Barnett, J. E. G., Holman, G. D., Chalkley, R. A., & Munday, K. A. (1975) *Biochem. J. 145*, 417–429.
Carruthers, A., & Melchior, D. L. (1983a) *Biochim. Biophys. Acta 728*, 254–266.
Carruthers, A., & Melchior, D. L. (1983b) *Biochemistry 22*, 5797–5807.
Duggleby, R. G. (1981) *Anal. Biochem. 110*, 9–18.
Faust, R. G. (1960) *J. Cell. Comp. Physiol. 56*, 103–121.
Foster, D. M., Jacquez, J. A., Lieb, W. R., & Stein, W. D. (1979) *Biochim. Biophys. Acta 555*, 349–351.
Geck, P. (1971) *Biochim. Biophys. Acta 241*, 462–472.
Ginsburg, H., & Stein, W. D. (1975) *Biochim. Biophys. Acta 382*, 353–368.
Gorga, F. R., & Lienhard, G. E. (1981) *Biochemistry 20*, 5108–5113.
Hankin, B. C., Lieb, W. R., & Stein, W. D. (1972) *Biochim. Biophys. Acta 288*, 114–126.
Holman, G. D. (1980) *Biochim. Biophys. Acta 599*, 202–213.
Jacquez, J. A. (1983) *Biochim. Biophys. Acta 727*, 367–378.
Naftalin, R. J., & Holman, G. D. (1977) in *Membrane Transport in Red Cells* (Ellory, J. C., & Lew, V. C., Eds.) pp 257–300, Academic Press, New York.
Pigman, W., & Anet, E. F. L. J. (1972) in *The Carbohydrates* (Pigman, W., & Horton, D., Eds.) pp 165–194, Academic Press, New York.
Regen, D. M., & Tarpley, H. L. (1974) *Biochim. Biophys. Acta 339*, 218–233.
Rich, G. T., Sha'afi, R. I., Romualdez, A., & Solomon, A. K. (1968) *J. Gen. Physiol. 52*, 941–954.
Vieira, F. L., Sha'afi, R. I., & Solomon, A. K. (1970) *J. Gen. Physiol. 55*, 451–466.
Widdas, W. F. (1952) *J. Physiol. (London) 118*, 23–39.
Widdas, W. F. (1980) *Curr. Top. Membr. Transp. 14*, 165–223.

Boundary Centrifugation in Isovolumetric and Isokinetic Cesium Sulfate Density Gradients: Application to Cartilage Proteoglycans and Other Macromolecules†

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ABSTRACT: A boundary sedimentation methodology is described that avoids plateau dilution and simplifies the calculation of centrifugal parameters. The technique is designed for the preparative ultracentrifuge and uses a newly developed sectorial cell. It is based on previous developments of the transport method and depends on isokinetic or isovolumetric Cs_2SO_4 density and viscosity gradients. These gradients are prepared with a single-chamber mixing device, and the only two parameters required for their calculations are presented in a tabulated form for general use with most available rotors and cell sizes. Conditions are specified (1) to assure that the density and shape of the sedimenting molecules remain invariant through the selected electrolytic gradient, (2) to monitor the gradient profiles, and (3) to verify attainment of isokinetic or isovolumetric sedimentations. A set of equations is presented to calculate the average and transport sedimentation coefficients and the differential sedimentation coefficient distribution for both the isokinetic and isovolumetric centrifugal regimes. The method was applied to slowly diffusing polydisperse proteoglycan monomers, to a paucidisperse DNA from bacteriophage PM2, and to a diffusible monodisperse system (purified bovine serum albumin). In all cases, the expected results were obtained.

In earlier publications, we described the use of the transport method for the centrifugal characterization of cartilage proteoglycans and other biological macromolecules in the preparative ultracentrifuge (Pita & Muller, 1972, 1973a,b; Pita et al., 1978, 1979, 1983). The present work describes a velocity sedimentation technique that is based on the same principles and incorporates the use of specially designed exponential gradients in combination with a newly developed sectorial cell (Pita et al., 1983). The gradient can be made to avoid plateau dilution during centrifugation and to substantially simplify the mathematical analysis of the sedimenting boundaries. Special care will be taken in this work to select a gradient medium that will not erratically affect the sedimenting molecules during sedimentation. The present technique has been especially designed to study polydisperse and slowly diffusing molecules such as cartilage proteoglycans, DNA, hyaluronate, etc. The practical parameters given below in Table III, however, have been calculated with sufficient generality as to render them useful for a variety of biological molecules within a density range of 1.25–1.85 g/cm^3, and for almost all available centrifugation cells and swinging-bucket rotors.

Following a different approach, preparative rather than analytical, several researchers have used, previously, exponential gradients in connection with zonal velocity centrifugation. The latter technique has been used in the fractionation of complex cell particles and biological macromolecules in the presence of sucrose isokinetic or equivolumetric gradients (Schumaker, 1967; McCarty et al., 1968; Spragg et al., 1969; Noll, 1969; Pollack & Price, 1971; Vanduffel et al., 1975). Although there is no reason why zonal centrifugation could not be used as an analytical technique, the usual approach, however, for studying differential distribution functions of s

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Table I: Equations for Boundary Sedimentation in Isokinetic and Isovolumetric Gradients[a]

definition	isokinetic gradients	eq no.	isovolumetric gradients	eq no.
	$v = s\omega^2X$ = constant	IA	$vX = s\omega^2X^2$ = constant	IB
	$s\omega^2X = s_i\omega^2X_i$	IIA	$s\omega^2X^2 = s_i\omega^2X_i^2$	IIB
X, ρ, and η	$X/X_i = W_i\eta_x/(\rho_P - \rho_x)$	IIIA	$(X/X_i)^2 = W_i\eta_x/(\rho_P - \rho_x)$	IIIB
sedimentation coefficient	$s_i = (1/\omega^2t)(X/X_i - 1)$	IVA	$s_i = (1/2\omega^2t)(X^2/X_i^2 - 1)$	IVB
			$s_i = (1/2\omega^2t)(N\Delta V/K_i)$	IVB′
transport s value	$\bar{s} = (1/\omega^2t)(1 - C_F/C_i)H_N/X_i$	VA	$\bar{s} = (1/\omega^2t)(1 - C_F/C_i)N\Delta V/K_i$	VB
radial position	$X = X_i(1 + s_i\omega^2t)$	VIA	$X^2 = X_i^2(1 + 2s_i\omega^2t)$	VIB
plateau dilution	$C_p/C_i = X_i/X$ or $=1$	3b	$C_p/C_i = 1$	
polydispersity function	$g(s) = \omega^2tX(dC/dX)$	VIIA	$g(s) = \omega^2t(X_i^2/X)(dC/dX)$	VIIB
	$g(s) = \omega^2tX_i(dC/dX)$	VIIA′	$g(s) = (\omega^2t2K/\Delta V)(dC/dN)$	VIIB′

[a] The meaning of the subscripts is as follows: B, bottom; P, sedimenting particle; p, plateau; F, final; i, initial (position or concentration); x, position X; 0, meniscus; n, aliquot number. The variables are as follows: η, viscosity; ρ, density; ω, angular velocity; t, time; ΔV, aliquot volume; N, aliquot number; v, sedimenting speed; C, macromolecular concentration; c, medium concentration. Group variables are $W_i = (\rho_P - \rho_i)/\eta_i$, $H_P = X_P - X_i$, $H_N = X_N - X_i$, $K = {}^1/_2EaX_0$ (see Figure 1), and $K_i = {}^1/_2EaX_i$.

values in polydisperse systems such as proteoglycans and DNA has relied mostly upon boundary velocity sedimentation. To apply this technique to the study of proteoglycans by the transport method seems to exclude the use of sucrose gradients since this sugar would interfere with the analysis of hexuronate concentration at the end of centrifugation. Resort to radioactive labeling of the macromolecule to evade this interference would restrict the technique to study macromolecules synthesized in cell or tissue cultures or only to study newly synthesized macromolecules in vivo. For these reasons, it seems simpler to select an adequate gradient medium, such as Cs_2SO_4, that could establish an isokinetic or an isovolumetric boundary sedimentation regime.[1]

Noll (1967) has described a simple device for easily preparing isokinetic gradients in cylindrical cells. It will be demonstrated later in this paper that when such gradients, which impart a constant velocity to the sedimenting particles, are discharged into a sectorial cell instead of a cylindrical one, an isovolumetric gradient results. An attractive property of isovolumetric gradients is that the concentration of the sedimenting particles in the plateau region will remain constant during centrifugation. This feature can eliminate most of the difficulties connected with plateau dilution.

To test the technique, the use of the equations and practical parameters given below will be exemplified, under Results, with an extensive study of bovine nasal proteoglycan monomers. In a less detailed manner, results for a diffusible monodisperse system, bovine serum albumin, and a slowly diffusing paucidisperse one, DNA from PM-2, are also presented to illustrate the general usefulness of the methodology.

Theory and Calculations

All mathematical equations needed for the calculation of the relevant centrifugal properties in isokinetic and isovolumetric gradients are summarized in Table I. The meaning of the symbols and subscripts are also given as a footnote to the table.

Theoretically Required Gradients. The mass transport equation for the centrifuge, originally deduced by Lamm (1929) in terms of the concentration change, dC/dt, for a solute having sedimentation coefficient s and diffusion coefficient D, is

$$\partial C/\partial t = (1/X)\frac{\partial}{\partial X}\left[D\frac{\partial C}{\partial X}X - Cs\omega^2X^2\right] \qquad (1)$$

where ω is the angular velocity and X is the radial position of a point showing a solute concentration C. In the plateau region, $dC/dX = 0$, and the first term in the brackets disappears. The remaining term predicts a radial dilution law when integrated between an initial concentration (C_i) and a final one (C_p) according to the well-known formula $C_p/C_i = e^{-s\omega^2t} = (X/X_i)^2$. If, however, $s\omega^2X^2$ could be made constant, then $dC/dt = 0$, and the plateau concentration will not change during centrifugation. Since by definition

$$s\omega^2X^2 = vX \qquad (2)$$

this would imply vX = a constant as expressed in eq IB in Table I for an "isovolumetric" or "isoconcentration" regime of sedimentation. Similarly, if instead of vX, only the sedimentation speed v is made constant (eq IA), an "isokinetic" mode of centrifugation will result. In the latter case, eq 1 for the plateau region yields $dC/dt = vC/X$ and integrating from C_i to C_p as before

$$\ln (C_p/C_i) = e^{-s\omega^2t} \qquad (3a)$$

which is a linear dilution law, also expressible as

$$C_p/C_i = X_i/X \qquad (3b)$$

when sedimentation of the boundary has occurred from an initial position X_i to a final position X.

For generalization purposes, it will not be assumed that X_i necessarily coincides with the meniscus radial coordinate, X_0. Any reference point X_i, where the viscosity η_i and density ρ_i of the medium are known, and where the sedimentation coefficient has the value s_i as affected by those conditions of the medium, can serve to evaluate the constant value of $s\omega^2X^2$ in the isovolumetric case and of $s\omega^2X$ in the isokinetic one. Thus, we can write eq II of Table I. The latter equations express the ratio between s_i and s_x (the subscript x is added to emphasize the connection with point X) and the respective positions X_i and X in the isokinetic case or the square of those positions in the isovolumetric regime. On the other hand, the ratio between s_x and s_i can be expressed by the well-known relation

$$\frac{s_x}{s_i} = \frac{\eta_i(\rho_P - \rho_x)}{\eta_x(\rho_P - \rho_i)} \qquad (4)$$

in terms of the respective densities and viscosities at the points X and X_i involved. Combining eq 4 with eq II of Table I results in eq III of the table, which finally summarizes the values of η_x and ρ_x that are needed for each position ratio X/X_i in order to achieve the conditions expressed by eq I and II. As noted, the distribution of densities and viscosities depends also upon the particle density, ρ_P. This density might not coincide with the reciprocal of the partial specific volume as

[1] The term "isovolumetric" is used here with the same meaning as the equivolumetric term found in the literature. We have preferred to use the prefix "iso" for boundary sedimentation, leaving "equi" to denote the context of zonal centrifugation.

determined, for example, by classical pycnometry in a diluted solution of the particle. What is important is that the effective density in the selected medium be known (for example, by isopycnic centrifugation) and that its value, as well as the shape of the particle, remains invariant throughout the entire concentration range of the medium used. A way to assess this invariancy is illustrated below for cartilage proteoglycans in Cs_2SO_4.

Calculation of Sedimentation Coefficients. If in eq IA the speed v is expressed as dX/dt and also referred to the initial conditions, then $dX/dt = s_i\omega^2X_i$. Integrating from X_i to a certain final position X attained by the particle in time t yields eq IVA in Table I. For the isovolumetric case, a similar integration of eq IB leads to eq IVB. Equation IVB′ is useful when the total volume, $(X^2/X_0^2 - 1)K$, is equated to N times the aliquot volume (ΔV). If the conditions at X_i are too far from the standard conditions (water at 20 °C), the standard correction formula still has to be applied to get

$$s_{20,w} = s_i \frac{\eta_i(\rho_P - \rho_{20,w})}{\eta_{20,w}(\rho_P - \rho_i)} \quad (5)$$

s Value Determination by the Transport Method. If at the end of centrifugation a total solution column of length H_P ($=X_p - X_0$ extending from the meniscus (X_0) to a point in the common plateau region (X_p) is extracted and its homogenized final solute concentration (C_F) is found and compared to the initial concentration (C_i) as detailed in the previous publications (Pita et al., 1978, 1979), a transport average s value can be calculated by using eq VA and VB in Table I for each sedimentation regime, respectively. For simplicity, eq VB has been expressed in terms of the cell constant, $K = {}^1/_2E\theta a$ (see below). Also, the final concentration C_F can be found as the average resulting when N aliquots of volume ΔV are individually analyzed for final concentration C_f. Hence, $C_F = \sum C_f/N$. Such a point to point determination of the various C_f's is necessary to obtain the sedimentation boundary profile by the transport method. With this profile, the previous s value equations can be evaluated from the second moment of the boundary profile or from the "peak" value found by stepwise differentiation.

In eq VA, a simplification was also introduced by assuming, in spite of the linear dilution law predicted by eq 3b, that no such dilution has occurred. In practice, this has been found to be the case when an isokinetic sedimentation, conducted in cylindrical cells, is analyzed by the transport method. The reason is the restoration of the initial concentration in the plateau when, upon removal of each aliquot, the material that has collided with the cell walls is also recovered and is included in the final aliquot concentration, C_f. Thus, the similarity between eq VA and VB is not surprising.

Polydispersity Determination by the Transport Method. A major goal of the present methodology is to find the polydispersity differential distribution function, defined as

$$g(s_{20,w}) = dC/ds_{20,w} \quad (6)$$

This can be found, as previously described (Pita et al., 1983), by the transport method from a plot of several C_f's (usually divided by C_i) vs. the position coordinate X or vs. the aliquot number N corresponding to each stepwise extraction along the boundary profile. Mathematically, the $g(s)$ function can be broken down into several terms:

$$g(s_{20,w}) = (dC_i/dC_f)(dC_f/dX)(dX/ds_i)(ds_i/ds_{20,w}) \quad (7)$$

The first term corrects every aliquot concentration, C_f, to eliminate the plateau dilution effect, if any. In isovolumetric regimes, we have seen that this factor is unity (no radial dilution). However, in isokinetic cases, it might follow a linear dilution law (eq 3b), or also it might be absent as explained before for the transport method. The second term of eq 7 refers to the macromolecular concentration gradient along the boundary, and it is found by numerical differentiation of the boundary as mentioned above. The third term can be found by, first, solving X (or N) from eq IV (Table I). This leads to eq VIA and eq VIB which, in turn, are differentiated with respect to s_i, yielding

$$dX/ds_i = \omega^2tX_i \quad (8a)$$

and

$$dX/ds_i = \omega^2t(X_i^2/X) \quad (8b)$$

The final term of eq 7 expresses the correction of each s_i to standard conditions, if needed. If the latter correction is neglected and eq 3, 8a, and 8b are combined into eq 7, the polydispersity differential distribution function finally results, for each case, as expressed by eq VII of Table I. The duality of eq VIIA and VIIA′ for the isokinetic case results from the duality of plateau dilution behavior in cylindrical cells mentioned before. On the other hand, eq VIIB′ is the counterpart to IVB′ in terms of N.

Usually the $g(s)$ functions are evaluated and plotted vs. the corresponding s values as given by eq IV in the table. When the various s values are separated by equal intervals, Δs, the "cumulative" or integral distribution function, defined as $G(s) = \int g(s)\,ds$, can be obtained by successively adding the previous $g(s)$ functions. Each partial addition is divided by the total sum, and the "normalized" $G(s)$ functions result.

Correlation between Isokinetic Gradients in Cylinders and Isovolumetric Gradients in Sectorial Cells. Suppose that an isokinetic gradient is introduced in a cylindrical cell having a volume V_{cyl} (from the meniscus coordinate $X_{0_{cyl}}$ to a position X_{cyl} down the cell). Suppose that the same volume of gradient is transferred, point by point, to a sectorial cell where it now extends from $X_{0_{sect}}$ to X_{sect}. (For generality's sake, we will assume that both meniscus positions, $X_{0_{cyl}}$ and $X_{0_{sect}}$, are different.) We will prove that the transferred isokinetic gradient becomes an isovolumetric gradient in the sectorial cell.

For a cylinder of radius R, the discharged volume can be expressed as

$$V_{cyl} = \pi R^2(X_{cyl} - X_{0_{cyl}}) \quad (9a)$$

and for the sector

$$V_{sect} = {}^1/_2E\theta(X_{sect}^2 - X_{0_{sect}}^2) \quad (9b)$$

Solving for the coordinate ratio in each case:

$$X_{cyl}/X_{0_{cyl}} = 1 + V_{cyl}/\pi R^2X_{0_{cyl}} \quad (10a)$$

and

$$(X_{sect}/X_{0_{sect}})^2 = 1 + V_{sect}/{}^1/_2E\theta X_{0_{sect}} \quad (10b)$$

Since the two volumes V_{cyl} and V_{sect} are assumed equal and since the meniscus coordinates can be chosen at will such that

$$\pi R^2X_{0_{cyl}} = {}^1/_2E\theta X_{0_{sect}}^2 \equiv K \quad (11)$$

the two equations, 10a and 10b, are equal, so

$$X_{cyl}/X_{0_{cyl}} = (X_{sect}/X_{0_{sect}})^2 \quad (12)$$

However, this is precisely the condition deduced from eq III in Table I when the term $W_i\eta_x/(\rho_P - \rho_x)$ is identical in both cases (i.e., when the same η_x and ρ_x values exist in each cell). Thus, by correlating the cells as stipulated by the constant K of eq 11, an isokinetic gradient in the cylinder of radius R and

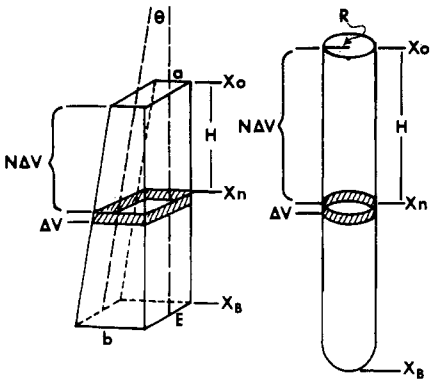

FIGURE 1: Comparison of sectorial and cylindrical centrifugation cells. Both cells are made of polycarbonate and include a bulky mass of this plastic (not shown) to cover the hemispherical portion of the swinging-bucket holders. In both cases, the effective bottom coordinate was X_B = 142 mm. The portions in braces indicate the volume change, ΔV, corresponding to a single aliquot withdrawn or added to the cell. For the sectorial cell, the constant dimension E was equal to b (for maximal capacity) and equal to 6.11 mm. Thus, tan θ = 0.04304 and θ = 0.04300 rad. At the top, X_0 = 95.7 mm and $a = X_0 \tan \theta$ = 4.12 mm.

starting at X_0 will be transformed, automatically, into an isovolumetric gradient if discharged in a sectorial cell of dimensions E and θ, and starting at $X_{0_{sect}}$.

In practice, a small problem exists due to the fact that eq 9b refers to the volume of a true sector and this applies only when the cell and its contents are subjected to centrifugal forces (in which case the meniscus surface becomes slightly curved). However, when the gradient is being formed by discharging the medium inside a static cell in the laboratory bench, the actual shape of this volume coincides not with a circular sector but with a trapezoid as illustrated in Figure 1. According to the dimensions given in the figure (the ones actually used for this paper), the angle θ is so small (about 2.5°) that it can be replaced by its tangent. At position X_0, $\tan \theta = a/X_0$. So the total volume of the trapezoidal cell, with bottom coordinate $X_B = b/\tan \theta$, can also be expressed by substituting $X_B = b/\theta$ and $X_0 = a/\theta$ in eq 9b as

$$V_T = \tfrac{1}{2}E(a + b)(X_B - X_0) \qquad (13)$$

Likewise, the correlation constant K in eq 11 can be approximated by

$$K = \tfrac{1}{2}EaX_{0_{sect}} \qquad (14)$$

Properties and Selection of the Gradient Medium. As mentioned above, it is necessary to know the effective particle density of the macrosolute to be sedimented in a given gradient medium. This can be done by conventional isopycnic flotation experiments. Two possibilities arise: (1) the flotation density coincides with the reciprocal of the partial specific volume, $1/\bar{V}$, as determined by pycnometry; (2) the medium hydrates or dehydrates the particle, changing its density to smaller or larger values than $1/\bar{V}$. In the first alternative, it is possible to start the gradient with a medium concentration equal to zero and then increase it exponentially as needed. In the second case, it is necessary to select a minimum concentration c_i at the initial point of the gradient so that beyond this value the density of the macromolecule is not further affected. This will ensure that all throughout the concentration range in the gradient the effective particle density will remain invariant.

Regardless of what happens to the particle density, it is also necessary to guarantee that the shape factor of the macromolecule remains constant throughout the selected gradient range, so that the frictional resistance increases, and the s value decreases, with increased medium viscosity. This will validate the applicability of eq 4.

A practical way to determine if all these conditions of invariancy are satisfied is to centrifuge the particle in several progressively increasing medium concentrations, without any gradient. The corrected s values should all yield the same $s_{20,w}$ when the observed s values are inserted in the standard correction formula, eq 5 (the subscript i now refers to the constant concentration in each case). For proteoglycans, it will be seen below that Cs_2SO_4, but not CsCl, fulfills all these conditions.

Having thus selected the electrolytic medium (Cs_2SO_4 in our case), it is necessary to have sufficient information on the density and viscosity of the medium. It is also necessary to have mathematical expressions that relate these properties to the concentration c of the medium.[2]

For Cs_2SO_4, the densities at 20 °C were taken from the International Critical Tables (1926–1930). For mathematical correlation with concentration, an equation of the form given by Ludlum & Warner (1965) at 25 °C was reformulated in terms of weight fraction (c = weight of medium/weight of solution) and adapted to fit the densities at 20 °C. The resulting expression is

$$\rho_{20} = (1 + 0.0442c)[1 + 0.78(c/1 - c) - 0.133(c/1 - c)^2] \qquad (15)$$

For a similar correlation with the viscosity, an empirical equation was found by using the data published recently by Muller & Pita (1983). The equation has the form

$$\ln \eta_{20} = \alpha(c/1 - c) + \beta c^2 + \gamma c^3 \qquad (16)$$

where the constants depend on the concentration range as follows: α = 0.2738, β = 0.4810, and γ = 0 for $c > 0.40$; α = 0.1905, β = 1.6758, and γ = 1.8614 for $0.40 < c < 0.50$.

Calculation of Gradient Curves. Once the medium concentrations, c, are related to η and ρ by means of equations like eq 15 and 16, it is possible to relate these concentrations to each point ratio X/X_i according to eq III of Table I. Unfortunately, the complexity of eq 15 and 16 does not allow the writing of explicit algebraic functions of c in terms of X/X_i. However, using computers or programmable electronic calculators, it is easy to select several concentrations (c_1, c_2, c_3, ...), to find the corresponding paired sets of viscosity and density values (η_1, ρ_1; η_2, ρ_2; and η_3, ρ_3), to insert these values in eq III, and then to find the corresponding $(X/X_0)_1$, $(X/X_0)_2$, $(X/X_0)_3$, ... values. (Here, we have changed the subscript i to 0, implying, for the moment, that the initial centrifugation point will coincide with the gradient meniscus, X_0, as discharged into the cell.) When these computations are done, for Cs_2SO_4 and for several particle densities within the range 1.25–1.85 g/cm^3, the curves of Figure 2 result. For more precision, the same data are given in Table II for selected points, J, I, and K. For monitoring purposes, the density values are also included.

It is important to notice that all these data have been calculated only at 20 °C and with the restriction that $c = 0$ at the gradient meniscus, where $X/X_0 = 1$. However, since the centrifugal equations can be referred to any initial position, X_i, within the gradient, it is possible in practice to start a boundary centrifugation at any place where $c_i \neq 0$, i.e., where $X_i/X_0 > 1$. A practical way of doing this is to remove a top section of the gradient comprised between $c = 0$ and $c = c_i$, after having discharged it in the cell. In this case, a distinction

[2] A lower case c is used here to distinguish the medium concentration from the macromolecular concentration C.

Table II: Cs_2SO_4 Concentrations and Densities at Points I, J, and K of Figure 2

sedimenting particle density (g/cm³)	point J, X/X_0 = 1.1324		point I, X/X_0 = 1.648		point K, X/X_0 = 1.972	
	% concn (w/w)	density (g/cm³)	% concn (w/w)	density (g/cm³)	% concn (w/w)	density (g/cm³)
1.25	6.825	1.057	10.54	1.092	12.96	1.116
1.30	7.963	1.067	12.23	1.109	15.05	1.138
1.35	9.004	1.077	13.85	1.125	17.06	1.156
1.40	10.005	1.087	15.38	1.141	18.96	1.180
1.45	10.935	1.096	16.85	1.157	20.75	1.200
1.50	11.845	1.105	18.24	1.172	22.43	1.219
1.55	12.71	1.114	19.57	1.187	23.99	1.238
1.60	13.54	1.122	20.83	1.200	25.35	1.255
1.65	14.33	1.130	22.01	1.214	26.68	1.271
1.70	15.08	1.138	23.10	1.227	27.99	1.287
1.75	15.81	1.146	24.13	1.240	29.20	1.303
1.80	16.51	1.153	25.05	1.252	30.39	1.320
1.85	17.17	1.160	25.94	1.263	31.51	1.336

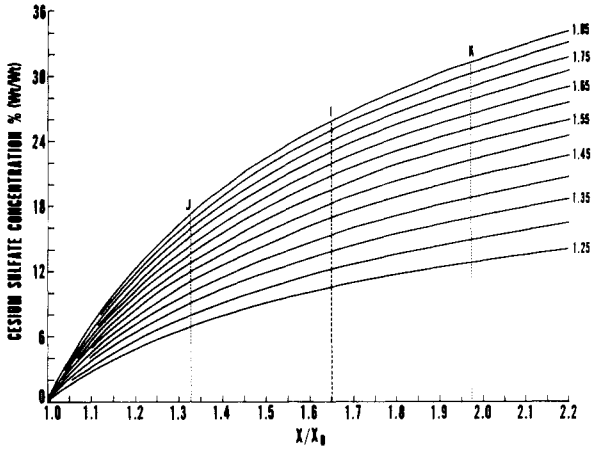

FIGURE 2: Theoretical isokinetic (and isovolumetric) Cs_2SO_4 gradients for various particle densities. The curves depict the Cs_2SO_4 concentrations plotted vs. "normalized" positions, X/X_0, for isokinetic gradients in cylinders. For isovolumetric gradients in sectors, the horizontal axis must be contracted by taking the square root of the abscissas. The vertical dashed lines correspond to three arbitrary points, I, J, and K, whose concentrations and normalized coordinates are given more precisely in Table II. These values can be used for monitoring the practical gradients obtained with the device of Figure 3.

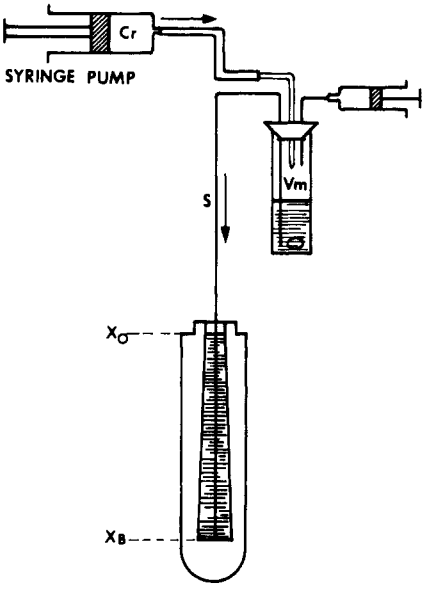

FIGURE 3: Diagram of the closed mixing chamber used for exponential gradient preparation. The essentials in the functioning of the mixing chamber are given in the text. The syringe pump is adjusted to deliver the total cell volume in 10–15 min. The small syringe to the right is used to load the mixing chamber by applying suction through siphon S and to regulate the initial pressure. In this way, the siphon, a narrow and rigid stainless-steel tubing, is also loaded with the chamber solution without dripping into the cell. For each volume V entering the chamber from the syringe pump, an equal volume is displaced into the cell. Stirring is done with a small magnet inside the chamber. The magnet is propelled by a larger magnet underneath attached to a long rotating rod (not shown). Stirring should be vigorous as to effectively homogenize the solution before transference occurs. Air bubbles should be avoided. On top of the gradient, an aliquot volume of distilled water is layered to avoid any restricted diffusion against the meniscus.

will exist between X_0, now a fictitious point in the open air, and X_i, the true physical meniscus from which the boundary will start to sediment. Exploitation of this technique will render the data of Figure 2 and Table II usable even for those cases where $c = 0$ has to be avoided.

Another feature of the curves of Figure 2 is that they have been calculated up to $X/X_0 = 2.2$, so the bottom coordinate, X_B, never exceeds $2.2X_0$ for isokinetic gradients (or $\sqrt{2.2}\,X_0$ for the isovolumetric ones). In practice, this is not a very severe limitation. Though X_0 can vary considerably from one commercial rotor to another, the ratio X_B/X_0 rarely falls outside the range 1.7–2.2. According to the data surveyed by Molloy & Rickwood (1978), the X_B/X_0 ratio for about 30 of the most commonly used swinging-bucket rotors averaged around 2.14. Hence, the curves of Figure 2 should also prove to be useful for practically all available rotor sizes and cell volumes.

Practical Preparation of Gradients. The case of isokinetic gradients will be described first. The one mixing chamber device to prepare this type of gradient has been described by Noll (1967) and is illustrated in the sketch of Figure 3. From the reservoir (a syringe pump) containing solution with a medium concentration c_r, a total volume V_T is dropwise discharged into the mixing chamber. The chamber contains a volume V_m at an initial medium concentration c_0. The volume V_m remains constant, but its concentration in Cs_2SO_4 increases asymptotically, approaching c_r when V_T has been completely transferred into the centrifuge cell through siphon S. The instantaneous medium concentration c in the chamber, when a given volume V has been transferred, is, according to Noll

$$c = c_r - (c_r - c_0)e^{-V/V_m} \quad (17)$$

When the volume V is discharged along the X coordinates of a cylinder and when c_0 is restricted to a zero value, the above equation produces a concentration profile very much like those of Figure 2. For a given total cylindrical volume

$$V_T = \pi R^2(X_B - X_0) \quad (18)$$

the parameters V_m and c_r can be chosen so as to follow any particular curve in Figure 2 depending upon the desired particle density. If, on the other hand, a nonzero c_0 concentration were required, eq 17 could produce a parallel curve,

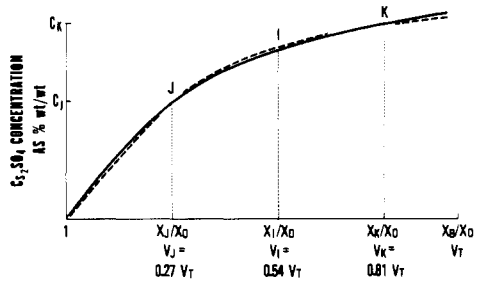

FIGURE 4: Adjustment of a practical to a theoretical isokinetic gradient curve. The practical curve (---) is adjusted to coincide with the theoretical one (—) at points J and K by selecting the needed parameters, c_r and V_m, in eq 9 (see text). J and K correspond to X/X_0 coordinates that determine 27% and 81%, respectively, of the total cylindrical volume V_T (as measured from X_0). These coordinates are, therefore, $X_J/X_0 = 0.27(2.2 - 1) + 1 = 1.324$ for point J and $X_K/X_0 = 0.81(2.2 - 1) + 1 = 1.972$ for point K. Point I is not an adjustment point. It is given in Table II for monitoring purposes, and its coordinates are the mean of the previous ones, i.e., 1.648.

shifted c_0 units upward, by the mere expedient of using $c_0 \neq 0$ in the mixing chamber to begin with. The required theoretical gradients of Figure 2, however, would not, in general, move up by an additive amount all along each curve, and the mismatch between the practical gradient and the theoretical one would require a redetermination of c_r's for each particle density and for each c_0. For simplicity, we have preferred to restrict eq 17 to $c_0 = 0$ and resort to the technique mentioned above of withdrawing part of the gradient top portions if c_i must be finite. Suppose, for example, that a particle of density 1.25 g/cm^3 has to be centrifuged isokinetically with concentrations lying above $c_0 = 6\%$ in Cs_2SO_4. Inspection of the lowest curve in Figure 2 indicates that the actual gradient should start at $X/X_i = 1.3$. If the cell was originally filled with gradient solution to its top edge at, e.g., $X_0 = 60$ mm, this means that a volume covering $(1.3 \times 60) - 60 = 78-60 = 18$ mm down the cell must be eliminated. This, of course, would reduce the length available for centrifugation. If the cell is a small one, having, for example, $X_B = 102$ mm and a ratio $X_B/X_0 = 1.7$, the shortening would reduce the gradient length from $X/X_i = 1.3$ to $X/X_0 = 1.7$. A better procedure in this case is to eliminate a certain volume from the top such that upon continued refilling of the cell with more gradient solution, the relationship of $X_i = 60$ mm to the hypothetical X'_0 coordinate attained by the withdrawn column's meniscus "in the air" (as if it were still part of the cylindrical cell) would be in a ratio of 1.3 to 1. The "fictitious" meniscus, X'_0, should have been, then, at $60/1.3 = 46$ mm from the rotor center. So in reality, the withdrawn gradient column should be only of $X_i - X'_0 = 60 - 46 = 14$ mm in length. In this way, when this 14-mm length is replaced with extra volume pumped into the bottom of the cell, a concentration of 6% will appear at the top edge having a coordinate ratio of 1.3 with respect to the fictitious meniscus at 46 mm. Likewise, the bottom concentration will correspond now not to the old coordinate ratio of 1.7 but to the new one of $X_B/X'_0 = 102/46 = 2.22$, which is practically the highest point attainable in Figure 2 for $\rho_P = 1.25$ g/cm^3.

Calculation of the Practical Parameters c_r and V_m. Figure 4 illustrates a typical plot of any of the curves of Figure 2 with a superimposed dashed trace plot of eq 17 in which $c_0 = 0$, namely

$$c = c_r(1 - e^{-V/V_m}) \tag{19}$$

Since eq 19 has two degrees of freedom, c_r and V_m, an exact agreement between this equation and the solid line of Figure 4 can be obtained at any two points, for example, at points J and K in the figure (point I is not a coincidence point). These points are arbitrarily selected to be at a ratio of 3 to 1 in terms of their corresponding volumes, $0.81V_T$ and $0.27V_T$, along the cell, as compared to the total volume V_T (see Discussion). Thus, for J and K, eq 19 becomes

$$c_J = c_r(1 - e^{-0.27V_T/V_m}) \tag{20a}$$

and

$$c_K = c_r(1 - e^{-0.81V_T/V_m}) \tag{20b}$$

Equation 20a can now be raised to the third power and subtracted from eq 20b to yield

$$c_r = [3c_J + \sqrt{9c_J^4 + 4(c_K - 3c_J)c_J^3}]/2(3c_J - c_K) \tag{21}$$

Substituting this value into eq 20a:

$$V_m = -0.27V_T/\ln(1 - c_J/c_r) \tag{22}$$

Using now the numerical values of c_J and c_K indicated in Table II, we can calculate the corrsponding sets of c_r's and V_m's for each particle density via eq 21 and 22. The c_r's thus obtained are gathered in Table III. The V_m values, however, are still dependent upon each V_T and, hence, upon the particular cell used (see eq 22). For generalization purposes, it is better to express V_T as in eq 18 and to impose the practical condition mentioned before, $X_B = 2.2X_0$. Hence, eq 22 becomes

$$V_m = KM \tag{23}$$

where

$$M \equiv 0.324/\ln(1 - c_J/c_r) \tag{24}$$

and K has been defined in eq 11; $K = \pi R^2 X_0$ for the cylindrical cell. The various M values are computed by using the corresponding c_r and c_J values and can be tabulated as shown also in Table III. The investigator, then, can find the required V_m for each particular case by simply using eq 23 for a cylindrical cell of radius R and gradient meniscus position X_0 (or X'_0).

The practical parameters c_r and V_m can be found also by using the empirical equations given in the footnote of Table III.

Isovolumetric Gradients in Sectorial Cells. All the previous considerations apply also to the preparation of an isovolumetric gradient in a sectorial cell. The c_r and V_m values are selected from Table III according to the sedimenting particle density, just like for isokinetic gradients. The only difference lies in the calculation of the mixing chamber volume, V_m, from eq 23, where the correlation constant, K, obviously should be

Table III: General Parameters for Preparation of Cesium Sulfate Isokinetic and Isovolumetric Gradients at 20 °C[a]

density of sedimenting particle, ρ_P (g/cm^3)	% c_r (w/w)	M	density of sedimenting particle, ρ_P (g/cm^3)	% c_r (w/w)	M
1.25	15.938	0.57955	1.60	30.738	0.55821
1.30	18.429	0.57262	1.65	32.161	0.54932
1.35	20.924	0.57586	1.70	33.589	0.54368
1.40	23.271	0.57647	1.75	34.934	0.53774
1.45	25.504	0.57860	1.80	36.245	0.53318
1.50	27.508	0.57528	1.85	37.461	0.52845
1.55	29.322	0.57020			

[a] c_r, the Cs_2SO_4 percent concentration in the syringe reservoir (Figure 3), and M, defined in eq 24 (see text), can be calculated also with the following empirical equations (where ρ_P is in units of grams per cubic centimeter): $c_r = 47.948\rho_P - 43.917$ for $1.25 < \rho_P < 1.45$, $c_r = (1972.4\rho_P - 2205)^{1/2}$ for $1.45 < \rho_P < 1.60$, $c_r = 26.978\rho_P - 12.350$ for $1.60 < \rho_P < 1.85$, $M = 0.7212 - 0.1044\rho_P$ for $1.63 < \rho_P < 1.85$, and $M = 0.6628 - 0.0625\rho_P - 0.0089 \cos[1028.6(\rho_P - 1.30)]$ for $1.25 < \rho_P < 1.62$.

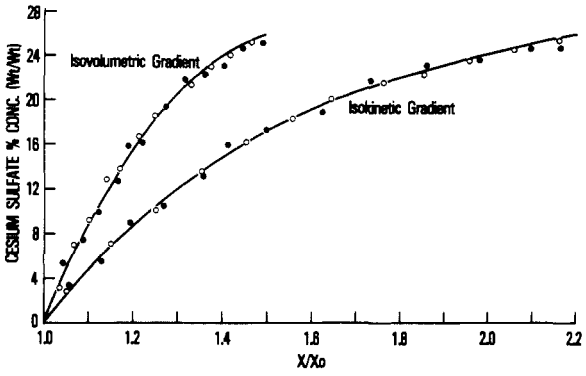

FIGURE 5: Comparison of theoretical and practically observed isokinetic and isovolumetric gradients. The solid lines correspond to the theoretical gradient of Figure 2 for $\rho_p = 1.55$ g/cm^3. The open circles indicate the actual gradient analyzed immediately after being prepared with the device of Figure 3. The closed circles correspond to the gradient observed after centrifugation for 2 h at 21 000 rpm. The two sets of circles do not differ appreciably, proving the stability of the gradients.

calculated by using the term $^1/_2E\theta X_0^2$ of eq 11 or its very good approximation, eq 14.

The gradient curves resulting when the contents of the isokinetic gradient mixer are discharged into a sectorial cell are not those of Figure 2, but are "contracted" in the horizontal axis by taking the square root of their corresponding X/X_0 points. Hence, the maximal X_B/X_0 would be $\sqrt{2.2} = 1.483$ instead of 2.2. In spite of this, the resolving power of the sectorial cell is not necessarily diminished since the actual difference between X_B and X_0, the total cell path available, is independent of the ratio X_B/X_0. If a cell, however, cannot accommodate the entire gradient length from 1 to 1.483, the theoretical curves will be still valid for that cell, but the gradients will not extend beyond a certain coordinate ratio smaller than 1.483.

Gradient Verification and Stability. Gradients that result from the practical parameters calculated before are very good approximations to the theoretically required gradients for each particle density. In practice, a verification must be done by monitoring, for example, the obtained density values at selected points along the gradient. (These could be the J, I, and K points of Table II.) For a particle density of $\rho_P = 1.55$ g/cm^3, we have done such a verification as shown in Figure 5 both immediately after producing the gradient (open circles) and after a 2-h centrifugation at 21 000 rpm (closed circles). The agreement with the theoretical gradients (solid lines in the figure) corroborates the previous considerations and approximations and verifies, indirectly, the empirical eq 15 and 16 for Cs_2SO_4.

Criteria for Judging the Attainment of Isovolumetric Conditions. To verify that a given macromolecular solute has sedimented isovolumetrically, two centrifugal profiles are obtained at two different centrifugation conditions, $(\omega^2t)_I$ and $(\omega^2t)_{II}$. If the boundaries have behaved isovolumetrically, then (1) their plateaus should remain essentially equal to C_i (or unity in the C_f/C_i plot), and (2) the abscissas of the two curves should be proportional to ω^2t when plotted vs. aliquot number N. This is true for a polydisperse system with negligible diffusion, as exemplified in Figure 6B. (3) In the case of a monodisperse and diffusible system, the two curves should appear approximately parallel if the centrifugation times are the same and the angular velocities different (Figure 6A). Thus, in the case of Figure 6B, for each point of curve II, e.g., N_{II}, there is an isoconcentration point, N_I, in curve I such that the ratio N_{II}/N_I is equal to the centrifugal ratio $C = (\omega^2t)_{II}/(\omega^2t)_I$. However, for Figure 6A, only the boundary centers (indicated by the vertical arrows) will be proportional to the centrifugal ratio C. The centers are calculated as the second moments of the boundaries in terms of X or, equivalently, as the first moments in terms of N, i.e., as $N = \int N\, dC_f/\int dC_f$.

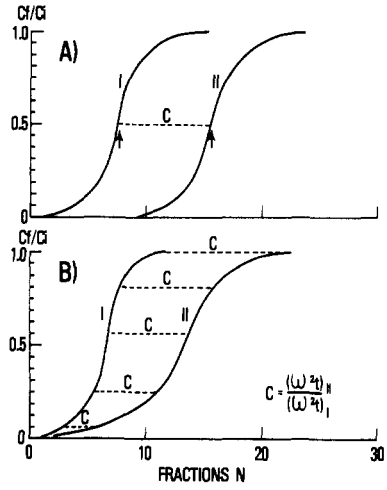

FIGURE 6: Ideal isovolumetric behavior of a boundary when examined at two different ω^2t's. (A) Case of a monodisperse and diffusible system. The two curves are nearly parallel. (B) Case of a polydisperse slowly diffusing system. The curves are point by point proportional to the centrifugal ratio C. In both cases, there is no plateau dilution.

Experimental Procedures

Materials, Centrifugation, and Analytical Assays. Proteoglycan monomers A1D1[3] from bovine nasal cartilage were obtained and purified by the classical technique reported by Sajdera & Hascall (1969) and Hascall & Sajdera (1969). The preparation was further purified by rate zonal centrifugation as used in Pita et al. (1981). Only the molecules with s values between 5 and 65 S were used in this paper to avoid the possible diffusion effects of the very small species and the heterogeneity contributed by the larger aggregates usually found as a 5–10% impurity in A1D1 preparations.

The DNA (a bacteriophage PM2 DNA, lyophilized) and bovine serum albumin (purified to contain only monomers) were from Boehringer Mannheim Biochemicals. The Cs_2SO_4 were density grade from Gallard-Schlesinger Chemical Manufacturing Corp. The CsCl, from Fisher Scientific Co., was density gradient certified. The protease inhibitors were from Aldrich Chemical Co., and the antibiotics, streptomycin sulfate and penicillin G, were from Calbiochem-Behring. All other chemicals were analytical reagent grade from Mallinckrodt Inc.

Centrifugations were conducted at 20 °C in a Sorvall OTD-2 ultracentrifuge provided with an ω^2t integrator and an ARC-1 acceleration rate control. For isopycnic equilibrium, the speed was 40 000 rpm during 72 h. For average s values, determination by the transport method from 30 to 60 min at 30 000 rpm was used as described by Pita et al. (1978). For boundary centrifugations, the speeds and times are given under Results. The rotors used were the SW 41 Ti, SW 50.1, and SW 65 Ti. At the end of each boundary centrifugation, 25–30 aliquots of volume ΔV microliters were aspirated from the top by using the technique described by Pita et al. (1978, 1983). For each aliquot, the corresponding homogenized final concentration, C_f, was determined and divided by the initial

[3] The notation A1D1 follows the nomenclature introduced by Heinegard (1977).

concentration C_i. The obtained C_f/C_i ratios were plotted either vs. the corresponding aliquot radial position, X_N, or vs. the aliquot number N. (The latter is more convenient for isovolumetric centrifugations.) From this plot, all the centrifugal properties can be calculated.

For boundary sedimentation, the proteoglycan A1D1 preparation was dissolved in the buffer solution described by Pita et al. (1983). The hexuronate concentration was determined with the modified carbazole method (Bitter & Muir, 1962). The DNA was dissolved in a buffer of pH 7.20 containing NaCl, Na_2HPO_4, and KH_2PO_4 (totaling an ionic strength of 0.16 M) and was protected with 100 μg/mL streptomycin sulfate and 100 units/mL penicillin G. Bovine serum albumin was dissolved in the same buffer used for DNA. Both DNA and serum albumin were assayed by their absorbances at 260 and 280 nm, respectively. A continuous MR1 D (Zeiss) flow cell was used in a Zeiss PMQ III spectrophotometer.

Centrifugation Cells. The sectorial cell used was previously described by Pita et al. (1983) and is schematically illustrated in Figure 1. For comparison purposes, a cylindrical cell is also included in figure. By use of the dimensions given in Figure 1 for the sectorial cell, the correlation constant K is $^1/_2EaK_0$ = 1204.5 mm^3 and the total volume V_T = 1.446 mL.

Summarized Experimental Procedure. To use the data of Table III in order to centrifuge isovolumetrically in sectors or isokinetically in cylinders a given macromolecule in Cs_2SO_4, the following steps are required:

(1) Determine the particle's density, ρ_P, by pycnometry and also its flotation density, ρ_f, by equilibrium centrifugation. If both coincide, proceed directly to step 4. If not, perform step 2.

(2) Determine its $s_{20,w}$ value in several densities of the medium. (Use ρ_f for the particle density in the denominator of eq 5.)

(3) Select the concentration range within which all previous $s_{20,w}$ values coincided, to guarantee invariancy of the effective density and shape of the particle.

(4) Select a given cell and determine its geometrical parameters for a given rotor (i.e., a, b, X_0, X_B, and E for the sectorial cell and X_0, X_B, and R for the cylinder). Calculate the needed correlation constant K from eq 11. Find the ratio X_B/X_0 and the total volume V_T (eq 9).

(5) From Table III, find the parameters c_r and M for the effective particle density found before.

(6) Find the mixing chamber volume V_m from eq 23.

(7) If c_i = 0, proceed to prepare the gradient, being sure that the initial macromolecular concentration C_i is identical both in the syringe reservoir and in the mixing chamber solutions.

(8) If c_i must be higher than zero, determine, from the curves of Figure 2, the initial ratio $Q = X/X_i$ corresponding to the selected c_i. (For sectors, use the square root of this ratio.) Equate X_i to the cell's X_0 (its top edge) and find the fictitious meniscus $X'_0 = X_i/Q = X_0/Q$. Determine what volume must be withdrawn from the top section of the gradient after being formed. For the cylinder, this is $\pi R^2(X_i - X'_0)$, and for the sector, $^1/_2E(X_i^2 - X'^2_0)\theta$, where $\theta = a/X_0 = b/X_B$. After this volume is withdrawn, it is replaced with more gradient solution from the mixing device.

(9) Select $\omega^2 t$ and perform the centrifugal run. (If a sector is used, be sure to place its parallel sides so that they become horizontal when the bucket is lifted by centrifugal forces.)

(10) After centrifugation, withdraw the cell from the holder, avoiding shaking and touching it with warm fingers. Keep it always in a vertical position to extract its content either by a continuous flow process or by discrete aliquot extractions as described above.

(11) Perform the required chemical analyses and construct the C_f/C_i plot.

(12) Calculate the relevant centrifugal parameters by using the pertinent equations of Table I.

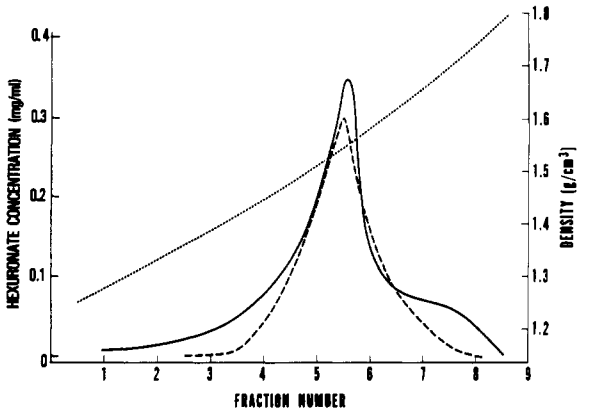

FIGURE 7: Equilibrium isopycnic centrifugation of proteoglycans A1D1 in Cs_2SO_4. The dotted line gives the density profile after 72-h centrifugation at 40 000 rpm. In both cases (dashed and solid lines), the hexuronate concentration peak was found very close to 1.55 g/cm^3.

RESULTS

Particle Density Determination and Selection of Gradient Medium. The pycnometric density and the flotation density of the proteoglycans in Cs_2SO_4 were very similar. Isopycnic flotation equilibria for a 0.23 mg/mL solution of A1D1 proteoglycan run for 72 h as explained under Experimental Procedures gave the hexuronic and density profiles shown in Figure 7. The peaks are located at a density value of 1.55 g/cm^3. At the same time pycnometric determinations of the same proteoglycan at a concentration of 17 mg/mL in a 0.15 M KCl solution yielded a value of 1.555 g/cm^3.

Additionally, the average s value of proteoglycans in progressively increasing concentrations of Cs_2SO_4 followed the standard correction of eq 5. Following the procedures described under Experimental Procedures, the $s_{20,w}$ value was found first, yielding 28.7 S. Substituting this value with ρ_P = 1.555 g/cm^3 and with $\rho_{20,w}$ = 1 and $\eta_{20,w}$ = 1 in eq 5 gives

$$s\eta_i = 80.41 - 51.71\rho_i$$

A plot of the latter equation is given in Figure 8 (solid line). Using now several Cs_2SO_4 concentrations, we found a series of experimentally observed s values, shown as circles in the same figure. The agreement between the continuous line and the experimental points proves that the proteoglycans behaved, indeed, very much according to eq 5 in Cs_2SO_4.

In contrast to this, the flotation of the same proteoglycans in CsCl yielded a flotation density much higher than 1.55 g/cm^3, perhaps 1.9 g/cm^3 or even more since the peak almost coincided with the saturation point of CsCl.

Isovolumetric Centrifugation of Bovine Nasal Proteoglycans. The proteoglycan A1D1 preparation was purified and further selected by zonal centrifugation as described under Experimental Procedures. From Table III, the values of c_r and M corresponding to a particle density of 1.55 g/cm^3 are seen to be 19.3% and 0.57, respectively. Thus, the mixing chamber volume required is, from eq 23, $V_m = KM$ = 1204.5 × 0.57 = 687 μL, or about 0.69 mL. This is roughly half the total volume to be discharged, V_T = 1.446 mL. Sufficient solution, therefore, was placed in the syringe pump at a concentration level of 29.3% in Cs_2SO_4, and exactly 0.687 mL

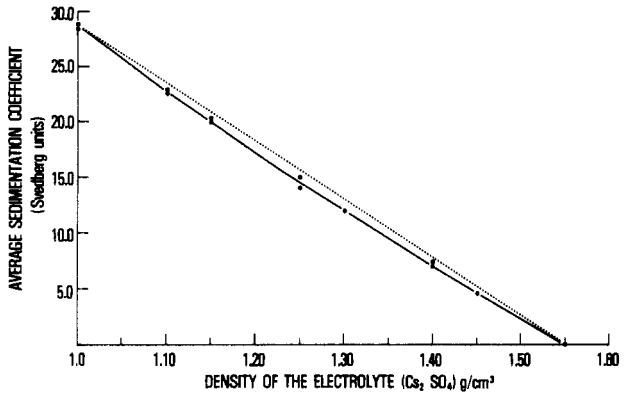

FIGURE 8: Decrease of the average $\bar{s}$ value of proteoglycans A1D1 with increasing densities of Cs_2SO_4. The circles indicate the average $\bar{s}$ values observed in cells loaded with the uniform densities shown on the horizontal axis. The dotted line corresponds to the linear decrease of the s value when only the buoyant density term of eq 4 is taken into consideration (see text). The solid line takes both the density and viscosity terms into consideration. Coincidence of the circles with the solid line proves that eq 4 is valid for proteoglycans in Cs_2SO_4.

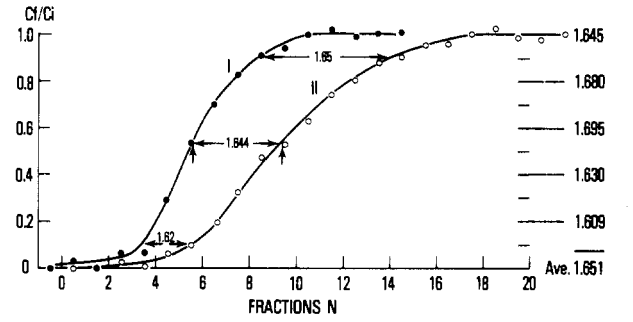

FIGURE 9: Centrifugation profiles of proteoglycans A1D1 in isovolumetric Cs_2SO_4 gradients. Curve I was obtained after centrifugation at 37 747 rpm for 37 min and curve II at 34 503 rpm for 73 min. The ω^2t ratio is $C = (5.72 \times 10^{10})/(3.47 \times 10^{10}) = 1.648$. The observed position ratios between corresponding fractions are shown at three levels of the curves and also at the right-hand side of the figure. The vertical arrows indicate the location of the boundaries' first moments in terms of N, their ratio being very close to the expected 1.648. The space to the left of the zero fraction corresponds to the water usually layered on top of the gradient to avoid restricted diffusion against the meniscus (if any).

was placed in the mixing chamber with no Cs_2SO_4 at all. Care was taken to have the same proteoglycan concentration, C_i = 0.18 mg/mL, in both containers. The total volume was dropwise discharged into the mixing chamber and simultaneously transferred into the sectorial cell in about 15 min. After centrifugation, 25 aliquots, 50 μL each, were withdrawn from the top and analyzed for uronic acid concentration. Upon division of each concentration by the initial value, the plots of Figure 9 were obtained. Two centrifugations were run at two different ω^2t values as indicated in the legend of Figure 9. In both cases, the plateau region reached a unity concentration ratio fairly well. The actually observed ratios between the points N of curve II with respect to those of curve I are shown in the right-hand side of the figure. They all lie very close to 1.65 as expected from the ω^2t ratio used (see legend). The first moments of the boundaries in terms of N were N = 9.40 for curve II and N = 5.72 for curve I. Their ratio was 1.643, again very close to the centrifugal ratio used, C = 1.648.

Figure 10 shows the polydispersity distribution function, $g(s_0)$, calculated independently from each profile of the previous figure. The coincidence of the two sets of points also demonstrates that good isovolumetric behavior of both complete boundaries has been attained. Finally, the weight-average

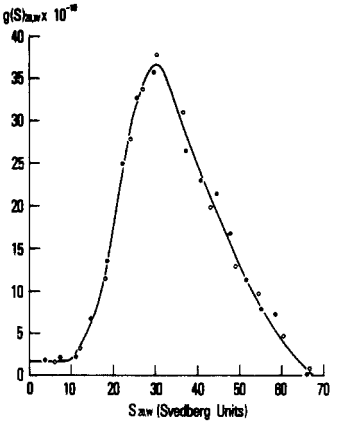

FIGURE 10: Polydispersity distribution functions corresponding to the centrifugal profiles of Figure 9. The closed and open circles correspond to curves I and II, respectively, of Figure 9.

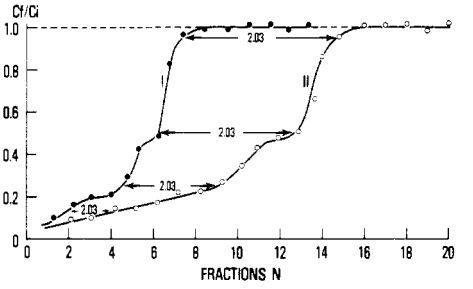

FIGURE 11: Isovolumetric centrifugation of PM2 DNA. Curve I was obtained at $\omega^2t = 3.49 \times 10^{10}$ and curve II at $\omega^2t = 7.09 \times 10^{10}$, the centrifugal ratio being C = 2.03. This ratio was achieved at several levels of the two curves.

sedimentation coefficients defined as $s_{wt} = \sum sg(s)/\sum g(s)$ were calculated, giving 34.51 and 34.55 S for curves I and II, respectively. These were almost identical with the transport average coefficients calculated through eq VB′. For curve I, C_f was 0.537 up to N = 12, yielding s_{trans} = 34.09 S, and for curve II, C_f was 0.478 up to N = 18, yielding s_{trans} = 34.51 S.

Isovolumetric Centrifugation of DNA and Serum Albumin. The DNA from PM-2 virus was first centrifuged isopycnically in Cs_2SO_4 to determine its flotation density in this salt. The value found was ρ = 1.415 g/cm³. This agrees well with previously published values (Cummings & Mondale, 1966). Hence, from Table III, the required reservoir concentration for DNA is 23.9% in Cs_2SO_4 and M = 0.577. When the same sectorial cell is used as before, the volume in the mixing chamber should be, from eq 23, $V_m = KM = 1204.5 \times 0.577$ = 695 μL or about 0.7 mL. The DNA was kept at an absorbance level of 0.600 in both solutions. Again, two centrifugations were run at different ω^2t's, namely, 3.49×10^{10} and 7.09×10^{10}, the centrifugal ratio being C = 2.03. Figure 11 shows the two profiles obtained. Both curves reached unity ratio at the plateau concentration level, both evidenced the attainment of C = 2.03 at various concentration levels, and both showed the existence of three (paucidisperse) components of coefficients equal to 12, 32, and 43 s, respectively. These coefficients were obtained from the peaks of the calculated $g(s)$ functions (not shown).

Finally, a sample of purified bovine serum albumin was centrifuged at a concentration of 1 mg/mL. To prepare the isovolumetric gradient for this protein, a density of 1.37 g/cm³ (reciprocal of V = 0.73 cm³/g) was assumed. This leads to a reservoir concentration of 21.77% from Table III and to M = 0.576. Hence, a volume of $V_m = KM = 1204.5 \times 0.576$

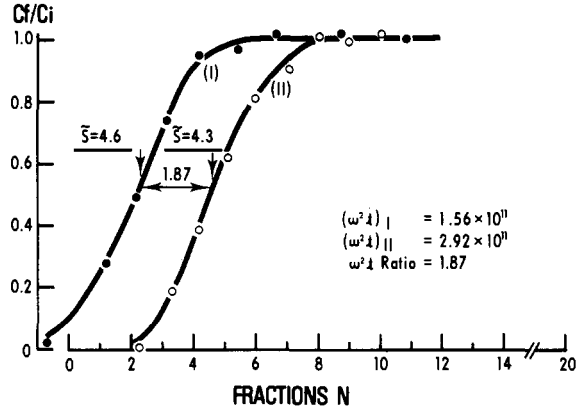

FIGURE 12: Isovolumetric profiles of bovine serum albumin. The two curves, affected only by diffusion, are nearly parallel. At the first moments of the boundaries, in terms of N (vertical arrows), the sedimentation coefficients gave respectively 4.6 and 4.3 S.

= 694 μL was used in the mixing chamber. Figure 12 shows the two $\omega^2 t$ values used. In both cases, the centrifugation time was 5.5 h. The two profiles were nearly parallel, as expected for a purely diffusible monodisperse system. The first moments in terms of N (arrows in the figure) gave a ratio of 1.87 as expected. They did not coincide, however, with the 50% concentration level of the boundaries. The figure also shows the s values obtained with eq IVB at the first moment centers.

Discussion

The sedimentation profiles obtained in this paper for the various macromolecules studied conformed to the criteria established for isovolumetric centrifugations: a plateau equal to the initial concentration was obtained in all cases; quasi-parallelism was obtained for two equal-time centrifugations of a diffusible monodisperse system like serum albumin; proportionality to $\omega^2 t$ was valid for PM2 DNA and the A1D1 proteoglycans. In the latter case, this allowed calculation of the $g(s)$ function with great reproducibility.

The three components found for the DNA sample are in reasonable agreement with the three components reported by Espejo & Canelo (1966) for the same type of DNA. The proteoglycans also showed an almost identical coefficient of 34 S when calculated from the boundary profile (weight average) as from transport considerations (eq VB), for both curve I and curve II. This agreement is by no means trivial, and it might not occur in conventional velocity sedimentation subjected to progressive plateau dilution. This clearly demonstrates the advantage of isovolumetric sedimentation. The Johnston–Ogston effect, however, might still be present, distorting both $g(s)$ distribution profiles in similar fashion, i.e., proportionally to their corresponding $\omega^2 t$. The observed isovolumetric behavior also proved that the effective density used of 1.55 g/cm^3 was valid for the entire polydisperse population of proteoglycan molecules. Small departure from their density value, however, might not impair the isovolumetric regime too much, as discussed previously by Noll (1967).

For bovine serum albumin, the density value used (1.37 g/cm^3) is somewhat higher than the 1.22 g/cm^3 reported by Mashburn et al. (1974) for the same protein in Cs_2SO_4. Yet, Figure 12 showed that the isovolumetric centrifugation condition was satisfied at the boundary centers. In a similar fashion, a density variation of 0.1 g/cm^3 might be tolerated in the case of proteoglycans. Thus, it might be possible to centrifuge in a single isovolumetric run an A1 proteoglycan preparation that contains not only monomers of density 1.55 g/cm^3 but also aggregates of density 1.45 g/cm^3. On the other hand, wide deviations in density, shape, or both, caused perhaps by degradation or by some abnormality in the synthesis of these macromolecules, might lead to observable distortions in the comparative isovolumetric patterns at different $\omega^2 t$'s. If that is the case, the distortions themselves could be used to explore the intrinsic abnormalities affecting the molecules.

The experimental results have also evidenced the advantage of using Cs_2SO_4 as the electrolytic medium for isovolumetric centrifugations. Not only does this salt not interfere with the uronic acid analysis as done by sucrose or by the chloride ion of CsCl (Kimata et al., 1982) but also its density and viscosity allowed the preparation of exponential gradients as designed in this paper through the simplified version of Noll's equation. The value selected for K (81% of V_T in Figure 4) followed the findings of McCarty (1968), showing a best fit for K around 76–83% of V_T. Resorting then to a ratio of 1 to 3 between J and K (i.e., $V_J = 0.27 V_T$), it was possible to find an analytical solution to eq 20a,b, leading to the computation of the c_r and M parameters of Table III. This procedure represents an improvement over the more complex computations required by the transcendental equations when sucrose gradients are used (Noll, 1967; McCarty et al., 1968).

The simplification of centrifugal calculations is an obvious advantage of the isokinetic and isovolumetric techniques. This is clearly reflected in the simplicity of eq IV of Table I. The mathematical complexity leading to long computer programs required by other gradient sedimentations (Steensgaard et al., 1978) is avoided. This simplification derives in part from the relationship established between s_x and s_i in eq II. As noted under Theory and Calculations, eq IVA, IVB, and IVB′ give the value of s_i, even when the boundary might have reached a position X where the density and viscosity of the medium are much greater than those at the meniscus.

Though in the present work great flexibility is possible for choosing among different cell volumes and rotor sizes, there is, however, a natural restriction of using the isokinetic gradients always in connection with cylindrical cells and the isovolumetric gradients with the sectorial ones. In this respect, a comparison between the two types of gradient–cell combinations is interesting. For a given rotor size and swinging-bucket holder, a cylindrical cell will, normally, accommodate more volume than a sectorial one. For example, the SW 41 rotor used in these studies, with an effective bottom coordinate of X_B = 142 mm for either cell type, will determine an X_0 of $142/2.2^{1/2}$ = 95.7 mm for the sector whereas for the cylinder X_0 = 142/2.2 = 64.5 mm. For comparable widths for each cell, e.g., $E = b$ = 6.11 mm for the sector and R = 5 mm for the cylinder, the volume capacity would be about 1.5 and 5 mL, respectively. Since the mixing chamber volume is usually about half the total volume used, this would imply V_m's of 0.7 and 2.5 mL, respectively. The former is somehow too small, and it might entail some difficulties for the mechanical construction and operation of the mixing chamber and its collateral connections. In this respect, the bigger cylindrical volumes render this type of cell more manageable. On the other hand, centrifugations in cylindrical cells will be affected by the imprecisions related to the recovery, or not, of the material colliding with the cell walls described above and which can yield (1) no apparent plateau dilution at all, (2) a linear dilution law, (eq 3b), or (3) an intermediate behavior. Unpublished data from this laboratory, however, give a strong indication that the absence of plateau dilution is the most frequent behavior when $\omega^2 t$ did not exceed 10^{11} s. Fourteen isokinetic centrifugations of an A1D1 proteoglycan preparation under these conditions yielded an average plateau dilution of

1.0005 ± 0.02. These results implied that the proteoglycan molecules that have collided with the cell walls had been recovered with the extraction of each aliquot from the top of the column as required by the transport method. The presence of concentrated salt might have helped such a recovery. This situation, however, might not be reproducible for other solutes, for other media, or for cells made of different materials. Still, these imprecisions might not preclude the use of cylindrical cells for preliminary work or when no sectorial cells are available.

A last comment will be made concerning the possibility of applying the isovolumetric methodology described in this paper to rate zonal centrifugations. For this purpose, it seems necessary to withdraw part of the formed gradient as described above in order to produce a meniscus concentration bigger than zero. Upon this concentration, then, the zone could be layered without incurring density instabilities. If, on the other hand, $c_0 = 0$ is desired, the only alternative seems to be the use of the inverted or "triangular" zone first suggested by Britten & Roberts (1960). In both instances, a theory of polydisperse boundary analysis will be needed if the profile resulting from the spreading of the zone is to be translated into a $g(s)$ distribution function. Efforts are being made presently in our laboratory in this direction to obtain an analytical rate zonal methodology in isovolumetric density gradients. This seems to be especially possible for the slowly diffusing proteoglycan macromolecules studied in the present paper.

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REFERENCES

Baldwin, R. L. (1954) *J. Am. Chem. Soc. 76*, 402.

Birnie, G. D. (1978) in *Centrifugal Separations in Molecular and Cell Biology* (Birnie, G. D., & Rickwood, D., Eds.) Chapter 6, Butterworths, London.

Bitter, T., & Muir, H. M. (1962) *Anal. Biochem. 4*, 330.

Britten, R. J., & Roberts, R. B. (1960) *Science (Washington, D.C.) 131*, 32–33.

Cummings, D. J., & Mondale, L. (1966) *Biochim. Biophys. Acta 120*, 448.

Edsall, J. T. (1953) *Proteins (1st Ed.) 1*, 549.

Espejo, R. T., & Canelo, E. S. (1969) *Virology 37*, 495.

Handbook of Biochemistry (1970) (Sober, H. A., Ed.) 2nd ed., Chemical Rubber Publishing Co., Cleveland, OH.

Hascall, V. C., & Sajdera, S. W. (1969) *J. Biol. Chem. 244*, 2384.

Heinegard, D. (1977) *J. Biol. Chem. 252*, 1980.

International Critical Tables (1926–1930) (Washburn, E. W., Ed.) McGraw-Hill, New York.

Kimata, K., Kimura, J. H., Thonar, E. J.-M., Barrach, H. J., Rennard, S. I., & Hascall, V. C. (1982) *J. Biol. Chem. 257*, 3819.

Lamm, O. (1929) *Ark. Mat., Astron. Fys. 21B (2)*, 1.

Ludlum, D. B., & Warner, R. C. (1965) *J. Biol. Chem. 240*, 2961.

Mashburn, A. T., Jr., Hoffman, P., & Hsu, D.-S. (1974) *Biochim. Biophys. Acta 362*, 366.

McCarty, K. S., Stafford, D., & Brown, O. (1968) *Anal. Biochem. 24*, 314.

Molloy, J., & Rickwood, D. (1978) in *Centrifugal Separations in Molecular and Cell Biology* (Birnie, G. D., & Rickwood, D., Eds.) Chapter 9, Butterworths, London.

Müller, F. J., & Pita, J. C. (1983) *Anal. Biochem. 135*, 106.

Noll, H. (1967) *Nature (London) 215*, 360.

Noll, H. (1969) in *Techniques in Protein Biosynthesis* (Campbell, P. N., & Argent, J. R., Eds.) p 101, Academic Press, New York and London.

Pita, J. C., & Müller, F. J. (1972) *Anal. Biochem. 47*, 395.

Pita, J. C., & Müller, F. J. (1973a) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 32*, 1481.

Pita, J. C., & Müller, F. J. (1973b) *Biochemistry 12*, 2656.

Pita, J. C., Müller, F. J., Oegema, T., & Hascall, V. C. (1978) *Arch. Biochem. Biophys. 186*, 66.

Pita, J. C., Müller, F. J., Morales, S. M., & Alarcon, E. J. (1979) *J. Biol. Chem. 254*, 10313.

Pita, J. C., Müller, F. J., & Howell, D. S. (1981) *Semin. Arthritis Rheum. 11*, 23 (Suppl.).

Pita, J. C., Müller, F. J., & Pezon, C. F. (1983) *Anal. Biochem. 133*, 9.

Pollack, M. S., & Price, C. A. (1971) *Anal. Biochem. 42*, 38.

Sajdera, S. W., & Hascall, V. C. (1969) *J. Biol. Chem. 244*, 77.

Schumaker, V. N. (1967) *Adv. Biol. Med. Phys. 11*, 245.

Spragg, S. P., Morrod, R. S., & Rankin, C. T., Jr. (1969) *Sep. Sci. 4*, 467.

Steensgaard, J., Miller, N. P. H., & Funding, L. (1978) in *Centrifugal Separations in Molecular and Cell Biology* (Birnie, G. D., & Rickwood, D., Eds.) Chapter 5, Butterworths, London.

Vanduffel, L., Peeters, B., & Rombauts, W. (1975) *Eur. J. Biochem. 57*, 481.

Vinograd, J., & Hearst, J. E. (1962) *Fortschr. Chem. Org. Naturst. 10*, 372.