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FURTHER EXPERIMENTS ON A NEW, FAST METHOD FOR DETERMINING MOLECULAR WEIGHTS OF DIFFUSING SPECIES IN A LIQUID PHASE

CONRAD N. TRUMBORE*, MARK GREHLINGER, MICHELE STOWE and FRANCES M. KELLEHER
Department of Chemistry, University of Delaware, Newark, DE 19716 (U.S.A.)
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SUMMARY

Further experiments have been conducted on a previously reported method for determining empirically the molecular weight of a series of proteins on the basis of the shape of the concentration profile of the protein sample exiting from a capillary after injection of that sample in plug form into a mobile phase which exhibits laminar flow in the capillary. The method depends upon the influence of the relative amounts of convection and radial molecular diffusion in determining the shape of the concentration *vs.* time profile of the capillary effluent detected by a concentration detector at the capillary outlet. Two times on the concentration profile are chosen to reflect regions which are most strongly influenced by these two processes. The ratio, R, of the heights of the profile at these two times is correlated with molecular weights in an empirical fashion to construct a standard curve and the molecular weight of an unknown is found by interpolation. In the work reported here we have demonstrated the validity of this method for narrow-molecular-weight polystyrene standards in organic solvents as well as for a set of lower-molecular-weight, non polymeric species. On the basis of these and our previous experiments we believe the method to be general for any species which is able to undergo Brownian motion, whose diffusivity is a well behaved function of molecular weight for a series of compounds of interest, and which does not engage in the type of extreme aggregation or adsorption behavior which we have previously reported when high-molecular-weight biopolymers are subjected to high shear stresses in a narrow capillary. The chief advantages of the method are the speed and the low expense involved. Given a predetermined standard curve, the number average molecular weight of a polymeric system can be obtained in a matter of minutes. We suggest that, because of its simplicity and speed, this method be applied to kinetic studies of polymerization or depolymerization and to studies of the effects of various parameters, such as ionic strength and pH, on the state of aggregation of biopolymers and to other experiments where rapid determination or confirmation of molecular weight is needed.

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INTRODUCTION

In an earlier paper[1] we introduced the concept of determining the molecular weight of biopolymers by analyzing the shape of a chromatographic peak resulting from a plug injection of the chemical species of interest into a narrow capillary. The injection is made into a straight, stainless-steel capillary on the order of a meter long with an internal diameter of 0.25 or 0.50 mm. The capillary employed in our studies contained no packing material and did not have any special surface treatment or coating. We demonstrated that the nature of the double-peaked chromatograms for a single component, noted by previous investigators[2–5], could be employed at constant flow-rate of the mobile phase and at constant column length to reflect the different characteristic diffusion properties of the injected aqueous protein molecules in a capillary. We reasoned that some peak shape parameter should be empirically related to the molecular weight of the unknown species if the molecular shape of the molecular weight standards used to bracket an unknown were essentially the same as the unknown, as appeared to be the case with the proteins we had investigated. The peak shape parameter we found most convenient was the ratio of the heights of the peaks of the concentration profile at times reflecting predominantly the effects of convection and diffusion, respectively.

We have applied the above method to the characterization of the molecular weight of organic polymers and find the method again shows promise as a fast, empirical way to determine number average molecular weights. These preliminary investigations indicate the method may approach gel permeation chromatography in its accuracy and surpass it by an order of magnitude in speed for the determination of number average molecular weights.

The method is found also to be a convenient way to follow the rates of polymerization and depolymerization. We have also applied the method to the estimation of molecular weights of lower-molecular-weight, non-polymeric molecules and find that these species have much the same behavior as the higher-molecular-weight organic and biopolymers. On the basis of our investigations, we believe this empirical method for the determination of molecular weights is universal for those species which freely diffuse and do not aggregate[6] in the mobile phase which is undergoing laminar flow and for which there are suitable molecular weight standards.

EXPERIMENTAL

The apparatus used in this method is a conventional high-performance liquid chromatograph with the exception that the column is a 50–100 cm capillary tubing of the type ordinarily employed in high-performance liquid chromatography (HPLC) as connecting tubing. Our apparatus consisted of the following components: a Model 100 Altex HPLC pump, a 7000 psi Valco injection valve, a 0.025 or 0.5 mm I.D., 77 or 89 cm long stainless-steel capillary tube initially flushed with organic degreasing solvents, 1 *M* nitric acid, and distilled water, connected via a zero dead volume fitting to an LKB Uvichord S liquid chromatography detector operating at 254 nm. All polystyrene standards were obtained from Pressure Chemical. Injected sample concentrations were 5 mg/ml and sample volumes were 10 μl injections with a 10-μl Glenco microliter syringe unless otherwise stated.

RESULTS AND DISCUSSION

Details of the method

The method depends on the spatial concentration pattern produced by the relative amounts of convection and radial molecular diffusion of a plug sample injected into a solvent moving through a capillary with a parabolic flow profile. Fig. 1 illustrates the time development of the concentration profile of a sample injected under plug flow assuming no diffusion is allowed. The leading part of the sample, with an axial length "*l*", represents the central axial segment of the sample which generates the bulk of the leading edge of the concentration profile representing the time dependence of the concentration of the emerging sample detected downstream at the liquid chromatography detector. In a concentration *vs.* time profile for a non-diffusing sample, a nearly exponential tail of the profile following a sharp peak is due to successive elution of the parts of the sample which were initially injected increasingly closer to the capillary wall[7].

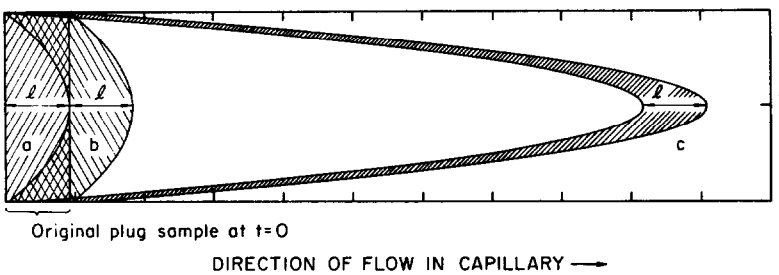

Fig. 1. Development as a function of time of a plug sample shown in cross section in (a) injected into a capillary with a mobile phase which maintains a parabolic flow profile. At later times the cross sectional profiles (b) and (c) of the sample are shown using the assumption of no diffusion, only convection of the sample with the mobile phase. *l* is the length of the capillary axis which is occupied initially by the injected sample.

Extensive diffusion of a sample as it passes through a capillary leads to a sample distribution pattern which is quite different from that obtained in the very low diffusion case and therefore leads to a radically different concentration profile. When diffusion dominates convection the usual gaussian concentration profile is obtained[7]. Under experimental conditions where convection and diffusion are both important, this complex diffusion pattern leads to chromatograms with double peaks or with a peak and a shoulder[2–5] such as those illustrated in Fig. 2. The "convection" peak, using our experimental apparatus and method, has been found empirically to be located from 1.2 to 1.3 times the initial peak breakthrough time, t_b. The peak of the "diffusion" component is taken as that found for a gaussian peak, namely 2.0 times the breakthrough time[7]. The heights of the chromatographic signal at these two times are defined as h_1 and h_2, respectively. Further, R, the ratio of these peaks is defined in eqn. 1 below:

$$R = h_1/h_2 \tag{1}$$

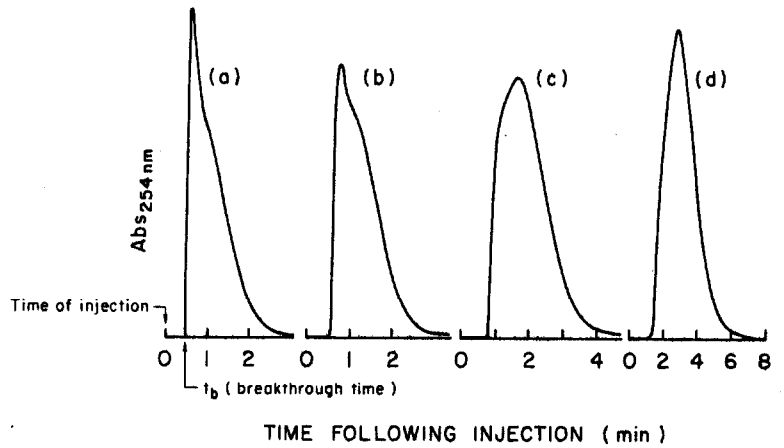

Fig. 2. Flow-rate dependence of elution profiles of a narrow-molecular-weight-range polystyrene standard injected into a stainless-steel capillary (85 × 0.050 cm I.D.). Injections (10 μl) of 5 mg/ml solution of a polystyrene sample whose number average molecular weight was 4000. Mobile phase and sample solvent were tetrahydrofuran. Flow-rates: (a) 0.25 ml/min; (b) 0.22 ml/min; (c) 0.15 ml/min; (d) 0.10 ml/min.

A standard curve of R values *vs.* molecular weight (or a non-linear function of molecular weight) is constructed and molecular weights of unknowns having similar hydrodynamic characteristics as the standards may be obtained by interpolation on the above standard curve. All determinations are carried out at a common flow-rate and in the same capillary column as that used in determining the standard curve.

Flow-rate dependence of R for a single component

The value of R (defined in eqn. 1) generally increases with flow-rate for a fixed capillary length. Fig. 3 illustrates the flow dependence behavior of R for three low-molecular-weight, low polydispersity polystyrene molecular weight standards. With a shorter residence time caused by a higher flow-rate in the capillary, the sample has less time for radial diffusion and the "convection peak" dominates the "diffusion peak", thus increasing the value of R. We have found that a linear or near linear dependence of R *vs.* mobile phase flow-rate is usually indicative of relatively few problems of the type we have previously reported[6] regarding polymer losses and other anomalous behavior when biological polymers are injected into flowing mobile phases in capillaries.

Fig. 4 illustrates the type of behavior found when the flow-rate dependent problems mentioned above are encountered. Such behavior commonly occurs with high-molecular-weight (> 50,000) polymers, especially in aqueous solutions. Our experiments reveal that biological polymers in aqueous solutions develop problems of this type at lower molecular weights than organic polymers of approximately the same molecular weight in organic solvents under otherwise identical conditions of flow-rate and column length. As we have previously reported[6], we believe phenomena such as those shown in Fig. 4 are caused by one or more of the following: flow-induced aggregation, diffusion toward lower shear rate zones, or shear-induced adsorption at the wall of the capillary.

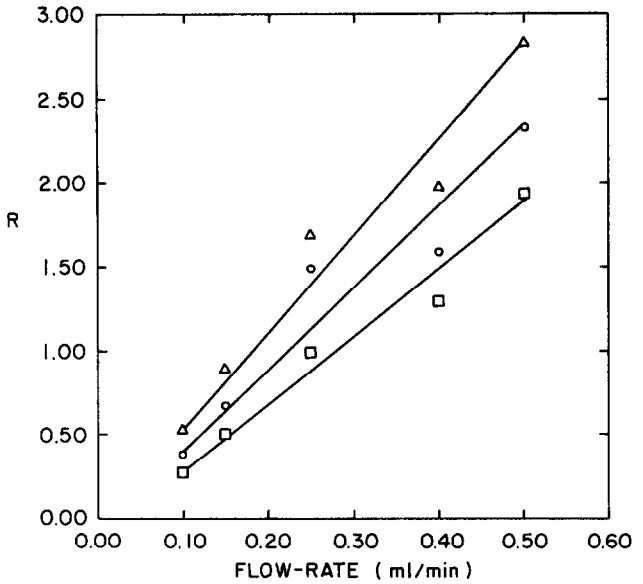

Fig. 3. Dependence of R values (see eqn. 1 and text for definition) on mobile phase flow-rate for three different narrow-molecular-weight-distribution polystyrene standards with weight average molecular weights of (△) 9000, (○) 4000 and (□) 2000. Mobile phase and sample solvent are both tetrahydrofuran. Same column as in Fig. 2. Sample injection volume 10 μl.

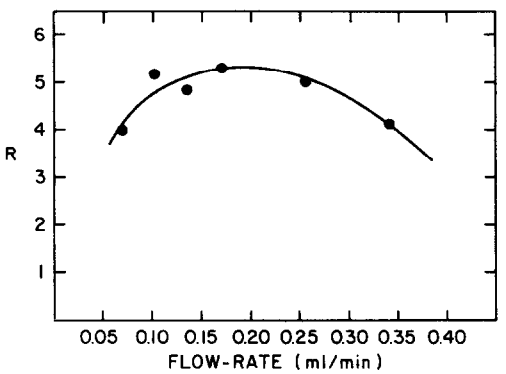

Fig. 4. Variation of R with mobile phase flow-rate indicating problems with non-conventional solute behavior during passage through a capillary. Sample: 1-μl injection of aqueous gamma globulin solution (5 mg/ml). Column: 77.5 × 0.025 cm I.D. Mobile phase: 0.1 M sodium acetate, 0.1 M sodium sulfate (pH 5.0).

Polystyrene standards

When aqueous solutions of narrow-molecular-weight-distribution, sulfonated polystyrene polymer standards (Pressure Chemical) were introduced into a number of different flowing aqueous mobile phases of different composition in a capillary, irreproducible results were obtained. However, when under the same flow-rate conditions similar non-sulfonated polystyrene samples were dissolved in and injected

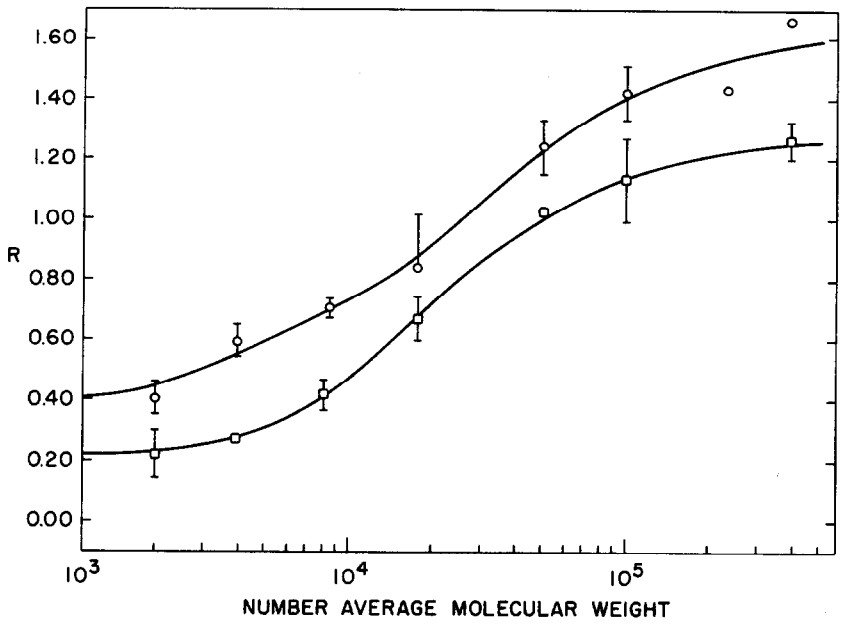

Fig. 5. Plots of *R vs.* log number average molecular weights for a number of polystyrene standards run under conditions of identical capillary length (85 cm) and diameter (0.050 cm I.D.), sample injection size (10 μl) and flow-rate (0.10 ml/min). Sample solvent and mobile phase: O, cyclohexane; □, tetrahydrofuran.

into two different organic mobile phases in the same capillary, quite reproducible results were obtained.

Fig. 5 illustrates the type of *R* value *vs.* log molecular weight plots obtained for the same set of polystyrene standards dissolved in two different organic solvents. Different *R* values are observed with different solvents, as might be anticipated since the diffusivities of the polymers are probably different in the two types of solvents. The implication from data in Fig. 5 is that for a given molecular weight the diffusion coefficient of the polystyrene standard is smaller in the more hydrophobic solvent. As illustrated in Fig. 6, the qualitative features of the elution curves are quite similar

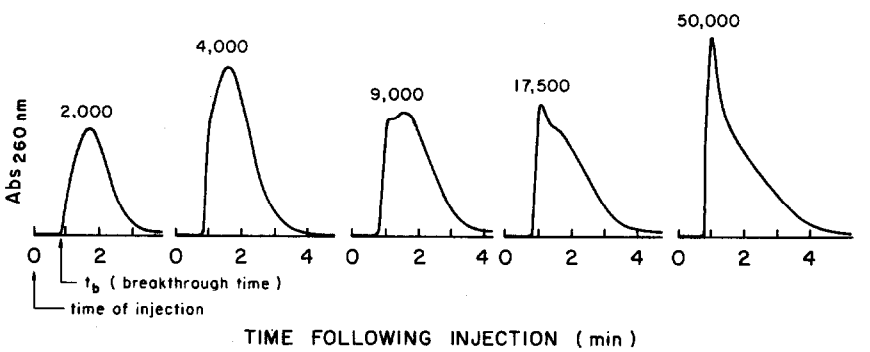

Fig. 6. Elution profiles of five different narrow-molecular-weight-distribution polystyrene standards injected into the same column as in Fig. 5 in separate experiments. Flow-rate, 0.15 ml/min; injection volume, 10 μl of polystyrene solutions (5 mg/ml) in tetrahydrofuran; mobile phase, tetrahydrofuran. Number average molecular weights of standards are listed above the curves.

to those previously obtained with biopolymers in aqueous solutions[1]. Again the shape of the elution curves is quite sensitive to molecular weight of the injected sample when the flow-rate of the mobile phase and the length of the capillary are both at critical values where double peak or peak plus shoulder behavior exists.

Ouano[8] reported similar types of elution concentration profiles with polystyrene standards but was unable to explain the bimodal nature of these profiles. The more recent explanations of this bimodal behavior[2-5] and unusual shear stress behavior of high-molecular-weight polymers near the capillary wall[6] readily account for the nature of the profiles shown in Fig. 6. With high-molecular-weight polymers there is again a limiting value of R where results become erratic and where indications are seen of polymer losses in the capillaries. Again plots of R values *vs.* molecular weights of polymer standards provide an opportunity to test the validity of interpolating R values of polymer unknowns to obtain molecular weights.

Effect of flow-rate on R values in standard curves

Fig. 7 illustrates the effect of flow-rate on the shape of the R *vs.* log molecular weight curves for polystyrene standards, using tetrahydrofuran as the sample solvent and mobile phase. The higher the flow-rate, the larger is the slope in the lower-molecular-weight regions. The flow-rate determines which molecular weight region will give chromatograms with two peaks or peaks with significant shoulders, since R values of such peaks will be in the neighborhood of 1.0. The R values in the molecular

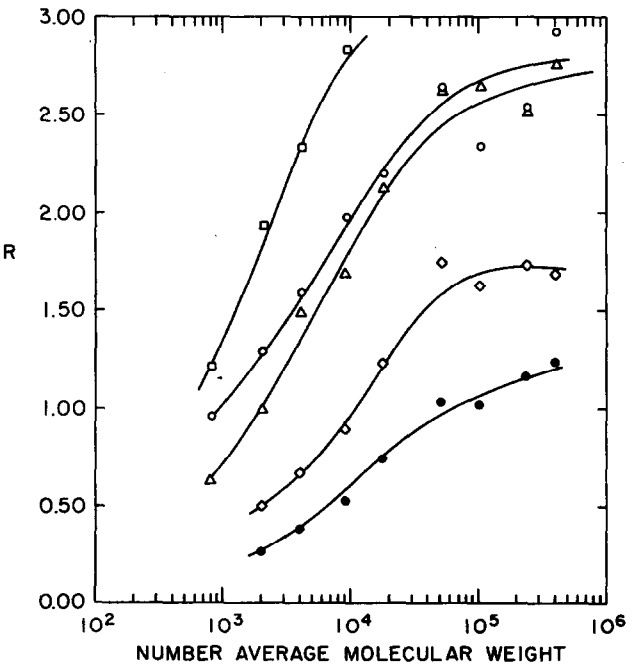

Fig. 7. Plots of R values *vs.* number average molecular weight as a function of flow-rate. Narrow-molecular-weight-distribution polystyrene standards with number average molecular weights as plotted. Solvent and mobile phase, tetrahydrofuran; column, same as in Fig. 5; mobile phase flow-rates as indicated. Flow-rates: ●, 0.10 ml/min; ◇, 0.15 ml/min; △, 0.25 ml/min; ○, 0.40 ml/min; □, 0.50 ml/min.

weight region of interest therefore may be "fine tuned" by adjusting the flow-rate. For example, in Fig. 7 the greatest sensitivity to change in low molecular weights is found at the 0.50 ml/min flow-rate whereas the 0.10 ml/min flow-rate range is the only one where molecular weights of greater than 10,000 can be discriminated from one another. The plateaus evident at the higher molecular weights are seen also with biopolymers and are probably indications of shear-related phenomena which complicate chromatograms which are otherwise primarily reflections of the competing processes of convection and diffusion. The results of Fig. 7 and related studies in our laboratory would appear to confirm the general nature of the proposed method for the empirical determination of molecular weights.

Potential method for determination of diffusion coefficients

Atwood and Golay[3] have recast Taylor's equation governing passage of a plug sample in laminar flow[7] through a capillary in the following form:

$$N = 24\pi DL/F \tag{2}$$

where N is the number of theoretical plates, D is the diffusion coefficient of the solute, L is the column length, and F is the flow-rate of the mobile phase. For a fixed length and flow-rate, N is dependent only on the diffusion coefficient of the solute. N is indicative of the shape of the peak. R is also a peak shape parameter and should therefore also be independent of the chemical nature of the species and dependent only on the relative importance of diffusion and convection for a fixed value of the flow-rate and column length.

Thus, in the ideal case, R is a function of N which, in turn, is only a function of D, L and F. Consequently, a measured value of R should correspond to a unique value of N, provided there are no complications such as shear-related aggregation or shear-induced migration, or losses to the wall[6]. Thus, known values of R, F and L should yield an accurate value of D from eqn. 1. We have attempted such an experiment with lysozyme. The calculated value of D was flow-rate dependent and agreement with reported literature values was reached by extrapolation to zero flow-rate of a plot of the calculated value of D *vs.* flow-rate. This possible extrapolation-to-zero-flow method of calculating diffusion coefficients would appear to offer a convenient and relatively fast method of calculating diffusion coefficients if further experiments prove the validity of the method.

Number average molecular weights of mixed polymer standards and polydisperse polystyrene samples

Since the types of molecules employed thus far in our method had been either of a single valued molecular weight or of a very narrow molecular weight range, it was of interest to investigate mixtures of the narrow-molecular-weight polystyrene standards to determine the type of molecular weight obtained by our method and also look at the effects of molecular weight distribution on the R values used in molecular weight determination. Table I illustrates typical results of such experiments. An analysis of the results indicates that number average molecular weight is obtained, even with a mixture of two samples of widely different, monodisperse molecular weight standard samples.

TABLE I

CALCULATED NUMBER AVERAGE MOLECULAR WEIGHTS OF POLYMER STANDARDS

Samples of different monodisperse polystyrene standards dissolved in tetrahydrofuran are mixed in varying proportions and are injected into a tetrahydrofuran mobile phase flowing at a flow-rate of 0.15 ml/min through the capillary utilized to obtain the data shown in Fig. 5. A number average molecular weight is calculated based upon the relative proportions and reported nominal molecular weights of the two narrow molecular weight distribution standards used in preparing the injected sample mixture.

Calculated mol.wt.	*Experimental R values*	*Estimated mol.wt.**	*Error (%)*
10,900	1.10 ± 0.01	11,200	3
13,000	1.24 ± 0.07	14,900	15
17,500	1.35	17,000	3
30,000	1.71 ± 0.15	34,500	15

* Estimated by interpolation using a standard curve of R values *vs.* molecular weight plotted for eight polystyrene standards of different number average molecular weights.

Elution profiles also have been obtained for polydisperse polystyrene samples analyzed independently by gel permeation chromatography. Number average molecular weights were estimated for these samples by using measured R values on Fig. 7 to interpolate a molecular weight value. Again it appears that this method is capable of analyzing the number average molecular weight of polydisperse samples with an accuracy closely approximating that of gel permeation chromatography in a time which is a small fraction of that taken by the gel permeation chromatography method. We believe it is possible to gain molecular weight distribution information from a more detailed analysis of the peak shapes and/or from analysis of the results of multiple injections of one sample at a number of different flow-rates.

Molecular weight determination of low-molecular-weight, monomeric compounds

We have measured R values of a number of aqueous solutions of low-molecular-weight organic compounds. The behavior of these molecules in capillaries is very much like that of higher-molecular-weight organic and biopolymers. That is, for a given flow-rate and a given capillary, the measured R values were found to increase regularly with increasing molecular weight and similar double-peaked elution profiles were found to vary with flow-rate as expected from the results of Atwood and Golay[3]. Also, as anticipated from eqn. 2, the flow-rates needed to obtain double peaks were higher than for higher-molecular-weight organic or biopolymers.

These results with low-molecular-weight species strongly imply that the proposed method is useful in a low-molecular-weight range with non polymeric systems as well as organic and biopolymer systems of much higher molecular weight.

Use of the method to follow rates of polymerization and depolymerization

Fig. 8 indicates another possible use of our molecular weight determination method in which the progress of a depolymerization, in this case an enzyme degradation of DNA, can be followed. Thus, the molecular weight integrity of biopolymer samples can be tested very quickly in an exceedingly short time with quick recovery of the tested sample.

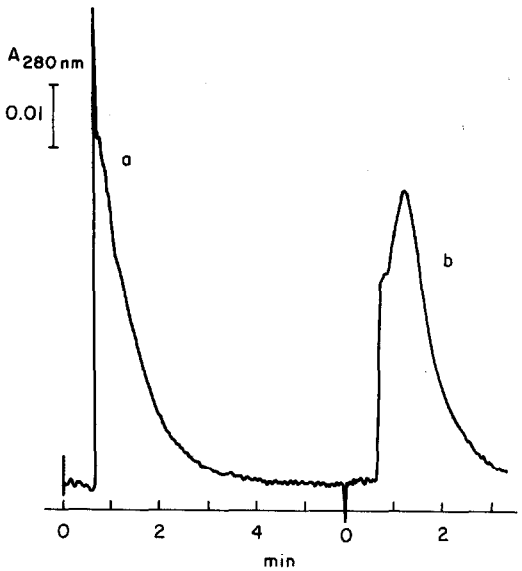

Fig. 8. Use of molecular weight method in monitoring DNA degradation. a, High-molecular-weight calf thymus DNA sample. b, Same DNA sample degraded by nuclease enzyme. Column, same as in Fig. 4; mobile phase, water; flow-rate, 0.07 ml/min.

One of the easiest molecular weight identifications possible by the above method is distinguishing between monomer and polymer. The above enzyme degradation experiment implies that the method also should be quite useful in following the progress of a polymerization reaction. An ideal polymerization monitoring system would be one in which part of the polymerizing solution is continuously circulated through the sample loop of a sampling valve attached to a capillary of the type used for molecular weight determination described above. The valve would be opened and sample the polymerizing solution at intervals during the polymerization. The sampling system and the capillary might have to be maintained at a temperature close to or equal to that of the polymerizing system because of problems related to polymer solubility or viscosity. For determination of very high molecular weights it may be necessary to dilute polymerization mixture on line before injection into the capillary. The speed of the molecular weight determination by this method is on the order of minutes.

Preliminary investigations in our laboratory with polystyrene polymerization have demonstrated that the system is indeed a very useful way to follow molecular weight changes during polymerization. We have seen indications of very subtle and quite abrupt changes during the polymerization process which are not observable in gel permeation chromatography of the same samples taken during polymerization. The origin of these abrupt changes is unknown at the present time. We suspect these are at least in part related to changes of the response of the growing polymer chains to the shear stress environment of the capillary.

Some additional notes regarding the method

The following are some observations regarding the method described above which we have found to be or believe might be useful:

(a) In our experience the convection peak occurs at between 1.2 and 1.3 times the breakthrough time (t_b) of the concentration profile and depends on the sample size and the length of the column, among other factors. It is most useful to select a flow-rate which produces a convection dominated peak with an R value greater than one and then to choose the convection peak height h_1 at the peak itself rather than the height of the concentration profile at exactly 1.2 or 1.3 times the breakthrough time, since these peaks are sometimes quite sharp. This makes the establishment of the breakthrough time less critical in the method. If there is no clear convection peak, then a value of 1.25 times the breakthrough time appears to be a reasonable choice for the measurement of the convection "peak" height measurement for R values below one.

(b) As indicated previously the best test for unwanted shear-related phenomena such as flow-induced wall adsorption or possible turbulence is to test the R value as a function of flow-rate. A steadily rising R value with flow-rate with no plateau is usually an indication that few problems will be encountered. If a plateau is encountered, the flow-rate must be lowered to a flow-rate below the plateau region and even then some problems with irreproducible peak shapes may be encountered. These problems may be solved by finding an appropriate rinse solvent or solution to flush the column with between injections[6].

(c) Our experimental evidence has led us to suggest that when problems arise with aggregation or adsorption, lower flow-rates and larger diameter tubing will help solve many problems of polymer losses and unusual peak shape behavior with higher-molecular-weight polymers. If such problems cannot be easily resolved, a new piece of tubing should be installed since the adsorbed polymer is very difficult to remove and the tubing is relatively inexpensive. However, a new piece of tubing may require a "breaking-in" period in which the compound of interest is injected several times until reproducible peaks are obtained[6].

(d) Our results thus far indicate that better results are obtainable in organic rather than aqueous solvents. Thus we would suggest the addition of organic solvents such as methanol to aqueous mobile phases used to analyze biopolymers by this method.

(e) The chromatographic system used in our studies has been far from optimal. Our pump was operating near the lower limit of reliability, the zero dead volume fittings probably distorted peak shapes to some extent and possibly introduced some turbulence at higher flow-rates, the detector had a rather large 8-μl cell volume, and the capillaries were not particularly selected or treated for their inner surface characteristics. With deliberate efforts to design a system which produces a true plug sample initially and maintains a laminar profile and steady flow-rate during the run, we believe far better accuracy and more reproducible standard curves can be obtained.

CONCLUSIONS

We believe the evidence presented above and in our previous report on biopolymers[1] is sufficient to suggest that the method outlined above for the empirical determination of molecular weights is quite broad in scope and is useful for many different systems in which there is free diffusion of individual molecules or possibly even particles which obey Brownian motion in fluids moving under laminar flow conditions. However, molecular weights of some particulate suspensions might not be accessible with this method because of the "tubular pinch" effect[9].

Our evidence with organic polymers indicates the method yields number average molecular weights. The range of molecular weights determined by this method is limited only by the unorthodox behavior of dilute solutions of polymers of high molecular weight under flow conditions[6]. We believe even this problem can be attacked by a combination of low flow-rates, larger diameter tubing, the appropriate choice of solvents for use in the mobile phase and in capillary washes between injections. In addition, special treatment of the inside surface of the capillaries either in the manufacture or through chemical treatment following manufacture may be necessary to eliminate the potentially serious problems of polymer adsorbtion and aggregation near the capillary walls, not only in our molecular weight system but in all forms of chromatography[6].

We believe this method has many advantages in the determination of number average molecular weights. An exceedingly useful feature of the method is its speed. Acquisition of the concentration profiles takes on the order of minutes. Thus studies of molecular weight as a function of many parameters such as temperature, pH, ionic strength, solvent composition, concentration of possible reactants with the molecule of interest, *e.g.* cofactors for enzymes, metal ions, etc. are possible in a very short time.

We also believe that, under some circumstances, species with very high molecular weights such as cellular components or colloids may be determined using this method. It would appear to us that the only limiting feature to determining molecular weights by this method would be non-Brownian motion behavior, lack of suitable standards, or unusual flow behavior in capillaries.

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