Computation of Molecular Weight and Weight Fraction of Five and Six Components in Mixtures from Model Equilibrium Ultracentrifugation Data

Shuryo Nakai* and Masahiko Nonaka

Department of Food Science, University of British Columbia, 6650 Northwest Marine Drive, Vancouver, British Columbia, Canada V6T 1Z4

Modified simplex optimization, with a constraint for eliminating negative weight fractions, was applied for maximizing the F ratio of multiple regression analysis of model equilibrium sedimentation patterns computed for mixtures of five and six macromolecular components. It was found that the best fit values for molecular weight and weight fraction of the components were obtained with deviations of less than 10% from the preset values if the sample volume and rotor speed were properly selected and the data from sedimentation runs using different rotor speeds were combined. It is possible to apply this method for computing association constants of interacting proteins.

INTRODUCTION

The quantitative characterization of reversible associations between macromolecules in solution is frequently required in biochemistry and molecular biology. Information on chemical equilibrium is important not only to explain the mechanism of protein functions which are caused by interactions with other compounds but also to predict the behavior of proteins during column chromatography, which is the most efficient technique for separating bioactive proteins. Analytical ultracentrifugation is superior to many other methods in interaction studies mainly because of its nondestructive nature, which can be readily utilized for simulating inteactions occurring in nature. However, lately the number of papers investigating protein interaction using ultracentrifugal sedimentation analysis has declined considerably. Reasons for the decline in the use of this important analytical technique are considered to include high costs of the equipment, inadequate resolution compared to more economical chromatography and electrophoresis, and long equilibration time reqiured for analysis. However, the recent release of a new Optima XL-A model from Beckman Instruments (Fullerton, CA) brings analytical ultracentrifugation within the reach of many laboratories. It may be important to point out that modern instruments have the capability to totally automate the sedimentation analysis including data processing for interaction study. This will, to some extent, compensate the drawback of slow process in equilibrium sedimentation analysis.

To avoid negative values in computed equilibrium concentrations of the component molecules when least-squares methodology is applied, Scholte (1969) introduced linear programing with nonnegative constraints for computation of molecular weight distribution (MWD) from sedimentation patterns. Van de Voort et al. (1979) modified the algorithm of Scholte (1969) for computation of MWD by replacing linear programing with multiple regression analysis. Negative weight fractions of components which were frequently encountered during multiple-regression analysis were forced to zero by successively eliminating the corresponding molecular weights from regression matrix. They also proposed a simplex optimization technique to search for the best fit values of weight-average molecular weight and composition of interacting component proteins. MWD values more accurate than those derived from the Scholte algorithm were obtained by this method. However, the examples used in their paper were for analysis of up to only three components. Although the interaction $A + B \rightleftharpoons C$ can be investigated, it is uncertain if more complicated interactions including more than three components can be analyzed with their method.

In this study, the computer program of Van de Voort et al. (1979) was rewritten for microcomputers for computing for mixtures of five and six components. It is customary to use mathematical models for assessing the efficiency of optimization methods (Hillstrom, 1977). There are many reasons for this practice: carrying out experiments is usually time-consuming; frequently the optimum of experiment is unknown, or even if it is known, various sources of errors may be involved in experiments which should be taken into consideration to obtain accurate estimates for the optimum. Since the reliability of the algorithm combining multiple-regression analysis and simplex optimization has been already confirmed by running equilibrium sedimentation experiments using real sample mixtures of three model proteins (Van de Voort et al., 1979), exercises using model sedimentation patterns for mixtures of five and six components are justified for the evaluation of the potential application of modern analytical ultracentrifuges in the investigation of complicated interactions of food proteins. No paper has been published on the analysis of interacting systems containing more than three components using sedimentation ultracentrifugation, probably because of the complexity in the computation of equilibrium constants.

The objective of this paper was to discuss the feasibility of analyzing up to six components, which may be adequate for studying inteactions of biologically important macromolecules. Thus, the drawback of low resolution of analytical ultracentrifugation may be partly resolved when used in protein interaction study. Possible utilization of this approach in interaction studies for food proteins was also discussed.

METHODS

As reported by Van de Voort et al. (1979), model equilibrium sedimentation patterns were drawn by plotting $c(\xi_n)/c_0$ vs ξ_n computed from

0021-8561/92/1440-0824$03.00/0

Table I. Models and Search Ranges Used for Optimization

component	MW	composition	search range[a] lower	upper
model 1			range 1	
1	15 000	0.2	14 000	27 000
2	45 000	0.1	40 000	76 000
3	100 000	0.2	113 000	216 000
4	250 000	0.3	320 000	612 000
5	800 000	0.2	905 000	1 730 000
model 2			range 2	
1	15 000	0.1	12 000	22 000
2	40 000	0.1	29 000	54 000
3	80 000	0.2	67 000	128 000
4	169 000	0.2	160 000	304 000
5	350 000	0.2	380 000	723 000
6	900 000	0.2	905 000	1 720 000
			range 3	
1			15 000	29 000
2			40 000	76 000
3			67 000	128 000
4			160 000	304 000
5			380 000	723 000
6			905 000	1 720 000
			range 4	
1			15 000	29 000
2			29 000	54 000
3			67 000	128 000
4			160 000	304 000
5			380 000	723 000
6			900 000	1 749 000

[a] These search ranges were used for computing the initial vertices for search for the optimal component weight fractions using simplex optimization.

$$c(\xi_j)/c_0 = \sum_j \sum_i f_i K_{ij} \quad (1)$$

where ξ is the reduced coordinate $(r_b^2 - r^2)/(r_b^2 - r_m^2)$ with r_m and r_b being radial distances from the center of rotation to the meniscus and to the cell bottom, respectively, $c(\xi)$ is the concentration at ξ, c_0 is the initial concentration of solute, f_i is the weight fraction of molecules with molecular weight M_i, and

$$K_{ij} = \lambda M_i \exp(-\lambda M_i \xi_j)/[1 - \exp(-\lambda M_i)] \quad (2)$$

where $\lambda = (1 - \bar{\nu}\rho)w^2(r_b^2 - r_m^2)/2RT$ with partial specific volume of solute $\bar{\nu}$, density of the solvent ρ, angular velocity ω, universal gas constant R, and absolute temperature T. Equation 1 is an extended form consisting of a series of linear equations of the Rinde equation representing the concentration distribution in a centrifuge cell at the equilibrium:

$$c(\xi)/c_0 = \sum_i f_i[\lambda M_i \exp(-\lambda M_i \xi)]/[1 - \exp(-\lambda M_i)] \quad (3)$$

The equation for computing model MWD is

$$f(M) = C \sum_n R_n \exp[-1/S_n(\ln M/M_n)^2] \quad (4)$$

where R_n is the proportion of n components present in the model distribution, S_n is a function of the standard deviation of MWD peaks assumed to follow log-normal distribution, and C is a constant to adjust the total weight fraction. As in the previous paper (Van de Voort et al., 1979), S of 0.1 for all n components and C to make $\sum f(M) = 1.0$ were chosen.

As shown in Table I, two model mixtures were used for subsequent optimization computation: model 1 was a mixture of five components with molecular weights of 15 000, 45 000, 100 000, 250 000, and 800 000 and weight fractions of 0.2, 0.1, 0.2, 0.3, and 0.2, respectively; model 2 contained six components with molecular weights of 15 000, 40 000, 80 000, 160 000, 350 000, and 900 000 and weight fractions of 0.1, 0.1, 0.2, 0.2, 0.2, and 0.2, respectively. Figure 1 shows the MWD patterns computed for those model mixtures. Figure 2 demonstrates examples of the model sedimentation equilibrium patterns computed for model 2 when centrifuged at different rotor speeds.

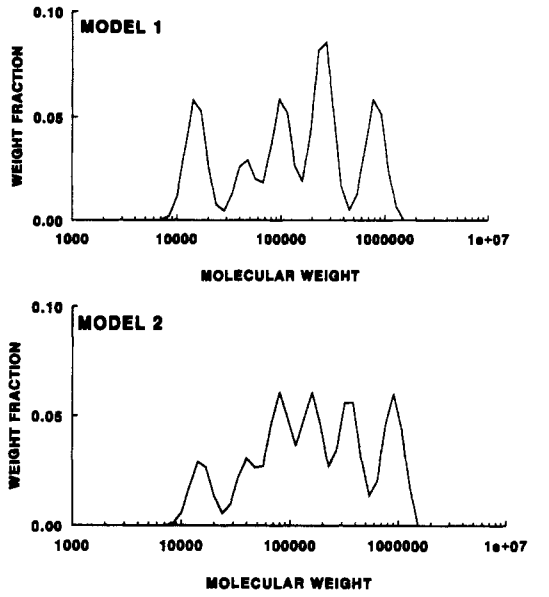

Figure 1. Molecular weight distribution patterns of model systems.

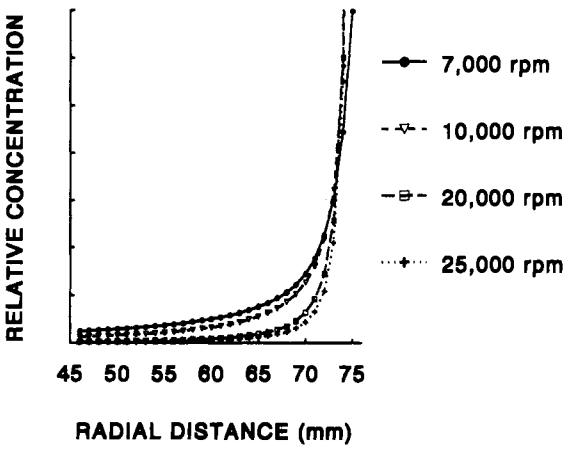

Figure 2. Calculated sedimentation equilibrium patterns of model 2 centrifuged at different rotor speeds.

The computer program used in the previous paper (Van de Voort et al., 1979) was modified to accommodate up to six components by writing a program in Microsoft Quick Basic for IBM-compatible microcomputers. The modified simplex optimization of Morgan and Deming (1974) was used after modifications to maximize the F ratio obtained from multiple-regression analysis as the response when the component molecular weights as the factors substitute into eq 1, thereby computing regression coefficients f_i which represent the weight fraction of each component.

Effects of sample volume and rotor speed on the accuracy of the values computed for molecular weight and weight fraction of components were compared with the preset values shown in Table I. To improve the accuracy of computation, different portions of the sedimentation patterns centrifuged at different rotor speeds were combined; also, different search areas were employed to construct the initial simplices for simplex optimization

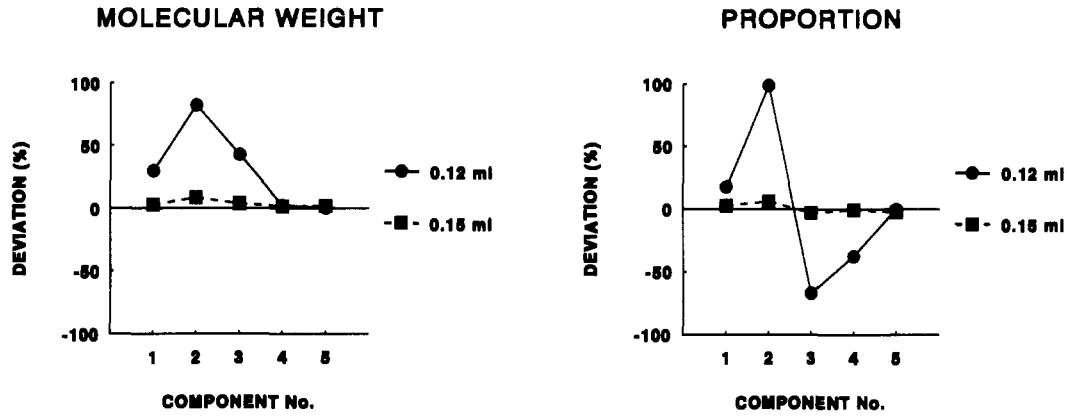

Figure 3. Effects of sample volume on deviation from theoretical values for molecular weight and component proportion of model 1. Rotor speed: 20 000 rpm.

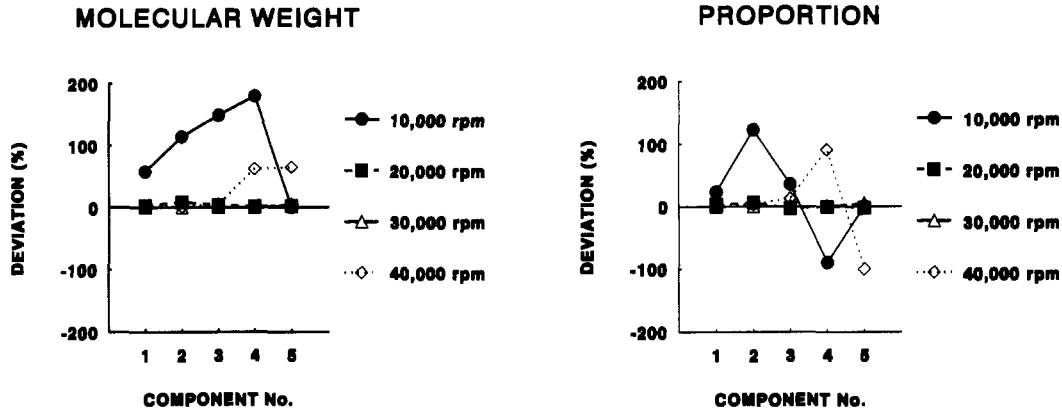

Figure 4. Effects of rotor speed on deviation from theoretical values for molecular weight and component proportion of model 1.

computation. To report the accuracy of computation, percent deviations of the computed values from the preset values were plotted to graphically illustrate.

RESULTS

It was found that the sample volume had to be increased from the regular 0.12 (Chervenka, 1969) to 0.15 mL to improve resolution of samples containing five or six components, although 0.12 mL was adequate to obtain accurate values for three components as reported previously (Van de Voort et al., 1979). This change in sample volume resulted in an increase in the solution column length in sector-shaped centerpiece from 61–75 to 46–75 mm in radial distance. Figure 3 demonstrates the effect of this volume increase. The deviations of computed values for molecular weight and proportion of five components from the preset values are substantially decreased by using a sample volume of 0.15 instead of 0.12 mL. The estimated values when 0.15 mL was used are highly accurate with deviations of less than 10% for all components. Large deviations observed for components 2, 3, and 4 of the 0.12-mL samples implicate lower resolution due to an inadequate number of data points and not due to selection of an inappropriate rotor speed.

The effect of changing rotor speed to four different ways for the five-component model is shown in Figure 4. The computed values for both molecular weight and proportion are in excellent agreement with the preset values within 5% deviations when rotor speeds are 20 000 or 30 000 rpm. As expected, at rotor speeds lower than the selected speeds, there is a propensity that smaller molecular components are inflicted by greater deviations from the predetermined values for molecular weight as well as proportion; in turn, at higher speeds, larger molecular components are affected by the same tendency. For a larger number of components (six) as shown in Figure 5, selection of rotor speed becomes more critical; 25 000 rpm is better than 20 000 rpm. When the data for molecular weight and proportion computed from the sedimentation patterns centrifuged at different rotor speeds were combined, the accuracy of the data was improved. An example, using data in Figure 5, is a combination of the best fit values for components 1–3 at 25 000 rpm with the values for components 4–6 at 10 000 rpm; the deviations are smaller than data separately computed from each pattern centrifuged at respective rotor speeds. The accuracy of data for components 1–3 may be further improved by using a higher rotor speed; however, as seen in Figure 2, higher concentrations of smaller molecular compounds should be used to avoid meniscus depletion with simultaneous loss of the information on larger molecular components due to elimination from solution by sedimentation to the cell bottom. This will make the total weight fraction computed for all components less than 1.0, as has been reported in the previous paper (Van de Voort et al., 1979).

Scholte (1975) stated that in the range of the lower λ values the concentration gradient in the cell is mainly determined by the larger molecules in the sample and vice versa. Since $\lambda = (1 - \bar{\nu}\rho)\omega^2(r_b^2 - r_m^2)/2RT$ is affected by rotor speed, the information from data of sedimentation conducted at various rotor speeds provides a better basis for the determination of the MWD. It is therefore reasonable to combine sedimentation equilibrium data in the meniscus area from a higher speed run and data in the cell bottom area from a lower speed run. Figure 6 shows the optimization results when two different portions of the sedimentation patterns centrifuged at two different rotor speeds are combined. Case 3 is the best result among

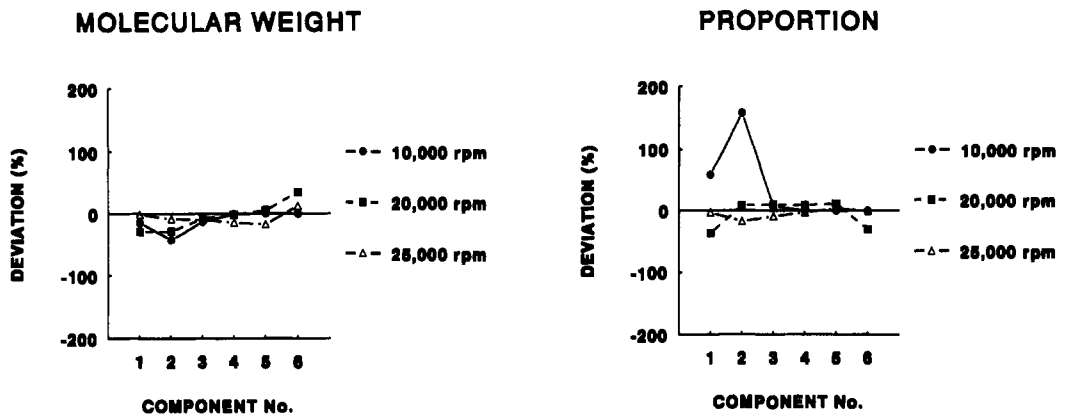

Figure 5. Effects of rotor speed on deviation from theoretical values for molecular weight and component proportion of model 2.

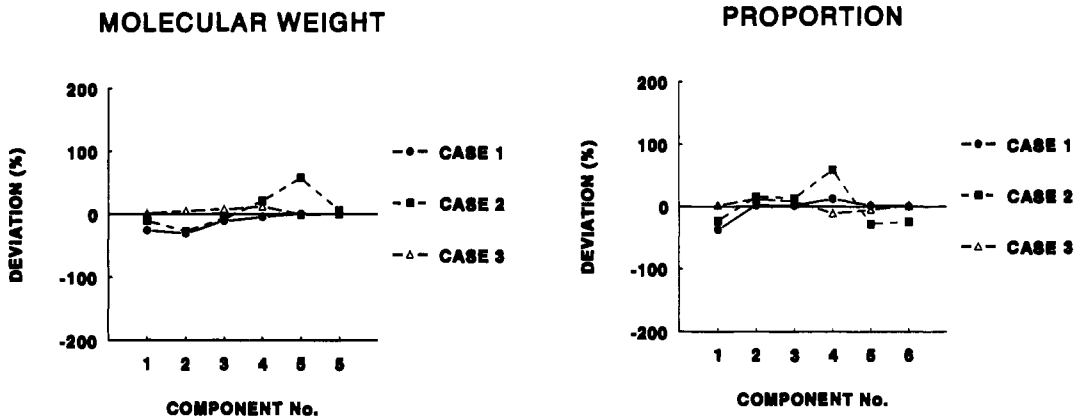

Figure 6. Deviation from theoretical values when absorbance data of two different rotor speeds were combined. (Case 1) rj = 46–65 at 20 000 rpm, rj = 66–75 at 7000 rpm. (Case 2) rj = 46–65 at 25 000 rpm, rj = 66–75 at 7000 rpm. (Case 3) rj = 46–70 at 25 000 rpm, rj = 71–75 at 7000 rpm.

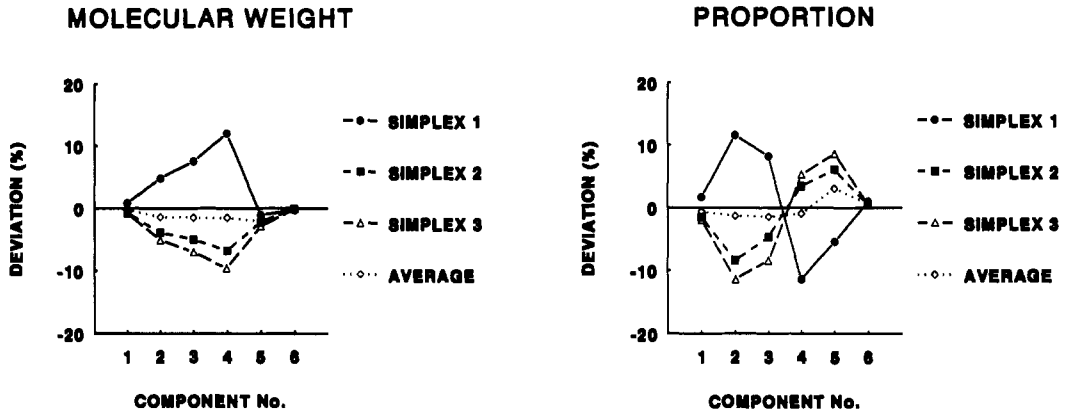

Figure 7. Deviation from theoretical values when different search ranges were used for optimization of model 2. For simplices 1, 2, and 3, ranges 2, 3, and 4 in Table II were used as the search ranges for optimization.

all three combinations with smallest deviations from the preset values; however, it is evident that the selection of speed and location in the pattern is critical. These results indicate that accuracy similar to that obtained by properly combining the estimated values as shown in Figure 5 can be expected for the molecular weight and composition using data combination in a similar manner.

In optimization, it should, theoretically, home-in on the same optimum no matter what search ranges are initially chosen. However, in reality, it is not unusual to reach different optimum values depending mostly on the rules for the termination of sequential optimization procedure or on a boundary constraint, if any. This problem can be readily circumvented by taking an average of the values derived from computations using several different initial search ranges. Figure 7 demonstrates the results of optimization computation using different initial simplices by entering different search ranges as shown in Table I. When the estimated values from computation using three different initial simplices are averaged, the values obtained are highly accurate. This effect was confirmed by drawing the MWD pattern using the average values. The computed pattern almost perfectly matched with the original pattern shown in Figure 1.

DISCUSSION

Application of simplex optimization for maximizing the F ratio of multiple-regression analysis to fit equilibrium sedimentation patterns with a constraint for rejecting negative weight fractions yielded accurate estimates for molecular weight and composition of the components in mixtures of up to six components, if selection of sample

Table II. Association Constants of Protein Interactions

protein	counterpart	K_a, M^{-1}	analytical method
avidin	biotin	10^{15}	radioexchange on CM-cellulose[a]
monoclonal Ig	α-lactalbumin	10^{12}–10^{13}	ELISA–Scatchard plot[b]
antibody	BSA	10^{8}	fluorescence polarization[c]
Fab′	HLA antigen	10^{8}	radiotitration–Scatchard plot[d]
antibody	hapten	10^{4}–10^{6}	equilibrium dialysis[e]
phosphorylase	5′-AMP	10^{4}–10^{6}	dialysis[f]
protease	inhibitor	10^{6}–10^{8}	[g]
pepsin	BSA	10^{4}–10^{6}	electrophoresis[h]
			fluorescence polarization[i]
chymosin	κ-casein ($1/K_m$)	10^{6}	enzyme activity[j]
self-association		10^{5}	spectroscopy[k]
			gel filtration[l,m]
			equilibrium sedimentation[n]
heterologous association		10^{3}–10^{5}	equilibrium sedimentation[o]
			frontal gel filtration[p]
			fluorescence polarization[q]
ovalbumin	Mn^{2+}	10^{3}	NMR[r]
lectin	sugar	10^{4}	fluorescence polarization[a]
			NMR[t]
			equilibrium sedimentation[u]

[a] Green (1963). [b] Kuzmanoff et al. (1990). [c] Kierszenbaum et al. (1969). [d] Parham (1984). [e] Nisonoff and Pressman (1958). [f] Feldmann (1978). [g] Angeletti (1988). [h] Cann and Klapper (1961). [i] Weber and Young (1964). [j] Fox (1988). [k] Koren and Hammes (1976). [l] Stevens and Schiffer (1981). [m] Godfrey and Harrington (1970). [n] Swann and Hammes (1969). [o] Jeffrey et al. (1979). [p] Gilbert (1971). [q] Nakai and Kason (1974). [r] Goux and Venkatasubramanian (1986). [s] Khan et al. (1981). [t] Brewer et al. (1983). [u] Howlett et al. (1983).

volume and rotor speed was properly made for ultracentrifugation and the best portions of computed data or sedimentation patterns were combined or the values computed using different initial simplices in optimization were averaged out.

The following strategy may be useful in obtaining quantitative results. According to Van de Voort et al. (1979), the MWD computed by applying multiple-regression analysis and the values for molecular weight and weight fraction of components computed by simplex optimization mutually complement in terms of accuracy; the former (MWD data) is rather qualitative, while the latter (simplex optimization data) is quantitative. The best use of these two methods for unknown mixtures is first to utilize the former to determine the number of components and crude estimates of their molecular weights and then to carry out the latter for computation of more reliable quantitative values which can be used for equilibrium constant computation.

Some precaution is needed for multicomponent analysis: (a) A limitation of sedimentation analysis of MWD is that each interval in the molecular weight scale of adjacent components should differ by a factor of 2 (Scholte, 1975). However, if the molecular weight of each component is known a priori, quantitative estimation of the weight fraction of each component could be valid. (b) In principle, the number of components in a sample mixture is not limited, although only up to six components have been tried in this study from the practical point of view. Since 15 factors were successfully computed in a similar application of simplex optimization (Nakai et al., 1991), the number six is not an absolute limit in capacity of the algorithm used in this study, although the running conditions of ultracentrifugation would become more restrictive in the determination of the feasibility of analysis. For mixtures of larger numbers of components, the greater gradients are required for sedimentation patterns using larger sample volumes, which need longer equilibration time, as well as high capacity of the scanner for monitoring larger absorbance values.

Equilibrium constants of protein interactions can be computed from the molar concentration of components in a sample solution computed from sedimentation data. Since the weight fraction computed according to the method proposed in this paper is the equilibrated concentration of the components in sample solution, those values can be used directly for substituting into the equation for computing the equilibrium constant. For cases complicated by more than three-component interactions such as $A + B \rightleftharpoons C$, e.g., $A + B \rightleftharpoons C + D$, $A + B \rightleftharpoons C + (C)_2$ as four-component interaction, and $A + B \rightleftharpoons C + (A)_2 + (A)_3$ as five-component interaction, curve fitting of possible equilibrium equations to the computed MWD would be useful for the estimation of equilibrium constants. Since the information on the molecular weight of interacting components is available a priori in most of the protein interaction studies, stoichiometry of various possible forms of the interaction can be postulated, which may be used to set search spaces for curve fitting using simplex optimization to find the best fit stoichiometry of the interaction as has been already applied to the α_{s1}–κ-casein interaction in the paper of Van de Voort et al. (1979). When the association constant of this interaction was calculated using data of mixtures with different concentration ratios assuming monomer–monomer interaction, an average value of 6.7×10^4 M^{-1} was obtained. This value was of about the same order as the values measured by fluorescence polarization (Nakai and Kason, 1974) of 3.2×10^4 M^{-1} and by frontal gel chromatography of Cann and Winzor (1987) with values of $3–9 \times 10^4$ under similar interaction conditions.

Some data of the association constants of protein interactions are collated in Table II to verify the data obtained for various protein interactions, e.g., the α_{s1}–κ-casein interaction. The strongest noncovalent association so far found in nature, i.e., avidin–biotin complex formation, has a K_a value of 10^{15} M^{-1}. In general, antigen–antibody interaction with a K_a of 10^8 M^{-1}, especially monoclonal antibody with up to 10^{12} M^{-1}, is stronger than enzyme–substrate interaction with a K_a up to 10^8 M^{-1}, which is stronger than lectin–sugar interaction with K_a values in the order of 10^5 M^{-1}. These data may be used for predicting chromatographic resolution, especially for affinity chromatography, as well as the specificity of different assay methods which utilize protein interaction phenomena, e.g., immunoassay and enzymatic assay.

Advancement in instrumentation has intensified the analytical capability of ultracentrifugation. The ability

to measure absorbance over a broad wavelength range in the scanner of mordern analytical centrifuges (190–800 nm for Optima XL-A) is a great advantage, since the behavior of nonprotein samples mixed with proteins can be seaprately analyzed or a specifically labeled protein can be analyzed in a mixture with other proteins. This enables analysis of a variety of heterologous associations, e.g., ligands, polysaccharides, or lipids interacting with proteins. There is no need for high rotor speeds for sedimentation equilibrium runs for protein interaction study; it is true that stable runs at rotor speeds below 10 000 rpm are preferable over high-speed runs for analysis of polymers larger than 1 000 000 as in the study of protein gelation. Therefore, a relatively low-cost analytical ultracentrifuge equipped with all of the necessary computing facility, if available, should realize completely automated analysis. All experimenters need to do is to place a sample into the instrument.

ACKNOWLEDGMENT

This work was supported by Operating Grant OGP0003641 from the Natural Sciences and Engineering Research Council of Canada.

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Received for review July 1, 1991. Revised manuscript received February 3, 1992. Accepted February 17, 1992.