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Practical on-line determination of biopolymer molecular weights by high-performance liquid chromatography with classical light-scattering detection

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ABSTRACT

Most biopolymer molecules are much smaller than the wavelength of light used in classical light-scattering experiments (*ca.* 500 nm), and thus the simple Rayleigh equation and a 90° light-scattering photometer are sufficient to determine their molecular weight. In combination with high-performance liquid chromatography (HPLC), it is demonstrated that a simple HPLC fluorimeter can be used as a 90° light-scattering detector for biopolymer molecular weight determinations. To simplify data handling, only relative molecular weights are measured. Three mathematical assumptions are adopted, and their validity for proteins is shown. To place the work in perspective, the relative advantages and limitations of this 90° light-scattering detector are compared with the more commonly used low-angle laser light-scattering detector. Two examples of protein molecular weight determinations are given to illustrate the broad utility of 90° classical light scattering to the study of biopolymer structures and interactions.

INTRODUCTION

The determination of the molecular weight of a biopolymer such as a protein or oligonucleotide is crucial to its identification. Mass spectrometry is the method of choice, but its usefulness is limited to a restricted range of molecular weights [1]. Another drawback of mass spectrometry is that it yields only the primary structure of proteins and cannot be used to study their behavior in solution, such as their non-covalent quaternary structure, and associations with other proteins.

The three most common physical methods for determining the molecular weight of biopolymers in solution are osmometry, analytical ultracentrifugation and classical light scattering. Osmometry and analytical ultracentrifugation are time consuming. In contrast, classical light scattering can be performed rapidly, although it requires careful and sometimes difficult clarification of the solutions. Techniques such as size-exclusion chromatography (SEC) and dynamic light scattering (DLS) measure the size of a biopolymer (*i.e.*, its hydrodynamic radius) and that result is often used to infer molecular weight. The approach has great utility but it can be unreliable owing to the lack of a unique relationship between molecular size and molecular weight, even under strongly denaturing conditions [2,3]. Denaturing polyacrylamide gel electrophoresis is the most commonly used method to estimate the apparent molecular weights of biopolymers. This technique may be inaccurate owing to the poorly defined relationship between both the charge and the residual biopolymer structure on the one hand and molecular weight on the other.

In this context, the link between classical light scattering and high-performance liquid chromatography (HPLC), particularly SEC for biopolymers, is natural [4–14]. Chromatography clarifies the

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biopolymer solution and separates its components to allow a more detailed analysis. A classical light-scattering photometer connected to the chromatographic column allows the molecular weight of the biopolymer to be determined on-line. Depending on the type of chromatography, this analytical system can simultaneously provide additional information such as size, charge and hydrophobicity of the biopolymer.

Most classical light-scattering biopolymer molecular weight determinations have been performed with low-angle laser light-scattering (LALLS) detectors in combination with refractive index and/or UV detectors. Takagi and co-workers applied this combination of detectors coupled to SEC to determine the molecular weight of biopolymers and have published extensively on molecular weight measurements of membrane proteins [8,12–14]. Krull and co-workers have demonstrated that these detectors can be used to determine biopolymer molecular weights when coupled with reversed-phase [7] and hydrophobic-interaction [10] chromatography as well as SEC [6].

Although both groups used LALLS detectors, they developed different basic approaches to the performance of the classical light scattering experiment. Takagi and co-workers make a relative determination of molecular weight by measuring the detector response to biopolymers of known molecular weight and to the sample of unknown molecular weight. Krull and co-workers calibrated the equipment in an absolute manner, a process which yields absolute molecular weight. Both approaches have their advantages. Takagi and co-workers' method is simple and straightforward but requires that the known samples or standards be well characterized and appropriately chosen. Krull and co-workers' method yields results which do not rely on external standards but the calibration procedure is exacting. Both groups have published excellent reviews of the LALLS HPLC field [15,16].

This paper presents an alternative detection method for classical light-scattering experiments. In 1935, Putzeys and Brosteaux [17] demonstrated that a 90° light-scattering photometer could be used to measure the (relative) molecular weight of proteins in solution. In the Theory section, we briefly describe the background, advantages and inherent limitations of using 90° detection, the principle limitation being that the biopolymer must be small relative to the wavelength of light used in the scattering experiment. The theoretical framework is then used in an experimental demonstration that a simple 90° HPLC fluorimeter can be used as a 90° classical light-scattering detector for the on-line molecular weight determination of biopolymers [18]. This detector is shown to be sensitive, relatively immune to dust and column particulates and easy to use. Further, as this is a variable-wavelength device, the scattering wavelength can be optimized for the experiment at hand. It is also ubiquitous in modern analytical laboratories. To simplify the data analysis and the discussion, we shall adopt the relative molecular weight determination methodology of Takagi and co-workers. To illustrate the general utility of this approach, we describe the quaternary structure of RNase, lysozyme and bovine serum albumin using trifluoroacetic acid–acetonitrile reversed-phase elution and 90° classical light scattering. Finally, we show how the method can be used, in combination with SEC and DLS, to study the conformational changes induced in human Glu-plasminogen by the presence of a lysine analogue.

THEORY

The starting point for this brief discussion of classical light-scattering theory is the Rayleigh–Gans–Debye approximation [19]:

$$\frac{KC}{\bar{R}_\theta} \approx \frac{1}{\bar{M}_w P_{(\theta)}} + 2A_2C + O(C^2) + \ldots \qquad (1)$$

where K is the optical constant, C is the weight concentration of the solute, $\bar{R}_\theta$ is the excess Rayleigh ratio, $P_{(\theta)}$ is a size/shape scattering factor and the expansion is in powers of C with virial coefficients. The term $O(C^2)$ is shorthand notation for a term of second order of concentration. The quantity one usually wishes to determine is the molecular weight, which for polydisperse samples is given as the weight-average molecular weight, $\bar{M}_w$.

The quantity measured in classical light-scattering experiments is $\bar{R}_\theta$, the excess Rayleigh ratio. $\bar{R}_\theta$ is the differential light scattering intensity of the solution, $i_{solution}$, minus that of the pure solvent, $i_{solvent}$, divided by the incident light intensity, I_0, and multiplied by a constant, whose value is determined

by the photometer geometry and the solvent refractive index, g:

$$\bar{R}_\theta = \frac{i_{\text{solution}} - i_{\text{solvent}}}{I_0} \cdot g \qquad (2)$$

If one zeroes the output of the detector with the pure solvent in the scattering cell, then the output of the scatterometer, I_s, during the experiment will be proportional to $\bar{R}_\theta$.

In eqn. 1, the fundamental constant which relates the scattered light intensity $\bar{R}_\theta$ to $\bar{M}_w$ is the optical constant K, given by

$$K = \frac{2\pi^2 n^2 (\mathrm{d}n/\mathrm{d}c)^2}{\lambda^4 N} \qquad (3)$$

where n is the refractive index of the solvent, $\mathrm{d}n/\mathrm{d}c$ is the specific refractive index increment of the solute (*i.e.*, the biopolymer in that solvent), λ is the wavelength of the incident light and N is Avogadro's number.

The concentration of the solute, C, must be measured in an independent experiment in order to implement eqn. 1. The coefficients of the concentration terms on the right-hand side of eqn. 1 are functions of the virial coefficients, which are related to the non-ideal nature of the solvent. In more practical terms, they are measurements of the solute's propensity in that solvent to self-associate. The second virial coefficient, A_2, is proportional to the excluded volume of the solute.

The final variable in eqn. 1 that needs to be described is $P_{(\theta)}$, which alone is explicitly dependent on molecular size. This variable is a dimensionless size/shape scattering factor, a function of the size of the scatterer relative to the wavelength of incident light and of the scattering angle:

$$P_{(\theta)} = 1 - \frac{16\pi^2 n^2 <r_g^2>_z \sin^2(\theta/2)}{3\lambda^2} + O^2\left[\frac{n^2 x^2 \sin^2(\theta/2)}{\lambda^2}\right] \ldots \qquad (4)$$

where $<r_g^2>_z$ is the z-averaged radius of gyration of the scatterer (biopolymer), θ is the scattering angle measured from the forward direction and x is a function of a size parameter characteristic of the shape of the scatterer (*e.g.*, for a sphere x is simply the radius). Thus, in general, $\bar{R}_\theta$ will have an angular dependence due to the size dependence of $P_{(\theta)}$.

In the most general application of eqn. 1, $\bar{R}_\theta$ is measured as a function of C and θ with a variable-angle or fixed multi-angle detector. These data are reduced using the methodology of Zimm and yield the excluded volume, which is proportional to A_2, the size, $<r_g^2>_z$, and the weight-average molecular weight, $\bar{M}_w$ of the scatterer [20]. Specifically, it is the angular component of the scattering data that is used to determine $<r_g^2>_z$. Alternatively, one can measure $\bar{R}_\theta$ at angles as low as 5–7°, where $\sin^2\theta/2$ is approximately zero and consequently $P_{(\theta)} = 1$. This method eliminates the size dependence of $\bar{R}_\theta$ at low-angles and $\bar{R}_\theta$ needs to be measured only as a function of C yielding A_2 and $\bar{M}_w$ [21]. The size information is lost but the low-angle experiment is considerably simpler, and usually only $\bar{M}_w$ is of interest. This is the principle of the LALLS detector.

However, most biopolymers of common interest are very small relative to the wavelength of light used in a scattering experiment, *i.e.*,

$$\frac{<r_g^2>_z}{\lambda^2} \ll 1 \qquad (5)$$

Substituting relationship 5 in eqn. 4 reveals that the cumbersome terms with angular dependence will be much less than 1 and therefore, even at large scattering angles such as 90°, $P_{(\theta)}$ will be very close to 1. For example, an immunoglobulin M (IgM) of 10^6 dalton molecular weight with a typical partial specific volume of 0.73 ml/g and a roughly globular structure has a radius of *ca.* 7 nm. Its radius of gyration will therefore be *ca.* 5 nm (the relationship between the radius, r, and the radius of gyration, $<r_g^2>$, is $<r_g^2> = (3/5)r^2$ [19]). Thus, for IgM, in aqueous solution ($n = 1.33$) when 500-nm light is scattered at 90°,

$$P_{(90°)} \approx 1 - 0.005 \qquad (6)$$

Even for this large protein, $P_{(90°)}$ differs from 1 by less than 1%. For smaller proteins $P_{(90°)}$ will be even closer to 1, and for all scattering angles $P_{(\theta)}$ will also be close to 1. Therefore, for most compact proteins, $P_{(\theta)}$ will be *ca.* 1 throughout the range of experimentally accessible scattering angles, *i.e.*, from 5 to 150°. Measuring $P_{(\theta)}$ at multiple angles and then plotting $P_{(\theta)}$ against $\sin^2(\theta/2)$ would yield an essentially flat curve, giving little information about molecular size, $<r_g^2>_z$. $\bar{R}_\theta$ will then be sensitive to scattering angle only by virtue of its dependence on the geometry of

the light-scattering photometer (g in eqn. 2) and not by its relation to size through $P_{(\theta)}$. In other words, for compact biopolymers of molecular weight less than *ca.* 1×10^6 dalton, $<r_g^2>_z$ cannot be determined by measuring $\bar{R}_\theta$ as a function of angle, and the choice of scattering angle can be based on simplicity of experimental design, equipment availability and sensitivity. In deference to these experimental considerations, we have chosen to use a 90° light-scattering photometer and the approximation that $P_{(90°)} = 1$ throughout this work.

As the molecular weight of a biopolymer increases past 1×10^6 dalton for compact structures, the size dependence at 90° in the size/shape factor $P_{(\theta)}$ and hence in eqn. 1 is no longer negligible. For a highly denatured protein, which assumes a random coil configuration [22], a 1% deviation from $P_{(\theta)} = 1$ will be evident at a molecular weight of *ca.* 2.5×10^5 dalton [this value was estimated using polyglutamic acid as a model compound for a denatured protein and eqns. 5 and 7; the value for σ (eqn. 7) was taken from ref. 23]. For double-stranded DNA, which can be described as a worm-like coil, a 1% deviation will occur at *ca.* $5 \cdot 10^4$ dalton (*ca.* 80 base pairs) (the relationship between molecular weight and $<r_g^2>$ for DNA was taken from ref. 24). Under these conditions, the radius of gyration must be either measured in an independent experiment or expressed as a function of $\bar{M}_w$. In the latter case, the size/shape factor $P_{(\theta)}$ and in turn the Rayleigh–Gans–Debye approximation become a function of $\bar{M}_w$ without any explicit size dependence. For example, the relationship between $\bar{M}_w$ and $<r_g^2>$ for a random coil is

$$\bar{M}_w = \sigma <r_g^2> \tag{7}$$

where σ is a constant whose value can be found or determined from the literature [23,24], or approximated. Using eqn. 7, the Rayleigh–Gans–Debye approximation for a random coil becomes a simple quadratic in $\bar{M}_w$. Using this mathematical technique, one can (at least in theory) extend the range of a 90° light-scattering photometer to several million daltons for compact biopolymers, *ca.* 10^6 dalton for the denatured proteins (random coils) and *ca.* $2.5 \cdot 10^5$ dalton DNA (worm-like coils).

Although 90° detection is limited to a lower molecular weight range than low-angle scattering, it has important advantages. One is that a 90° light-scattering photometer is not as sensitive as a LALLS detector to dust and other large particles. This relative insensitivity to particulates can considerably simplify the experimental design. Biopolymer solutions invariably contain particulate matter (*e.g.*, dust and other insoluble components) and bubbles which are relatively large and "massive" compared with most biopolymer molecules of common interest. Even following chromatography, the eluent contains column-derived particulates which are limited in size to *ca.* 0.5 μm by frits and other in-line filters. Air bubbles may also be present. These particulates scatter light strongly in the forward, low-angle, direction and only relatively weakly at 90° [19]. Thus they contribute much more to the noise in a LALLS experiment than they do in a 90° light-scattering experiment. This is an especially important consideration for experiments with small biopolymers at low concentrations, where scattering from these large particulates can obscure that from the biopolymers. In general, when using an HPLC LALLS detector, great care must be taken to filter particulates eluting from the column. No such special precautions are necessary using a 90° HPLC light-scattering photometer, making for much greater ease of system and sample preparation.

Another advantage of 90° light-scattering methodology is the availability of suitable detectors. As will be demonstrated below, the output of a simple fluorimeter (in this instance an HPLC detector), under specific conditions, obeys basic light-scattering relationships and can therefore be used to determine molecular weight. Further, if a variable- or multi-wavelength fluorimeter is used, the scattering wavelength can be optimized to minimize the undesirable effects of scatterer or solvent absorption, if any, or to match the scattering wavelength to that used in the available refractive index detector. Wavelength matching may be an important consideration for precision work [6]. In general, these HPLC detectors are robust, easy to use and sensitive.

Detector sensitivity is an especially important parameter in light scattering, as it sets the lower limit of detectable molecular weight. Additionally, under certain conditions, with a highly sensitive detector, experiments can be performed on solutions of low enough concentration that the virial expansion (eqns. 1 and 4) can be neglected. The Rayleigh–

Gans–Debye approximation [with $P_{(\theta)} = 1$] is then reduced to the simple Rayleigh relationship

$$\bar{R}_\theta = KC\bar{M}_w \tag{8}$$

The Rayleigh equation can be used to determine absolute molecular weight if the light-scattering and UV and/or refractive index detectors are calibrated in an absolute manner [6,21]. Alternatively, the light-scattering photometer can be calibrated relative to a solvent of known scattering power such as toluene [25]. However, for the purposes of this paper, the methodology of Takagi [15] will be used, in which the light-scattering photometer and concentration detector responses are referenced to or calibrated against well defined protein or DNA standards. According to Takagi's methodology, eqn. 8 is rewritten in the following form:

$$I_s = K'(\mathrm{d}n/\mathrm{d}c)^2\, C\bar{M}_w \tag{9}$$

where K' is a system constant which is determined empirically. If the concentration is measured using a UV detector and solving for $\bar{M}_w$, eqn. 9 becomes

$$\bar{M}_w = \frac{I_s A}{K''(\mathrm{d}n/\mathrm{d}c)^2\, UV} \tag{10}$$

where K'' is another empirical system constant, UV is the output of the UV detector and A is the absorptivity of the biopolymer. If a refractometer is used to measure concentration, eqn. 9 becomes

$$\bar{M}_w = \frac{I_s}{K'''(\mathrm{d}n/\mathrm{d}c)\, RI} \tag{11}$$

where K''' is a third constant and RI is the output of the refractometer.

If one is dealing with a class of compounds which all have, or are assumed to have, equivalent specific refractive index increments, *i.e.*, dn/dc values, then eqns. 10 and 11 can be simplified to

$$\bar{M}_w = \frac{I_s A}{\kappa''\, UV} \tag{12}$$

and

$$\bar{M}_w = \frac{I_s}{\kappa'''\, RI} \tag{13}$$

respectively, where κ'' and κ''' are two other empirical system constants. In the simplest mode, a well defined protein of known molecular weight, $\bar{M}_w^k$, is used as a standard to determine the molecular weight of an unknown protein, $\bar{M}_w^u$. If a ratio is made of the UV and light-scattering responses of two chromatographic runs, the system constant cancels out, to yield the equation

$$\bar{M}_w^u = \bar{M}_w^k \cdot \frac{I_s^u\, UV^k A^u}{I_s^k\, UV^u A^k} \tag{14}$$

where I_s^u and I_s^k are the light-scattering photometer response to the unknown and known protein, respectively, and A^u and A^k are the respective absorptivities of these proteins. When the HPLC peaks of interest have the same absorptivities (*i.e.*, when they are conformers of the same protein) or if a refractometer is used, eqn. 14 is similar to that for the RI detector, namely,

$$\bar{M}_w^u = \bar{M}_w^k \cdot \frac{I_s^u C^k}{I_s^k C^u} \tag{15}$$

where C^u and C^k are the respective concentration detector responses to these proteins. If the chromatogram containing the unknown peak also contains a peak of known weight-average molecular weight, the two peaks can be used in the calculations of eqns. 14 and 15 to yield directly a value for $\bar{M}_w^u$. Examples using both of these methods will be shown in the Results and Discussion section.

EXPERIMENTAL

Apparatus

Chromatographic system A, used to demonstrate light-scattering photometer linearity and sensitivity as functions of molecular weight and weight concentration, consisted of a Model 250 Bio-Series pump (Perkin-Elmer, Mountain View, CA, USA), a Rheodyne Model 7125 injector (Alltech, Deerfield, IL, USA) fitted with a 100-μl sample loop and a Model 650-15 fluorescence detector (Perkin-Elmer). The fluorescence detector contained a 12-μl flow cell and was modified for use as a 90° light-scattering photometer by placing a Schott GG435 yellow second-order filter (Melles Griot, Irvine, CA, USA) in front of the excitation window with monochromators matched at the xenon arc source emission maximum of 467 nm. The time constant was set to 3 s. An alternative configuration used a Fluorolog II double–double monochromator fluorimeter (Spex

Industries, Edison, NJ, USA) with the same filter and monochromator settings as described above and a 25-μl flow cell (Hellma Cells, Jamaica, NY, USA). A Model 235 diode-array detector (Perkin-Elmer), monitored at 280 nm, served as the concentration detector. The detectors were arranged in series, with the UV preceding the light-scattering photometer. Care was taken to minimize the delay volume resulting in band spreading between the detectors. The Omega data system (PE-Nelson Analytical, Cupertino, CA, USA) was used for real-time monitoring and data storage. When the Spex fluorimeter was used, the Omega system monitored and stored the UV data channel while the Spex DM3000 acquisition and analysis system acquired the light-scattering photometer output. Data processing and presentation were done using Lab Calc (Galactic Industries, Salem, NH, USA). The column used for SEC was a 25 cm × 9.4 mm I.D. Zorbax GF-250 (Mac-Mod Analytical, Chadds Ford, PA, USA), fitted with a Rheodyne Model 7315 column inlet filter (Alltech) and operated at ambient temperature. The isocratic aqueous mobile phase was 50 m*M* sodium phosphate–100 m*M* KCl (pH 7.1) at a flow-rate of 0.7 ml/min. Spectroscopic measurements of protein concentrations were made using an Aviv Model 14DS (Aviv Assoc., Lakewood, NJ, USA) spectrophotometer.

Chromatographic system B consisted of all the hardware described for system A less the UV detector. Concentration measurements were obtained from a Model 5902 interference refractometer (RI) modified for use at 488 nm, incorporating a Model 5911 1-mm path length measuring cell (Tecator, Hoganas, Sweden). The SEC column was placed inside a column water-jacket (Rainin Instrument, Emeryville, CA, USA). The column and refractometer were thermostated at 30.3 $\pm$ 0.1°C using a Model F4-K dual-compensation circulating water-bath (Haake Buchler Instruments, Saddle Brook, NJ, USA). Detectors were arranged in series with the refractometer ahead of the light-scattering photometer. The mobile phase was 20 m*M* sodium phosphate–100 m*M* Na_2SO_4 (pH 7.0) at a flow-rate of 0.7 ml/min.

Experiments were designed to simulate the earlier work of Stuting *et al.* [16] with the 90° HPLC light-scattering detector in place of the LALLS detector. System A hardware, consisting of pump, diode-array detector monitored at 220 nm and fluorimeter, were used for these experiments. In place of the C_8 column employed by Stuting *et al.*, we used a 214TP54 25 cm × 4.5 mm I.D. C_4 column from Vydac. The mobile phases, flow-rate and gradient were as described in ref. 16.

Plasminogen chromatographic experiments were carried out using system A. Two sets of data were obtained under two different mobile phase conditions. The first set used the mobile phase described for system A. The second set used the system A mobile phase containing 10 m*M* of the lysine analogue 6-aminohexanoic acid (6-AHA), which has been shown to affect the conformation of Glu-plasminogen [26]. Sample preparation for dynamic light-scattering (DLS) measurements required the use of a Model HSC10K high-speed centrifuge with a Model HSR-16 rotor (Savant Instruments, Farmingdale, NY, USA). DLS measurements were performed on plasminogen samples buffered under both mobile phase conditions, using a Nicomp 370 submicron particle size analyzer (Pacific Scientific Instrument Division, Silver Spring, MD, USA). The light source for DLS was an Innova 90 argon ion laser (Coherent Laser Products Division, Palo Alto, CA, USA) operating at a wavelength of 488 nm.

Materials

The criteria used for selecting proteins as reference materials were that they be of the highest available purity, have established molecular weights and absorptivities and yield a single peak, or at least resolvable peaks on SEC. Proteins meeting these criteria and used in these studies were immunoglobulin M (IgM) ($1 \cdot 10^6$ dalton, $A_{280}^{0.1\%}$ = 1.2 cm^2/mg)[a], immunoglobulin G (IgG) (150 000 dalton, $A_{280}^{0.1\%}$ = 1.4 cm^2/mg)[a], tumor necrosis factor (TNF, non-covalent trimer of 51 000 dalton, $A_{280}^{0.1\%}$ = 1.3 cm^2/mg)[a] and macrophage colony stimulating factor (M-CSF, covalent dimer of 49 700 dalton, $A_{280}^{0.1\%}$ = 0.72 cm^2/mg)[a] obtained from Cetus; human Glu-plasminogen (Pg, 94 000 dalton, $A_{280}^{0.1\%}$ = 1.69 cm^2/mg)[b] obtained from American Diagnostica (Greenwich, CT, USA); thyroglobulin (Tyr, 669 000 dalton, $A_{280}^{0.1\%}$ = 1.08 cm^2/mg) [27], bovine serum

[a] In-house determination.
[b] Data supplied by American Diagnostica.

albumin (BSA, 66 000 dalton, $A_{278}^{0.1\%}$ = 0.66 cm^2/mg) [28], ovalbumin (Oval, 44 000 dalton, $A_{280}^{0.1\%}$ = 0.74 cm^2/mg) [29], carbonic anhydrase (CA, 29 000 dalton, $A_{280}^{0.1\%}$ = 1.68 cm^2/mg) [29], soybean trypsin inhibitor (STI, 20 100 dalton, $A_{280}^{0.1\%}$ = 1.02 cm^2/mg) [29], lysozyme (Lys, 14 700 dalton, $A_{280}^{0.1\%}$ = 2.59 cm^2/mg) [28] and ribonuclease (RNase, 13 500 dalton, $A_{278}^{0.1\%}$ = 0.73 cm^2/mg) [28] from Sigma (St. Louis, MO, USA). Size-exclusion standards were obtained from Bio-Rad Labs. (Richmond, CA, USA). Potassium chloride and 6-aminohexanoic acid were obtained from Sigma, sodium sulfate from Fluka (Buchs, Switzerland), Acrodisc 13 0.2-μm low-protein-binding syringe filters from Gelman Sciences (Ann Arbor, MI, USA), acetonitrile from J. T. Baker (Philipsburg, NJ, USA); trifluoroacetic acid (TFA) from Pierce (Rockford, IL, USA), Centricon 10 microconcentrators from Amicon (Danvers, MA, USA) and disposable borosilicate glass culture tubes (50 × 6 mm I.D.) from Fisher Scientific (Pittsburgh, PA, USA). Water used for the preparation of all samples and solutions was Type 1, produced with a Lab 5 reagent-grade water purification system (Zyztech, Seattle, WA, USA).

Evaluation of light-scattering photometer response

(A) Experiments for evaluating the light-scattering photometer response as a function of molecular weight, using a UV detector as the concentration detector (system A), were conducted by injecting 15–50 μg of each standard protein. Three replicate injections were made for each protein standard. The volumes injected never exceeded 100 μl. Peak areas obtained from the UV and light-scattering chromatograms were used to generate detector response values (see eqn. 12). The three values obtained for each protein standard were averaged and plotted against their literature molecular weights using linear regression analysis. The plot obtained represents a weight-average molecular weight calibration graph, the inverse of the slope of which is the system constant κ''. This curve was used to establish the weight-average molecular weight for plasminogen in a subsequent experiment. All calculations were based on integrated areas.

(B) Light-scattering photometer response as a function of injected mass was evaluated using IgG and STI. IgG in the range 1.1–10.8 μg and STI in the range 7.7–77 μg were injected on to system A. The light-scattering photometer response, as quantified by integrated areas, was then plotted against the injected mass. Light-scattering photometer sensitivity, defined as the minimum amount injected necessary to achieve a signal-to-noise ratio of *ca.* 4, was *ca.* 1.1 μg for IgG and *ca.* 7.7 μg for STI. To unambiguously discriminate protein peaks from detector noise, larger amounts of protein were injected.

(C) Experiments evaluating the light-scattering photometer response as a function of molecular weight using an RI detector as the concentration detector (system B) were performed by injecting 10–75 μg of each standard protein. Experiments were conducted as described in (A). The ratio of light-scattering to RI peak areas constituted the detector response values (see eqn. 13), which were plotted against the literature molecular weights. The inverse of the slope in the linear regression analysis of these data correspond to the system constant κ'''.

Human Glu-plasminogen

Chromatography. Glu-plasminogen was supplied in sealed ampules containing 1 mg of protein plus excipients. One ampule was reconstituted with 1 ml of water and the concentration was measured spectroscopically. Replicate 25-μg injections of plasminogen were made on to System A. The detector response values obtained from the chromatographic data were averaged. Using the calibration graph generated as described in (A), weight-average molecular weights for the peaks observed in the chromatograms were determined by interpolation, using their detector response values. For comparison purposes, the molecular weight of Glu-plasminogen was estimated from a log–linear plot of molecular weight *versus* retention volume, using standard SEC methodology. The chromatograms used to construct the molecular weight-based calibration graph as described in (A) were also used to produce the retention-based calibration graph.

To assess the effects of conformational changes of Glu-plasminogen, calibration graphs of log (molecular weight) *versus* retention volume and molecular weight *versus* mass detector response were reconstructed, using the system A mobile phase containing 10 m*M* 6-AHA. Each reference protein used to construct the calibration graphs was spiked to

contain 10 mM 6-AHA. One ampule of plasminogen was reconstituted and spiked to contain 10 mM 6-AHA; triplicate 25-μg injections were then chromatographed on system A and the results averaged. Mass detector response values and retention volumes were recorded for both the native and the extended forms of plasminogen. These values were used to calculate the molecular weight of these species using both the log (molecular weight) *versus* retention volume and the molecular weight *versus* mass detector response curves.

Dynamic light scattering. Dimer was observed to be present in Glu-plasminogen at the level of 5%. To assess accurately the changes in monomer hydrodynamic radius accompanying the transition from native to extended forms, dimer was removed. Monomer and dimer were baseline resolvable under system A chromatography conditions. Therefore, in a series of injections, one ampule of 1 mg/ml plasminogen was chromatographed and the monomer fraction collected. Centricon 10 ultrafiltration at 4°C was used to concentrate the sample to 1 mg/ml. The concentrated plasminogen was then rechromatographed and shown to be exclusively monomer with a retention volume characteristic of the native form. For DLS measurements, a 300-μl aliquot of this monomeric plasminogen was transferred to a clean, dust-free culture tube, sealed and centrifuged at room temperature for 4 min at 13 000 g. A second 300-μl aliquot spiked to contain 10 mM 6-AHA was similarly prepared. These centrifuged samples were measured by DLS at 25.0 $\pm$ 0.1°C using 700 mW of single-mode power. The resulting hydrodynamic radius obtained by DLS was then used to estimate molecular weight using the equation

$$\bar{M}_{\mathrm{w}}^{\mathrm{est}} = 1.6\, R_{\mathrm{h}}^{3} \qquad (16)$$

where $\bar{M}_{\mathrm{w}}^{\mathrm{est}}$ is the estimated molecular weight, R_{h} is the hydrodynamic radius in ångstroms and the proportionality constant, 1.6, was empirically derived using the molecular weight and radii of hydration of the proteins listed in Table II in ref. 30. The uncertainty in this constant based on those data is $\pm$50%.

Reversed-phase HPLC of ribonuclease, bovine serum albumin and lysozyme

A solution consisting of BSA at 3.2 mg/ml, RNAse at 9.8 mg/ml and Lys at 10.1 mg/ml [16] was prepared by adding powdered lyophilized Lys and BSA to a dilute RNAse solution. The mixture was passed through a 0.2-μm Acrodisc 13 filter and immediately chromatographed. Detector response values were calculated as before using integrated peak-area ratios combined with the appropriate absorptivities (see eqn. 12). To establish the identity of the resulting peaks, each protein was also chromatographed separately. These single protein solutions were allowed to equilibrate in solution for at least 1 h prior to injection.

RESULTS AND DISCUSSION

Experimental validation of theory and methodology

The use of a simple HPLC fluorimeter as a 90° light-scattering photometer appears to be both novel and somewhat controversial [31]. The purpose of this section is to show that the output of such a device obeys the Rayleigh relationship (eqn. 8) and can therefore be used to determine, on-line, the molecular weights of biopolymers. Further, the applicability of three working assumptions is demonstrated in the context of our methodology. These assumptions are (1) that $P_{(\theta)} \approx 1$ for compact biopolymers of $\bar{M}_{\mathrm{w}} < 1 \cdot 10^{6}$ dalton; (2) that under normal HPLC conditions the virial expansion is negligible; and (3) that $\mathrm{d}n/\mathrm{d}c$ for all proteins under similar chromatographic conditions is the same. Although none of these assumptions is crucial to our methodology, together they allow the use of the simplified data handling techniques outlined in ref. 15 and incorporated here as eqns. 9–15. The validity of the three assumptions is not rigorously individually proved, but their plausibility and applicability are demonstrated below.

First, eqn. 9 predicts that the output of the 90° light-scattering photometer should be a linear function of concentration, with a slope proportional to the $\bar{M}_{\mathrm{w}}$ of the injected species. Hence there should be a linear relationship between the integrated light-scattering photometer output and the total injected protein mass. Fig. 1 shows that the integrated output of the light-scattering photometer for two proteins, soybean trypsin inhibitor (20 100 dalton) and an IgG (*ca.* 150 000 dalton) has a linear relationship with total injected mass ($r > 0.999$). The virial expansion in the Rayleigh–Gans–Debye approxi-

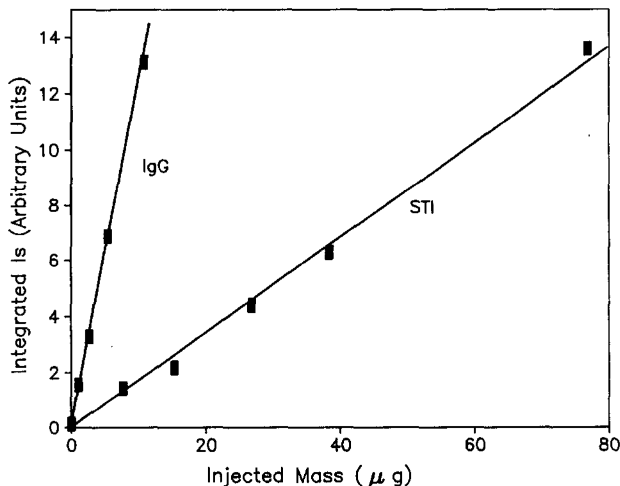

Fig. 1. Plot of the integrated output of the 90° light-scattering detector, I_s, *versus* the total injected mass for an IgG (molecular weight *ca.* 150 000 dalton) and for soybean trypsin inhibitor (STI, molecular weight 20 100 dalton).

mation (eqn. 1) gives rise to a higher than first-order (*i.e.*, non-linear) relationship between the scattered light intensity, I_s, and the concentration. The absence of any non-linearity in these data supports the assumption that the virial expansion is negligible over these concentrations. The ratio of the slopes for these two proteins, *ca.* 0.13, is equal to the ratio of their molecular weights (eqn. 15). Hence Fig. 1 shows that, over practical HPLC concentrations, I_s is proportional to injected mass and the virial expansion is negligible.

Second, eqn. 9 also predicts that the output from a 90° light-scattering photometer should be a linear function of biopolymer molecular weight. Fig. 2 demonstrates that the light-scattering photometer displays exceptional linearity ($r > 0.999$) with molecular weight, as predicted by eqn. 9, when the weight concentrations were measured using an on-line UV detector. The proteins ranged in molecular

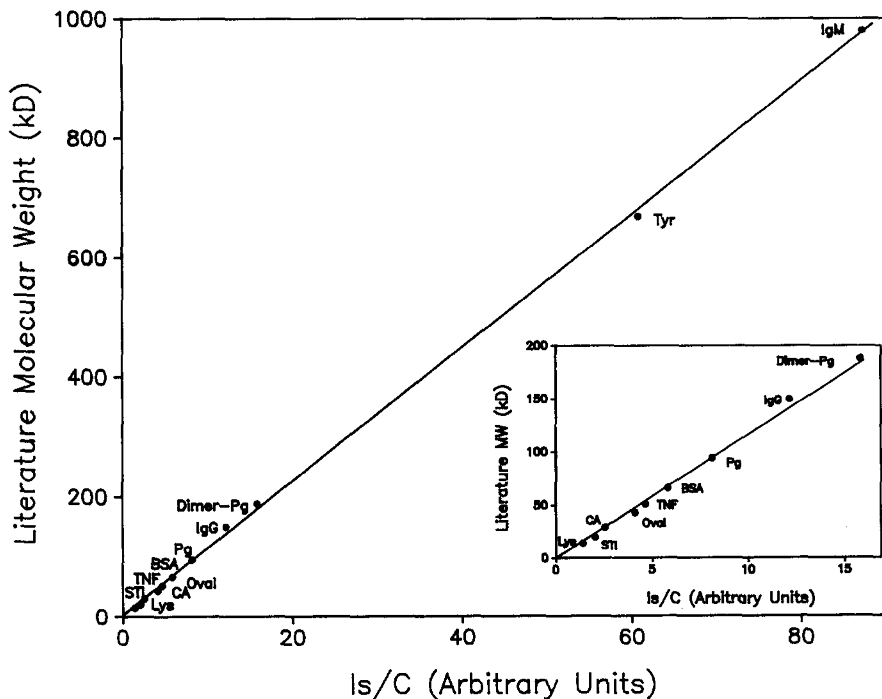

Fig. 2. Plot of literature molecular weight *versus* I_s/C for IgM (*ca.* $1 \cdot 10^6$ dalton), IgG (150 000 dalton), plasminogen (Pg, 94 000 dalton) and its dimer, bovine serum albumin (BSA, 66 000 dalton), tumor necrosis factor (TNF, non-covalent trimer of molecular weight 51 000 dalton), ovalbumin (Oval, 44 000 dalton), carbonic anhydrase (CH, 29 000 dalton), soybean trypsin inhibitor (STI, 20 100 dalton) and lysozyme (Lys, 14 500 dalton). The inset shows the lower molecular weight region expanded for clarity. The concentrations were measured using UV adsorption detection (eqn. 12). kD = kilodalton.

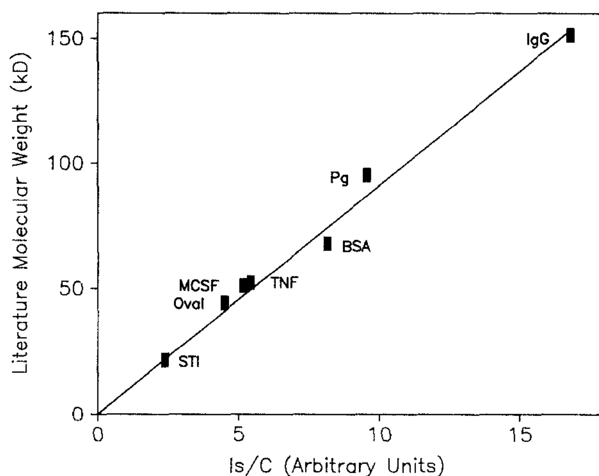

Fig. 3. Plot of literature molecular weight *versus* I/C for IgG (*ca.* 150 000 dalton), plasminogen (Pg, 94 000 dalton), bovine serum albumin (BSA, 66 000 dalton), tumor necrosis factor (TNF, non-covalent trimer of molecular weight 51 000 dalton), macrophage colony stimulating factor (M-CSF, covalent dimer of molecular weight 49 700 dalton), ovalbumin (Oval, 44 000 dalton) and soybean trypsin inhibitor (STI, 20 100 dalton). Concentrations were measured using a refractometer (eqn. 13).

weight from 14 500 to $1 \cdot 10^6$ dalton. Although smaller proteins yielded signal-to-noise ratios large enough to be detected, they were not quantifiable owing to the lack of chromatographic resolution between the protein and the inclusion volume of the column. Fig. 3 shows the same linear relationship between I_s and $\bar{M}_w$ when a refractometer is used to measure concentration (eqn. 11). Eqns. 1 and 4 taken together imply that a biopolymer large enough to cause $P_{(\theta)}$ to deviate from 1 would scatter less, on a weight basis, than a smaller biopolymer. This phenomenon would give rise to an upward curvature in the plot of molecular weight *versus* light-scattering photometer response. The linearity in Figs. 2 and 3 demonstrates that, in fact, for these proteins, $P_{(\theta)} \approx 1$.

Third, if the dn/dc values for various proteins were dissimilar, the value of $I_s/C\bar{M}_w$ would vary from protein to protein (eqn. 9). The goodness of fit of the data to the line in both Figs. 2 and 3 justify using the assumption that they are similar. Individual proteins vary slightly in dn/dc but, as a whole, this approach to data handling has broad applicability. From a theoretical viewpoint, assuming that proteins have similar dn/dc values is equivalent to assuming that, on a weight basis, they have similar amino acid compositions [32]. In fact, this is true for most proteins. To the extent that different length polymers of each type of nucleic acid (*e.g.*, double-stranded DNA) have the same average composition, any length polymer of that type will have the same dn/dc as any other length polymer of the same type under similar conditions. The dn/dc value is measured on a per weight basis and is, therefore, not a function of polymer length or number of basis pairs.

The assumption that the dn/dc values of the species of interest are similar simplifies not only the mathematics, but also the experimental design in that only two detectors are needed, a light-scattering photometer and concentration detector. However, this assumption should be used with caution. For example, a heavily glycosylated protein may have a dn/dc that is significantly different from that of a non-glycosylated protein. Whenever the composition of the biopolymer of interest is unusual (*e.g.*, a protein with an unusually high number of aromatic residues) or unknown (glycosylated?), the dn/dc should be measured. This can be done on-line in either an absolute or relative manner, using a UV detector for concentration and a refractometer for solution refractive index. The combined outputs are used to calculate the dn/dc which, when combined with the output of the light-scattering photometer, yields molecular weight (eqn. 9). These three detection methods (light-scattering photometer, UV detector and refractometer) are described in detail for relative measurements in ref. 12 and for absolute measurements in ref. 6. A 90° light-scattering detector such as that described here can be substituted for the LALLS detector in ref. 12 and, if it can be absolutely calibrated, for that in ref. 6.

The choice of concentration detector to be used in light-scattering experiments is mostly a matter of convenience. An HPLC fluorimeter, a UV detector or a refractometer (when using the assumption of similar dn/dc values) could be employed. The UV detector is useful for proteins and nucleic acids and is robust, easy to set up and applicable to gradient HPLC methods. However, the absorptivity of the biopolymer needs to be known (eqns. 10, 12 and 14). Also, the error in the dn/dc (arising, for example, from the failure of the assumption of similar dn/dc

values) produces an error in the calculated $\bar{M}_w$ that is proportional to the square of the error in dn/dc. In this context, a fluorimeter is very similar to a UV detector except that the fluorescence quantum yield of the biopolymer now needs to be known, in addition to its absorptivity. The product of the quantum yield multiplied by the absorptivity needs to be substituted for the absorptivity in eqns. 10, 12 and 14. If a refractometer is used, the absorptivity of the biopolymer need not be known and the error in dn/dc contributes only in a linear fashion to that of the $\bar{M}_w$ (eqn. 11). Unfortunately, refractometers are not as easy to use as UV detectors, owing to their temperature and pulse sensitivity, and they cannot be readily applied to gradient methods. The signal-to-noise ratio of the concentration detector is not of prime importance in light scattering, as the sensitivity of the light-scattering photometer is typically limiting.

In order to be practical, a light-scattering photometer must be able to detect reliably biopolymers at the injected masses commonly used in modern HPLC. Fig. 4 shows that the light-scattering photometer that we used, an HPLC fluorimeter, can reliably detect *ca.* 8 μg of soybean trypsin inhibitor (20 000 dalton) and 1 μg of IgG (150 000 dalton). Eqn. 9 establishes that the output of the light-scattering photometer, I_s, is proportional to the product of C and $\bar{M}_w$, explaining the differential sensitivities of the detector to these two proteins. The Spex Fluorolog II, which was used to cross-validate many of the above results (data not shown), had a sensitivity roughly equivalent to that of the PE 650-15.

Applicability of 90° HPLC light scattering in biochemistry

The theoretical and experimental results above confirm that a simple 90° fluorimeter can be used as a light-scattering photometer in a fairly straightforward manner to obtain the molecular weights of biopolymers eluted from HPLC columns. Therefore, these simple devices can be used to address many of the same problems investigated with LALLS photometers. To emphasize this point, two examples are given.

First, reversed-phase chromatography is widely used to purify and characterize proteins, and is capable of resolving conformers as well as monomers from aggregates. However, it is usually not possible, on the basis of retention volume or percentage of organic modifier alone, to distinguish between peaks that differ only in conformation and those which differ in their state of aggregation. Light scattering can accomplish this task.

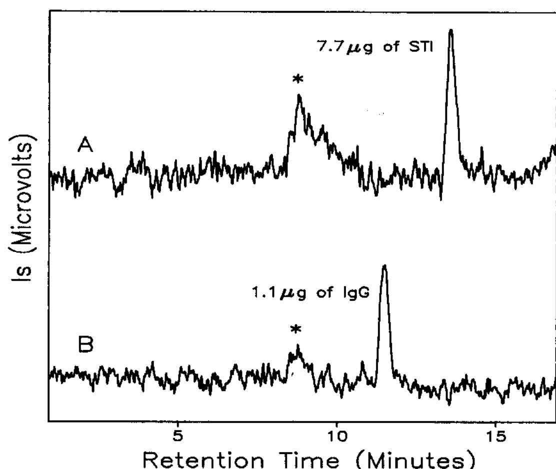

Fig. 4. Chromatograms of I_s (PE 650-15) *versus* retention time for (A) soybean trypsin inhibitor (20 100 dalton, total injected mass = 7.7 μg) and (B) IgG (150 000 dalton, total injected mass = 1.1 μg). Both chromatograms show early-eluting material marked with an asterisk.

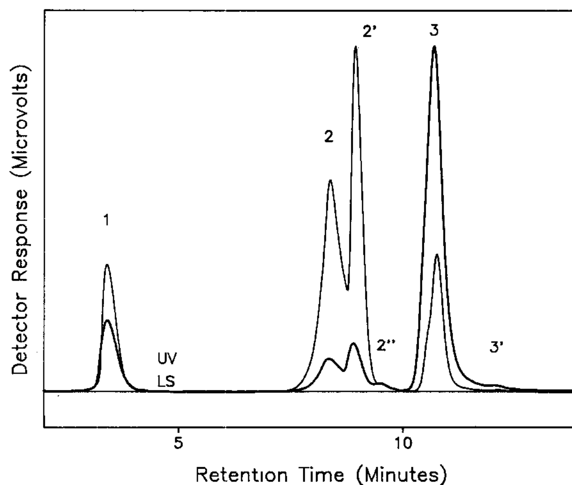

Fig. 5. Linear gradient reversed-phase HPLC of RNase (13 500 dalton), lysozyme (14 500 dalton) and BSA (66 000 dalton).

Fig. 5 shows the reversed-phase chromatography of a mixture of RNase, Lys and BSA [16]. Individual injections of each protein showed that whereas RNase appears to be eluted as a single monomeric species, Lys has three peaks and BSA two. The I_s/UV ratios of Lys peaks 2 and 2′ are equal and therefore the $\bar{M}_w$ of 2 and 2′ are equal, suggesting that they are separable as conformers of equivalent mass (eqn. 14). Further, the experimentally derived ratio (eqn. 15) of the molecular weights of Lys peaks 2 and 2′ to that of RNase is 0.97, while the theoretical ratio of the molecular weights of Lys (14 500 dalton) to RNase (13 500 dalton) is 1.05. The close agreement between the ratios establishes that peaks 2 and 2′ are forms of Lys monomer. The error between the theoretical value of 1.05 and the experimentally derived value of 0.97 may arise from the uncertainty in absorptivity and dn/dc values of these two proteins under the reversed-phase chromatographic conditions at which they are eluted. Again, using the simple ratio method embodied in eqn. 14, the material eluted under Lys peak 2″ is calculated to be approximately nine times as massive as that of peaks 2 and 2′. It is therefore aggregated material. BSA peaks 3 and 3′ are equally massive and are both approximately 4.5 times the molecular weight of RNase, indicating that they are conformers of monomeric BSA (66 000 dalton).

The identification of Lys peaks 2 and 2′ as resulting from conformational change rather than primary structural differences is supported by an experiment in which the dissolution time was increased prior to reversed-phase (RP) HPLC injection. The protein mixture chromatographed in Fig. 5 was prepared by adding lyophilized Lys and BSA to a dilute RNase solution and immediately injecting the mixture. When Lys was allowed to equilibrate in solution for *ca.* 1 h before being injected on-column, peak 2 almost disappeared and nearly all the mass was found in peak 2′ (Fig. 6). As 2 and 2′ are capable of interconversion, their separation is probably due to differences or changes in conformation. This inference is supported by previous work by Benedek *et al.* [33] and Lu *et al.* [34], who have shown that Lys can be eluted in RP-HPLC as two peaks, one of which has undergone a reversible conformational change while on the column. The absence of peak 2″ in Fig. 6 suggests that the additional time in solution prior to injection allowed the Lys aggregates to dissociate.

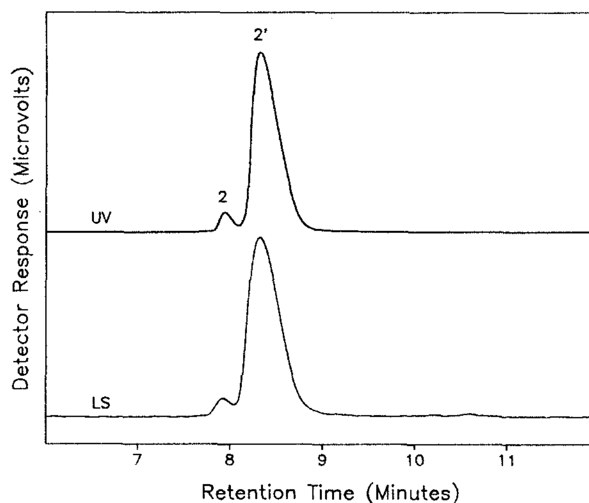

Fig. 6. Linear gradient reversed-phase HPLC of lysozyme (gradient and chromatographic system identical to Fig. 5). Peaks 2 and 2′ correspond to the similarly labeled peaks in Fig. 5.

The second example is used to illustrate the utility of combining classical light scattering with SEC and DLS to investigate protein conformational changes and protein–matrix interactions. Specifically, we studied the effects of the presence of the lysine analogue 6-AHA on the conformation and chromatography of human Glu-plasminogen (Pg). In the

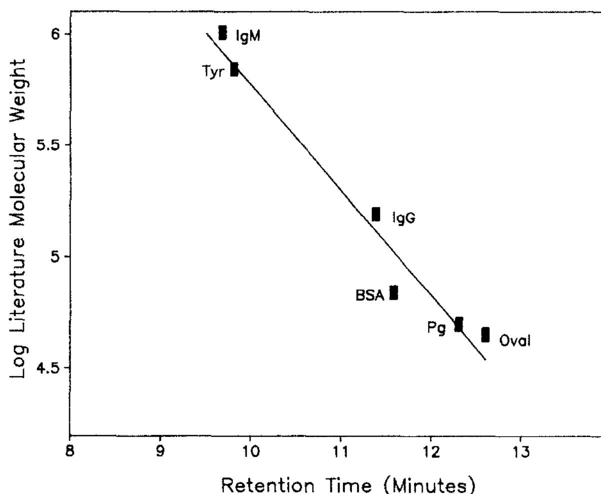

Fig. 7. Log(molecular weight) *versus* retention time for GF-250 SEC column used to estimate the molecular weight of plasminogen in the absence of the lysine analogue 6-AHA. Abbreviations of protein names as in Fig. 2.

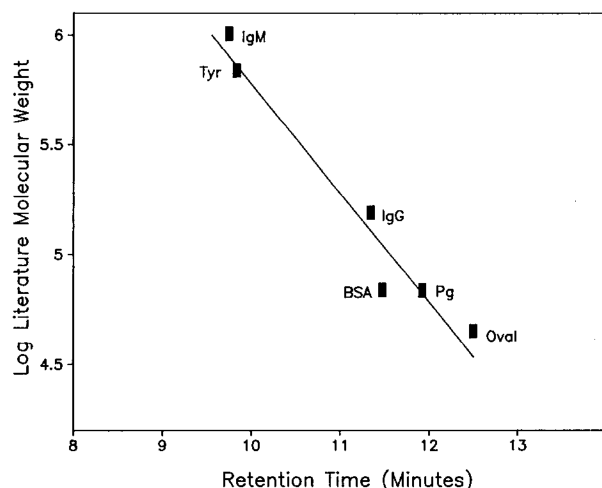

Fig. 8. Log (molecular weight) *versus* retention time for GF-250 SEC column used to estimate the molecular weight of plasminogen in the presence of 6-AHA. Abbreviations of protein names as in Fig. 2. The buffer used contained 10 m*M* 6-AHA.

absence of 6-AHA, the log (molecular weight) *versus* retention time curve for Pg on the GF-250 SEC column yields an estimated molecular weight of *ca* 50 000 dalton (Fig. 7). When Pg was chromatographed with 10 m*M* 6-AHA added to the eluent, the apparent molecular weight increased to *ca.* 61 000 dalton (Fig. 8). Both calibration graphs were derived using standard SEC methodology and the chromatography of the protein standards and Pg appeared normal (Fig. 9). Taken in isolation, these data are difficult to understand as the molecular weight of Pg is known to be 94 000 dalton [35].

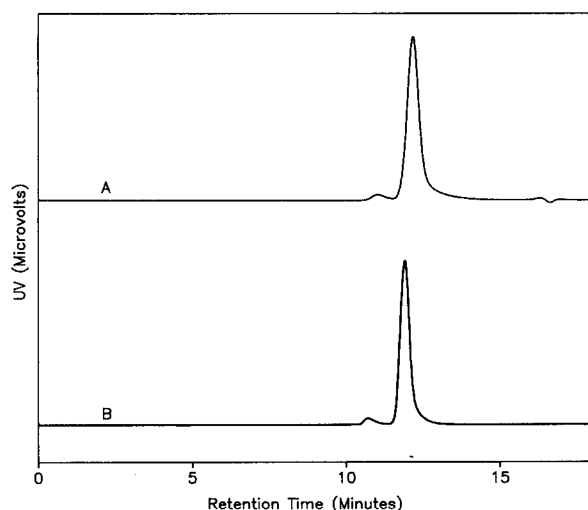

Fig. 9. SEC of plasminogen in (A) the absence and (B) the presence of 10 m*M* 6-AHA.

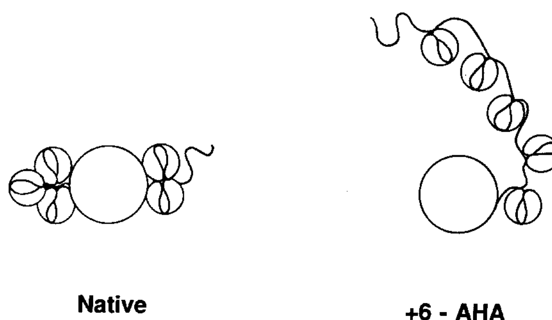

Fig. 10. Plasminogen (94 000 dalton) undergoes a transition from a compact native state to an extended structure in the presence of the lysine analogue 6-AHA [26].

DLS measurements determined a hydrodynamic radius of 3.8 nm for Pg in the absence of 6-AHA and of 4.7 nm when 6-AHA was added to the Pg solution. These data are consistent with either a dimerization of Pg (170 000 dalton, eqn. 16) or an unusually large conformational change of Pg monomer.

Using the 90° light-scattering photometer and the calibration graph shown in Fig. 2, the molecular weight of Pg in both the presence and absence of 6-AHA was determined to be 91 000 dalton (Pg was not included in the data used to generate the calibration graph). In the light of these results, the appropriate interpretation of the DLS data is that they show that Pg monomer undergoes a large unfolding on addition of the lysine analogue (Fig. 10). This is in fact the case, as has been reported previously [26]. The anomalous SEC data presumably are caused by shape and/or other secondary interactions between the protein and the column matrix which result in increased retention.

CONCLUSIONS

Starting from the Rayleigh–Gans–Debye theory of light scattering, we have shown that there is no appreciable size dependence of the scattering function at 90° for compact biopolymers of up to $1 \cdot 10^6$ dalton. For less compact structures, such as highly denatured proteins, the same principles should hold

true up to a molecular weight of $2 \cdot 10^5$ dalton and for double-stranded DNA up to molecular weight of $5 \cdot 10^4$ dalton. Therefore, a 90° light-scattering photometer can be used to determine the molecular weight of these biopolymers without having to measure their size. We have demonstrated this experimentally for native proteins using a simple 90° HPLC fluorimeter with matched excitation and emission monochromators as a 90° HPLC light-scattering photometer. The light-scattering photometer showed excellent linearity and goodness of fit with molecular weight and weight concentration.

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