used as calibrants, to Dr. I. O. WALKER for ultracentrifuge facilities, and to the Medical Research Council for the maintenance grant held by R.F.

*The Dyson Perrins Laboratory,*
*University of Oxford (Great Britain)*

R. FAIRWEATHER
J. H. JONES
J. K. WILCOX

1 J. ENGEL, J. KURTZ, E. KATCHALSKI AND A. BERGER, *J. Mol. Biol.*, 17 (1966) 255.
2 D. M. SEGAL, *J. Mol. Biol.*, 43 (1969) 497.
3 A. YONATH AND W. TRAUB, *J. Mol. Biol.*, 43 (1969) 461.
4 W. TRAUB, *J. Mol. Biol.*, 43 (1969) 479.
5 D. M. SEGAL, W. TRAUB AND A. YONATH, *J. Mol. Biol.*, 43 (1969) 519.

6 D. J. PROCKOP, K. JUVA AND J. ENGEL, *Z. Physiol. Chem.*, 348 (1967) 553.
7 E. ADAMS, S. ANTOINE AND A. GOLDSTEIN, *Biochim. Biophys. Acta*, 185 (1969) 251.
8 F. BOREK, J. KURTZ AND M. SELA, *Biochim. Biophys. Acta*, 188 (1969) 314.
9 D. F. DETAR in H. C. BEYERMAN, A. VAN DE LINDE AND W. MAASSEN VAN DEN BRINK (Editors), in *Peptides*, North-Holland, Amsterdam, 1967, p. 125.
10 S. SAKAKIBARA, Y. KISHIDA, Y. KIKUCHI, R. SAKAI AND K. KAKIUCHI, *Bull. Chem. Soc. Jap.*, 41 (1968) 1273.
11 H. DETERMANN, *Gel Chromatography*, Springer, Berlin, 1968, p. 120, and references there cited.
12 L. FISCHER, in T. S. WORK AND E. WORK (Editors), *Laboratory Techniques in Biochemistry and Molecular Biology*, Vol. 1, North-Holland, Amsterdam, 1969, p. 291, and references there cited.
13 H. FURTHMAYR AND R. TIMPL, *Anal. Biochem.*, 41 (1971) 510.
14 K. A. PIEZ, *Anal. Biochem.*, 26 (1968) 305.
15 R. FAIRWEATHER AND J. H. JONES, *J. Chromatogr.*, 58 (1971) 285.

Received December 15th, 1971