Experimental Determination of Sedimentation and Diffusion Coefficients Employing Concentration Changes as a Function of Time†

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ABSTRACT: Numerical methods have been developed for rapid simultaneous determination of sedimentation and diffusion coefficients from transient state sedimentation analyses based on computer simulated data. Application of this methodology to experimental data obtained for ribonuclease A and chymotrypsinogen A demonstrates that the values of the sedimentation and diffusion coefficients determined from this approach do not differ significantly from the values of these parameters obtained *independently* by conventional techniques. Such determinations allow rapid estimation of apparent molecular weights.

Sedimentation in the ultracentrifuge occurs in a sector-shaped cell in a nonuniform force field; resulting concentration changes as functions of time and space are described by the mass conservation equation (Lamm, 1929)

$$\frac{\partial c}{\partial t} = \frac{1}{r}\frac{\partial}{\partial r}\left\{Dr\frac{\partial c}{\partial r} - s\omega^2 r^2 c\right\} \quad (1)$$

Few explicit experimental attempts have utilized the dynamic aspects of this formulation in their totality although measurements of concentration changes with time throughout the liquid column have been used in the determination of the sedimentation coefficient of thallous sulfate (Creeth, 1962), and evaluation of such changes at the air–liquid meniscus has led to the development of several different approaches to determination of molecular weights of macromolecules (LaBar, 1965; Charlwood, 1967; Simpson and Bethune, 1970).

A recent theoretical investigation (McNeil and Bethune, 1973) of dynamic sedimentation analysis has indicated its possible use in the simultaneous estimation of the molecular parameters, *i.e.*, sedimentation and diffusion coefficients, governing such transport. This paper delineates the experimental application of these techniques to determination of the sedimentation and diffusion coefficients of ribonuclease A and chymotrypsinogen A.

Materials

Ribonuclease A was obtained as a phosphate-free lyophilized preparation from Worthington Biochemical Corp. (lot RAF 6080-90). Prior to use the protein was dissolved in pH 7.3, 0.01 M imidazole–0.1 M NaCl buffer and heated for 10 min at 60° to dissociate polymers which may have been formed during lyophilization (Crestfield *et al.*, 1962).

Chymotrypsinogen A was obtained as a lyophilized salt with 0.10% intrinsic chymotryptic activity from Worthington Biochemical Corp. (lot CG-OEA). After dissolution in 0.038 M Na_2HPO_4 (pH 7.2) buffer, equimolar diisopropyl fluorophosphate was added to inhibit activation of the zymogen, preventing the polymer formation exhibited by chymotrypsin (Massey *et al.*, 1955). The sample was dialyzed against two changes of buffer. All buffer salts were of reagent grade.

Experimental Methods

Sedimentation studies were performed in a Beckman Spinco Model E ultracentrifuge. The optics were aligned according to the method of Van Holde and Baldwin (1958), and between experiments the rotational position of the Rayleigh mask was checked (Van Holde and Baldwin, 1958) to ensure constancy of position after removal of the upper collimating lens for cleaning. The interference mask used had symmetric, parallel, 1-mm slits. A Kodak 77A Wratten filter in combination with a high-pressure A-H6 (General Electric) mercury arc constituted the light source.

A single centrifuge cell with a 12-mm filled epon centerpiece and sapphire windows was employed in an H rotor. The sample side contained 0.02 ml of FC-43 (Beckman Instruments) and 0.18 ml of solution; the reference side had 0.018 ml of FC-43 and 0.20 ml of dialysate. Hamilton microsyringes were used for loading.

Photographs were taken on Kodak IIg spectroscopic plates. Exposure times were 20 sec and development was performed in freshly prepared Kodak D-19 developer. To ensure uniformity of development an external water bath was used to maintain the processing temperature at 20°.

For chymotrypsinogen A photographs were taken every 200 sec from 600 to 2000 sec;[1] the speed was 21,740 rpm. For ribo-

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[1] The timer was started with the drive. Therefore, these times do not correspond to the instantaneous time of application of a uniform field. To obtain the latter it is necessary to account for the variations of the speed with time during acceleration (Trautman, 1964). Since acceleration gave a speed two-thirds of the final speed within 250 sec for ribonuclease A and within 200 sec for chymotrypsinogen A, these times would have to be subtracted from any of the times indicated to obtain the equivalent time at the final operational speed. However, since the time derivative and not the time itself appears explicitly in the Lamm equation, this correction is not required in the calculation procedures.

nuclease A photographs were taken at 200-sec intervals from 400 to 2000 sec; the speed was 25,980 rpm. The temperature was regulated at 20° with the RTIC unit. Corresponding buffer blanks were run at each speed.

Initial concentrations, J_0, as measured by fringe displacement were obtained from synthetic boundary experiments. The fractional fringe was measured from photographs taken immediately after boundary formation. For both proteins the initial concentration was 2.5–3.0 g/l. or 10–11 fringes.

Independent measurements of the sedimentation and diffusion coefficients were made for each protein at the concentration used for the simultaneous determination. Sedimentation coefficients were determined using a double-sector schlieren cell with sapphire windows at 67,770 rpm from least-squares analysis of ln (r) at $(\partial c/\partial r)_{max}$ against time. Diffusion coefficients were obtained from least-squares analyses of height and area measurements (Gosting, 1956) as a function of time, using a synthetic boundary cell.

Photographic plates were evaluated using a Mann two-dimensional projection comparator (Bethune, 1965; Richards *et al.*, 1968). In the case of interference photographs each plate was aligned in the horizontal direction by rotating the plate so that the junction of the horizontal and vertical cross hairs, placed on the center of a centripetal reference fringe, was also centered on the corresponding centrifugal reference fringe. Each plate was aligned in the vertical direction using the vertical cross hair and the reference wire. The position of the reference on the photograph was read with a precision of $\pm 2 \times 10^{-4}$ cm. This value was used as the primary radial reference. Concentrations (as fringe shifts) were determined in successive exposures at the same radical distance from the reference wire at positions designated 1, 2, 3, . . . , n. The distance between these positions on the plate was 0.011 cm, corresponding to a distance along the liquid column of 0.005 cm, the magnification factor being 2.19. Vertical displacements were measured from the center of the center reference fringe (Richards *et al.*, 1968). The identification of a given fringe was facilitated by the presence of a plateau at all times. At each radial level the vertical displacement of a given fringe was measured five times and averaged; where concentration gradients were large, two or more fringes were used for evaluation of vertical displacements. Schlieren photographs were evaluated as described previously (Bethune, 1965).

Mathematical Methods

Equation 13 of the preceding paper (McNeil and Bethune, 1973) provided the most accurate method for the calculation of s and D when the bounds of integration were varied at constant time of observation for simulations described by the Mason–Weaver (1924) equation; in these cases numerical smoothing techniques developed with synthetic data (McNeil and Bethune, 1973) for c, $\partial c/\partial r$, and the time derivative of the first moment were used. Methods of solution for s and D were developed for molecules exhibiting minimal concentration dependence of these parameters. Thus, proteins fulfilling this requirement were chosen for experimental investigation. The sedimentation coefficients of chymotrypsinogen A and ribonuclease A are minimally dependent on c, that for ribonuclease remaining essentially constant (Heins *et al.*, 1967) up to 10 g/l. while that for chymotrypsinogen decreases from an extrapolated value of 2.46 S at infinite dilution to 2.40 S at 5 g/l. (Dreyer *et al.*, 1955).

Although the numerical techniques were developed using an integral form of the Mason–Weaver equation, in the experimental situation an equivalent integral formulation of the Lamm equation is required

$$\frac{d}{dt}\int_{r_1}^{r_2} cr\,dr = \left(Dr\frac{\partial c}{\partial r} - s\omega^2 r^2 c\right)\Bigg|_{r_1}^{r_2} \qquad (2)$$

The congruence of these approaches is demonstrated by the agreement found between values of $c(r,t)$ obtained from the analytical solution of the Mason–Weaver equation and those obtained from Archibald's exact solution (1942) of the Lamm equation (Yphantis and Waugh, 1956). Moreover, numerical estimates of $\partial c/\partial t$ at the meniscus obtained from concentration arrays generated from the analytical solution of the Mason–Weaver equation have been successfully used in experimental evaluation of meniscus concentrations for sedimentation equilibrium experiments in a sector-shaped cell (Charlwood, 1965).

In application of the numerical techniques, concentrations near the centripetal portion of the cell, where $\partial c/\partial t < 0$, are smoothed in time using linearization involving coth (c), evaluated at a fixed radial position r. Near the centrifugal region, where $\partial c/\partial t > 0$, concentrations are smoothed in time using linearization involving coth ($1/c$), again at a fixed radial position (McNeil and Bethune, 1973). At any radial position sequential smoothing is performed over three times of observation and an average value of c obtained (McNeil and Bethune, 1973).

These calculated concentrations form the data base for all subsequent calculations, all derivative and integral functions being calculated numerically from this smoothed concentration array. In estimation of $\partial c/\partial r$, accurate application of the central difference formula for numerical differentiation (Conte, 1965) requires linearity between the independent variable (r) and the dependent variable (c) or some analytical function involving it. Under a variety of simulated experimental conditions ln (c) *vs.* r was found to be linear at the base of the liquid column and c *vs.* r to be linear near the air–liquid meniscus (McNeil and Bethune, 1973). Evaluation of $\partial c/\partial r$ at a fixed time, therefore, utilized these relations, the pertinent formulae being eq 39a and 39b (McNeil and Bethune, 1973). Integration is performed using the IBM scientific subroutine QSF; the time derivatives of the resultant integrals are estimated by the central difference formula, based on the theoretical linear relationship between the integral and time (McNeil and Bethune, 1973).

The variation of the bounds of integration, r_i ($i = 1, 2, 3, 4$), in eq 2 allows simultaneous estimation of s and D if the r_i are selected by the criteria elaborated from synthetic data (McNeil and Bethune, 1973). Thus, r_1, the lower bound of integration in the first integral, was set at r_m plus 0.015 cm, where r_m is the radial distance of the air–liquid meniscus from the center of rotation. The upper bound of this integral, r_2, was fixed in the plateau obtaining at the last time of observation. The lower bound of the second integral, r_3, was set equal to r_1, while r_4, its upper bound, varied from that location at which the concentration was equal to the plateau concentration (c_p) plus twice the anticipated error in plate reading,[2] to r_b, the radial

[2] The minimal value for r_4 was chosen from data obtained at the second time of observation, that being the earliest time for which values of $(d/dt)\int_{r_3}^{r_1} cr\,dr$, calculated by the central difference theorem, were available. This value for r_4 was used at all subsequent times. Thus, because of the decrease in the length of the plateau with time, the value of c_4 at this fixed location at later times differed from c_p by more than twice the error in reading (*vide infra*).

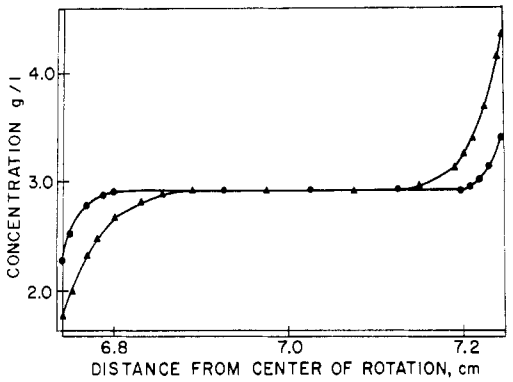

FIGURE 1: Concentration *vs.* position profile at the first (●, 400 sec) and last (▲, 1800 sec) times of experimentation for chymotrypsinogen A at a speed of 21,740 rpm, 20°. Initial concentration = 2.90 g/l. in 0.038 M Na_2HPO_4 (pH 7.2) buffer. Points were read every 0.005 cm along the liquid column for a total of 113 points; not every point is indicated.

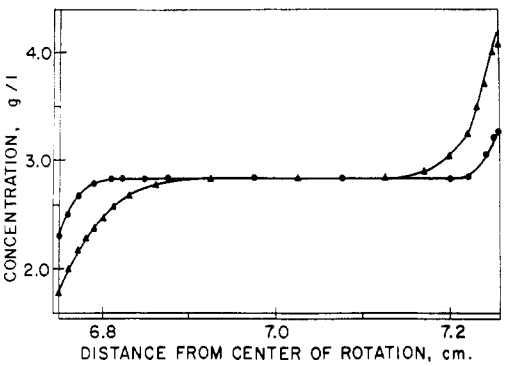

FIGURE 2: Concentration *vs.* position profile at the first (●, 600 sec) and last (▲, 2000 sec) times of experimentation for ribonuclease A at a speed of 25,980 rpm, 20°. Initial concentration = 2.83 g/l. in 0.01 M imidazole–0.1 M NaCl pH 7.3 buffer. Points were ready every 0.005 cm along the liquid column for a total of 112 points; not every point is indicated.

position of the base of the liquid column, minus 0.015 cm (LaBar, 1966b). Solution of the paired equations by a Gaussian elimination method (Conte, 1965) yielded values of s and D.

While r_1, r_2, and r_3 were fixed for any one experiment, r_4 varied, resulting in several sets of paired simultaneous equations, each of which was solved for s and D, thus generating an array of values of these parameters, implicitly as a function of r_4. In general, the results from a number of such independent calculations can be presented either in terms of an overall mean and standard deviation or in terms of a mean associated with a predetermined standard deviation. An iterative technique, therefore, was devised for isolation of a population of values with a predetermined standard deviation of the order of that customarily obtained from independent measurements of sedimentation and diffusion coefficients. In the first calculation stage the means (m_1) and standard deviations (σ_1) are obtained using all calculated values of s and D. In the second stage all values outside the range ($m_1 \pm \sigma_1$) are eliminated and new means (m_2) and standard deviations (σ_2) calculated. This procedure is repeated several times. A cluster of calculated values of defined standard deviation from the mean can thus be isolated. An initial sample population which is normally distributed will exhibit little fluctuation of the mean during the elimination procedure.

TABLE I: Values of the Mean and Standard Deviation for s and D for the Entire Population of Values Calculated for Chymotrypsinogen and for Subsets of the Original Population.

Elimination Stage	Sample Size (n)	Mean Value of Sed Coef (S)	SD of the Mean
0[a]	42	2.41	0.64
1	29	2.42	0.42
2	15	2.44	0.24
3	9	2.45	0.12
		Mean Value of Dif Coef (F)	
0[a]	42	9.66	0.66
1	28	9.66	0.38
2	16	9.61	0.20
3	9	9.57	0.10

[a] That is, for the entire population of values.

Results and Discussion

Experimental concentration distributions for chymotrypsinogen A and ribonuclease A exhibited a plateau both at early and late times of experimentation (Figures 1 and 2). The initial concentration, c_0, used for both proteins was low (<3.0 g/l), and, at the speeds selected, over the short time of experimentation concentration changes at the meniscus and base are of the order of 30% of c_0. The mean value (Table I) of the sedimentation coefficient (2.45 S) of chymotrypsinogen associated with a standard deviation of 0.12 S differs by 2% from that determined independently (2.50 S with E_s[3] = 0.14 S) while that of D, 9.57 F (Fick units) with a standard deviation of 0.10 F, differs by 0.74% from that determined independently (9.50 F with E_D[4] = 0.37 F); however, since the 95% confidence limits overlap in both cases the differences are not statistically significant. At each time for which time derivatives of the integral were available, *i.e.*, 6, 7 sets of simultaneous equations were solved, a total of 42 values of s and D being obtained.

The individually calculated values of these coefficients obtained for differing upper bounds of integration, r_4, were randomly distributed (Table II). No consistent variation in the magnitude of the constants with r_4 is apparent, thus suggesting sample homogeneity, since for heterogeneous samples an increase in s and a decrease in D occur as r_4 approaches the base of the liquid column (McNeil and Bethune, 1973).

The mean value (Table III) of the sedimentation coefficient of ribonuclease A (1.64 S) associated with a standard deviation of 0.10 S differs by 1.8% from that obtained independently (1.67 S with E_s = 0.01 S). The value of D with a standard deviation of 0.16 F, 10.71 F, differs by 1% from the independently determined value, 10.6 F (E_D = 0.36 F); however, since the 95% confidence limits overlap in both cases, the differences are not statistically significant. At each time for which time derivatives of the integral were available, *i.e.*, 7, 6 sets of simultaneous equations were solved, a total of 42 values of s

[3] E_s represents the 95% confidence limits for c calculated (Draper and Smith, 1966) from the standard error of the slope of ln (r) at $(\partial c/\partial r)_{max}$ *vs.* t (*vide supra*).

[4] E_D represents the 95% confidence limits for D calculated (Draper and Smith, 1966) from the standard error of the slope of $(A/H)^2$ *vs.* t (*vide supra*).

TABLE II: Values of s and D for Chymotrypsinogen Calculated as a Function of the Distance of r_4 from the Center of Rotation at a Fixed Time, 1200 sec, and at Fixed Values of r_1, r_2, and r_3.

r_4 (cm)	7.217	7.221	7.226	7.230	7.235	7.239	7.244
s (S)	2.51	2.35	2.32	2.61	2.92	3.04	2.84
D (F)	9.97	10.21	10.25	9.83	9.36	9.18	9.48

and D being obtained. Again the absence of a clear trend in the values of s and D as a function of position (Table IV) suggests the absence of heterogeneity.

Sedimentation and diffusion coefficients for chymotrypsinogen A and ribonuclease A have been determined previously. For chymotrypsinogen values of 2.54 S and 9.5 F were obtained (Schwert, 1951), while a lower value of s, 2.46 S, found in a study of the concentration dependence of s on c (Dreyer *et al.*, 1955) is in good agreement with the present data. For ribonuclease A a range of values of s and D have been reported, obviating meaningful comparison; values of 1.64 S and 11.9 F (Rothen, 1940), 2.00 S and 13.10 F (Edsall, 1953), and 1.74 S (Heins *et al.*, 1967) may be cited. The presence of dimers may account for such large variability, particularly in the earlier reported values.

The magnitude of the differences between the mean values of these parameters and the values obtained from independent determinations is a function of the number of times at which observations are made. To illustrate this, the elimination procedure (*vide supra*) was performed utilizing the results from the first set of simultaneous equations, then the results from the first set together with those from the second set, etc. For both the sedimentation coefficient (Figure 3) and the diffusion coefficient (Figure 4) the *absolute* difference drops dramatically as the number of sets increase and approaches 1%. These data indicate that the number of observations is a critical factor in determining the magnitude of these differences. In statistical terms, if $\bar{\omega}$ represents a population mean value and p, a random sample mean value, and ψ a measure of the approximation of $\bar{\omega}$ by p, then (Kendall, 1952)

$$\psi = \left|\frac{\bar{\omega} - p_i}{\bar{\omega} - p_j}\right| = \sqrt{\frac{n_j}{n_i}}$$

TABLE III: Values of the Mean and Standard Deviation for s and D for the Entire Population of Values Calculated for Ribonuclease A and for Subsets of the Original Population.

Elimination Stage	Sample Size (n)	Mean Value of Sed Coef (S)	SD of the Mean
0[a]	42	1.68	0.52
1	25	1.62	0.29
2	15	1.67	0.18
3	9	1.64	0.10
		Mean Value of Dif Coef (F)	
0[a]	42	10.77	0.85
1	27	10.80	0.48
2	18	10.80	0.30
3	9	10.71	0.16

[a] That is, for the entire population of values.

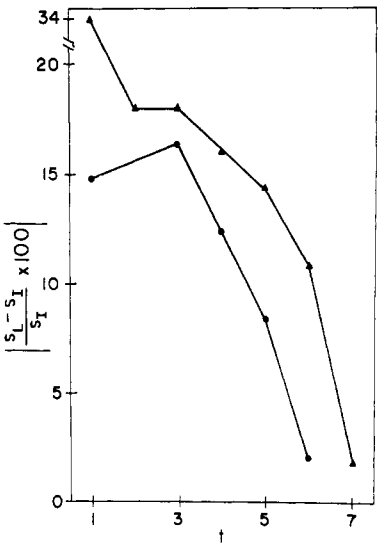

FIGURE 3: Effect of the number of observations made with time on the accuracy of the determination of the mean value of s. The abscissa represents the number of sets of simultaneous equations considered for the calculation. (The first and last times of observation do not contribute a set since the central difference theorem was used for the determination of temporal derivatives). On the ordinate the absolute value of the per cent difference between s_L, the sedimentation coefficient calculated from the integral form of the Lamm equation, and s_I, that measured independently, is plotted. While the percent deviation fluctuates randomly from positive to negative, here the absolute value is plotted to illustrate the dependence of absolute accuracy on the number of observations. (●) Chymotrypsinogen, (▲) ribonuclease.

where the sample size increases from n_i to n_j. Thus, as n_j increases, for fixed n_i, the sample mean value, p_j, approaches the population mean, $\bar{\omega}$, more closely. In the present context, if the independently determined sedimentation or diffusion coefficient is selected as the population mean value, then the estimation of this value from those determined by the present procedure should increase in accuracy as the number of observations increases (Table V). While such an increase is observed, the magnitude of the increase is greater than that predicted. This may be accounted for, in part, if the precision of the ith and jth sets differ, in which case the relevant formula is

$$\psi = \frac{h_j}{h_i}\sqrt{\frac{n_j}{n_i}}$$

where h is the precision factor, related to the probable error (Sokolnikoff and Redheffer, 1958). Variation in the relative magnitudes of h_j and h_i appears to be an explanation for a value of ψ higher than expected (Table V). The ratio h_j/h_i can be calculated using the actual values of ψ obtained and the values expected theoretically, for ψ a function of n_j/n_i only.

TABLE IV: Values of s and D for Ribonuclease A Calculated as a Function of the Distance of r_4 from the Center of Rotation at a Fixed Time, 1200 sec, and at Fixed Values of r_1, r_2, and r_3.

r_4 (cm)	7.222	7.227	7.231	7.236	7.240	7.245
s (S)	1.86	2.45	2.28	2.04	1.81	1.61
D (F)	11.51	10.20	10.58	11.12	11.62	12.08

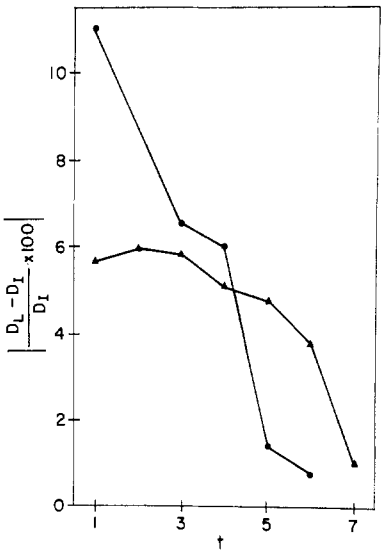

FIGURE 4: Effect of the number of observations made with time on the accuracy of the determination of the mean value of D. The abscissa represents the number of sets of simultaneous equations considered for the calculation. (The first and last times of observation do not contribute a set since the central difference theorem was used for the determination of temporal derivatives.) On the ordinate the absolute value of the per cent difference between D_L, the diffusion coefficient calculated from the integral form of the Lamm equation, and D_I, that measured independently, is plotted. While the per cent deviation fluctuates randomly from positive to negative, here the absolute value is plotted to illustrate the dependence of absolute accuracy on the number of observations. (●) Chymotrypsinogen, (▲) ribonuclease.

Such calculations yield values of h_j/h_i of 2.9 and 4.5 for the calculation of s and D, respectively, for chymotrypsinogen A, and of 6.4 and 2.2 for those of ribonuclease A. The average of all four values gives an overall enhanced accuracy, h_j/h_i, of $\sim$4. Such an improvement is not unexpected since at early times the values of $\partial c/\partial r_4$ at the lower limit of r_4 may fall outside the range in which numerical approximation of $\partial c/\partial r$ is most accurate (McNeil and Bethune, 1973). At later times and/or near the upper limit of r_4, however, the magnitude of $\partial c/\partial r$ is always within the desired range. Moreover, since the lower limit for r_4 in all calculations is chosen at the second time of observation, h_j is greater than h_i, and thus ψ can assume a value larger than that calculated assuming simple error analysis. Quantitatively, the percent difference between the plateau concentration, c_p, and the concentration c_4 at the smallest value of r_4 selected for each protein increased from $\sim$2% at the second time of observation to $\sim$15% at the penultimate time, on the average, for both proteins. Since the 2% difference was selected to correspond to twice the anticipated error in reading (*i.e.*, 2 σ), then the 15% corresponds approximately to about 7–8 times such error. Thus, on this basis alone an improvement in accuracy of 7- to 8-fold could be expected.

TABLE V: Theoretical and Actual Values of ψ.

	Actual	Theor
Chymotrypsinogen A[a]		
For s	7	2.45
For D	11	2.45
Ribonuclease A[b]		
For s	17	2.66
For D	6	2.66

[a] $n_i = 7, n_j = 42$. [b] $n_i = 6, n_j = 42$.

Determination of s and D under the same experimental conditions allows estimation of the apparent molecular weight, M_{app} (Svedberg and Pedersen, 1940) (eq 3).

$$M_{app} = \frac{s}{D}\frac{RT}{(1 - \bar{v}\rho)} \tag{3}$$

Since the s and D calculated here are associated with standard deviations (*vide supra*), any subsequent manipulation of s and D to yield a derived quantity, *e.g.*, M_{app}, is also accompanied by an associated standard deviation, in this case determined from (Hendee, 1970)

$$\pm\frac{s}{D}\sqrt{\left\{\frac{\sigma_s}{s}\right\}^2 + \left\{\frac{\sigma_D}{D}\right\}^2}\left\{\frac{RT}{1-\bar{v}\rho}\right\} \tag{4}$$

where σ_s and σ_D are the standard deviations for s and D, respectively. However, calculation of average values of s and D from a given experiment involved, in our case, a series of averages, first starting with the entire sample of calculated values of s or D to obtain an average value with an associated standard deviation and then continuing with an elimination procedure in which all values one standard deviation from the mean were eliminated and a new mean and standard deviation was calculated. Thus, an average s or D with a preset standard deviation of varying magnitude was obtained, the number of values used to determine the average varying with the size of the standard deviation involved (Tables I and III); *i.e.*, small standard deviations necessitated several elimination stages with resultant diminution of the sample size. Accordingly, both the derived value of M_{app} and its standard deviation are functions of the stage of the elimination procedure, *i.e.*, the size of the standard deviation selected. Since for both ribonuclease and chymotrypsinogen the mean values of s and D remained essentially constant during the elimination procedure while the standard deviations changed up to 6-fold, selection of appropriate values for use in eq 3 involves a compromise between the decreased reliability of the approximation of M_{app} as the size of the sample, n, from which it is obtained drops and the increased size of the standard deviation for M_{app} as n increases (as n increases both σ_s and σ_D increase).

Thus, for example, if elimination stage 2 for chymotrypsinogen is selected where $s = 2.44 \pm 0.24$ S, $D = 9.61 \pm 0.20$ F for $n \cong 15$, then M_{app} is 22,300 $\pm$ 2200 assuming a partial specific volume for chymotrypsinogen of 0.721 (Schwert, 1951). Similarly for elimination stage 3 where $s = 2.45 \pm 0.12$ S, $D = 9.57 \pm 0.10$ and $n \cong 9$, then $M_{app} = 22{,}500 \pm 1100$. The independently measured values yield $M_{app} = 23{,}100 \pm 1600$.

Similarly, for ribonuclease, if elimination stage 2 is selected where $s = 1.67 \pm 0.18$ S, $D = 10.80 \pm 0.30$ F, and $n \cong 15$, then $M_{app} = 12{,}400 \pm 1400$, assuming a partial specific volume for ribonuclease of 0.695 (Harrington and Schellman, 1956). If elimination stage 3 is selected where $s = 1.64 \pm 0.10$ S, $D = 10.71 \pm 0.16$ F, and $n \cong 9$, then $M_{app} = 12{,}300 \pm 770$. The independently measured values yield $M_{app} = 12{,}700 \pm 400$.

These molecular weights may be compared with those calculated from amino acid analysis data which should yield absolute molecular weights, M_0. However, in the present

context, where M_{app} is obtained, these will be related to M_0 through

$$\frac{1}{M_{app}} = \frac{1}{M_0} + Bc$$

where B is the second virial coefficient and c the concentration. Comparison between the two values requires not only extrapolation to zero concentration but, in addition, corrections for buffer and charge effects of the medium. Since this paper constitutes a prototype investigation of the utilization of dynamic aspects of transport, the essential comparison has been with s and D obtained by this procedure and with those obtained under identical conditions by independent measurements. Thus, any attempt to identify our derived values of M_{app} with M_0 must take these factors into account. Nonetheless, despite these limitations, the comparison is not unfavorable. The molecular weight of chymotrypsinogen, 25,767, as obtained from amino acid analysis data (Hartley, 1964) is within two standard deviations of the value of 22,300 found by either the present technique or from the independently determined values (*vide supra*). The molecular weight of ribonuclease, 13,683, determined from amino acid analysis data (Hirs *et al.*, 1956), falls within two standard deviations of the above values for M_{app} calculated either by the present technique or from the independently determined values.

For certain systems this technique, which allows a rapid estimate of M_{app}, should prove particularly valuable. For example, for time dependent systems in which polymerization, aggregation, or autolysis occurs, thus preventing attainment of equilibrium, this method would permit an estimate of molecular weights without the ambiguities inherent in the Archibald approach (LaBar, 1966a). Moreover, more rapid data acquisition should decrease the time of experimentation required and thus allow good weight-average estimates of M_{app} for all but the most rapidly reacting irreversible systems.

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