Allowance for boundary sharpening in the determination of diffusion coefficients by sedimentation velocity: a historical perspective

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Received: 12 December 2017 / Accepted: 13 December 2017 / Published online: 27 January 2018
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Abstract

This review summarizes endeavors undertaken in the middle of last century to employ the Lamm equation for quantitative analysis of boundary spreading in sedimentation velocity experiments on globular proteins, thereby illustrating the ingenuity required to achieve that goal in an era when an approximate analytical solution of that nonlinear differential equation of second order provided the only means for its application. Application of procedures based on that approximate solution to simulated sedimentation velocity distributions has revealed a slight disparity (about 3%) between returned and input values of the diffusion coefficient—a discrepancy comparable with that of estimates obtained by current simulative analyses based on numerical solution of the Lamm equation. Although the massive technological developments in the gathering and treatment of sedimentation velocity data over the past three to four decades have changed dramatically the manner in which boundary spreading is analyzed, they have not led to any significant improvement in the accuracy of the diffusion coefficient thereby deduced.

Keywords Analytical centrifugation · Sedimentation velocity · Sedimentation coefficient · Concentration dependence · Boundary spreading · Diffusion coefficient

Introduction

The potential for quantitative analysis of boundary spreading in sedimentation velocity experiments originated from the report of the continuity equation describing solute migration in a centrifugal field (Lamm 1929). Indeed, an approximate analytical solution of that nonlinear differential equation of second order soon followed for the situation in which the sedimentation coefficient (s) and translational diffusion coefficient (D) are both assumed to be independent of solute concentration c (Faxén 1929). Although experimental observations of a

negative, essentially linear concentration dependence of s for globular proteins have undermined the validity of the first of those assumptions, justification for the second has been provided by the observation of the essential concentration independence of D for globular proteins from experiments conducted under the constraints of constant temperature and solvent chemical potential (Cecil and Ogston 1949; Creeth 1952; Creeth et al. 1958; Scott et al. 2014)—the constraints also considered to pertain to sedimentation velocity experiments (Braswell 1987; Winzor et al. 2004a, b). In that regard the pronounced concentration dependence of translational diffusion coefficients obtained by dynamic light scattering can be attributed to contributions to D arising from protein interaction with buffer species, which must be regarded as additional solute components when chemical potential is being monitored under the constraints of constant temperature and pressure (Kirkwood and Goldberg 1950; Stockmayer 1950; Hill 1959) that operate in those experiments.

A major breakthrough in the analysis of boundary spreading in sedimentation velocity experiments was the report by Fujita (1956, 1962) of an approximate analytical solution to the Lamm equation for solutes exhibiting linear concentration dependence of s and concentration independence of D . Other approximate solutions (Weiss 1964; Weiss and Yphantis 1965;

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Billick and Weiss 1966) were precursors to the application of numerical simulation for solving the Lamm equation (Dishon et al. 1967) that finally generated concentration gradient distributions demonstrating the adequacy of the Fujita approximate analytical solution. Only limited advantage was taken of this breakthrough (Baldwin 1957; Fujita 1959; Van Holde 1960; Creeth and Winzor 1962; Inkerman et al. 1975) before the virtual demise of analytical ultracentrifugation soon thereafter—a technique that was only revitalized by the appearance of a new-generation instrument in the final decade of the twentieth century. A second factor was the movement away from the less sensitive schlieren optical system to UV absorbance optics—a change that allowed experimental data to be obtained at much lower protein concentrations (a lower limit of about 10 µg/ml), where the effects of hydrodynamic nonideality leading to boundary sharpening are negligible.

The availability of Beckman XL-A and XL-I analytical ultracentrifuges with online data capture of experimental solute distributions in the form of absorbance and Rayleigh interference patterns has certainly rekindled interest in the characterization of proteins by the equilibrium and velocity techniques of analytical ultracentrifugation. The consequent revival of interest in the analysis of boundary spreading in sedimentation velocity experiments has been slowly building (Behlke and Ristau 1997, 2002; Philo 1997, 2006; Schuck 1998, 2000; Schuck and Demeler 1999; Schuck and Rossmanith 2000; Stafford and Sherwood 2004; Demeler 2005) because it has coincided with the massive advances in computer technology that have rendered readily feasible the numerical solution of differential equations by finite element methods (Claverie et al. 1975; Holladay 1979; Cox and Dale 1981; Todd and Haschemeyer 1981). Furthermore, the fact that many industrial samples, such as monoclonal antibodies, are used clinically at high concentration dictates a need for their characterization at high concentration to test for the presence of aggregates—a matter of great concern to regulatory agencies. However, despite the existence of better algorithms and more powerful methods to describe sedimentation velocity distributions, only limited advantage (e.g. Schuck 2000; Solovyova et al. 2001; Li et al. 2012) seems to have been taken of the ability of these simulative procedures to incorporate the concentration dependence of s (and also of D) into the analysis of boundary spreading. Such seeming reluctance to employ the simulative procedures for the analysis of boundary spreading in sedimentation velocity experiments may well reflect difficulties in incorporating additional iteration into an already highly iterative process.

On grounds of historical significance it seems timely to review the literature on the much earlier practice

(Baldwin 1957; Fujita 1959; Van Holde 1960) of employing the approximate analytical solution (Fujita 1956, 1962) of the Lamm equation to determine the diffusion coefficient from sedimentation velocity distributions that reflect the boundary sharpening resulting from linear s - c dependence; and also to take advantage of a current numerical integration procedure (Schuck 1998, 2000) for solving the Lamm equation to provide simulated sedimentation velocity distributions that allow more critical assessment of the consequences of the enforced simplification on which the approximate solution is based. Although the effort and understanding required to employ those analytical procedures is likely to preclude any future applications, their review at this time serves to emphasize the advanced level of boundary spreading analysis that had been attained over 60 years ago. Indeed, in many respects the current simulative solutions provided by computer programs such as SEDFIT (Schuck 1998, 2000; Dam and Schuck 2004) and SEDANAL (Stafford and Sherwood 2004) merely provide alternative means of achieving those same goals.

Theoretical considerations

Solute migration in an ultracentrifuge is described comprehensively by the Lamm (1929) equation,

$$\left(\frac{\partial c}{\partial t}\right)_r = \frac{1}{r} \left[\left(\frac{\partial}{\partial r}\right) \left(rD \left(\frac{\partial c}{\partial r}\right) - \omega^2 r^2 s c \right) \right]_t \quad (1)$$

where c is the solute concentration at radial distance r , and t is the time of centrifugation at angular velocity ω . In the middle of the last century the only option available for taking advantage of this equation to characterize sedimentation velocity patterns was to obtain an analytical solution (albeit approximate) to Eq. (1).

The Fujita approach

Achievement of an analytical solution to the Lamm equation was based on an initial distribution wherein an infinitely sharp boundary between solvent and solution is located at radial distance r_o such that $c=0$ for $r < r_o$ and $c = c_o$ for $r > r_o$, consideration being restricted to solutes for which D is concentration independent and the concentration dependence of c can be expressed as $s = s^0(1 - k_s c)$. Introduction of the following dimensionless variables,

$$\theta = c/c_o \quad (2a)$$

$$y = (r/r_o)^2 \quad (2b)$$

$$\tau = 2\omega^2 s^0 t \quad (2c)$$

$$\varepsilon = 2D/(\omega^2 s^2 r_o^2) \quad (2d)$$

$$\alpha = k_s c_o \quad (2e)$$

allows Eq. (1) to be expressed as

$$\left(\frac{\partial \theta}{\partial \tau}\right)_y = \left(\frac{\partial}{\partial y}\right) \left[\varepsilon y \left(\frac{\partial \theta}{\partial y}\right) - y \theta (1 - \alpha \theta) \right]_\tau \quad (3)$$

which remains a nonlinear differential equation of second order (in θ). Its conversion to the linear differential equation,

$$\left(\frac{\partial u}{\partial \tau}\right)_y = y \left(\varepsilon \frac{\partial^2 u}{\partial y^2} - \frac{\partial u}{\partial y} \right) \quad (4)$$

is effected by means of the transform (Stockmayer 1951)

$$\theta = \left(\frac{\varepsilon}{\alpha}\right) \frac{\partial(\ln u)}{\partial y} \quad (5)$$

which, upon introducing the two further substitutions,

$$\eta = 2 \left\{ 1 - [\text{yexp}(-\tau)]^{\frac{1}{2}} \right\} \quad (6a)$$

$$\zeta = 1 - \exp(-\tau) \quad (6b)$$

allows Eq. (4) to be written as

$$\frac{\partial u}{\partial \zeta} = \varepsilon \frac{\partial^2 u}{\partial \eta^2} + \frac{\varepsilon}{2-\eta} \frac{\partial u}{\partial \eta} \quad (7)$$

In order to achieve an analytical solution of this linear partial differential equation of second order in u , Fujita incorporated an approximation that:

$$\left[\frac{\text{yexp}(-\tau)}{\left\{ \varepsilon (1 - \exp(-\tau)) \right\}} \right]^{\frac{1}{2}} = \frac{1 - \left(\frac{\eta}{2}\right)}{(\varepsilon \zeta)^{\frac{1}{2}}} \gg 1 \quad (8)$$

which was also used by Faxén (1929) to obtain an approximate analytical solution for the simpler situation with s and D both concentration independent. Resort to this approximation implies values of much less than unity for both $\tau = 2\omega^2 s^0 t$ and $\varepsilon = 2D/(\omega^2 s^2 r_o^2)$, this being a proviso that should at least be fulfilled during the early stages of sedimentation velocity experiments on globular proteins. Because $[\text{yexp}(-\tau)]^{-\frac{1}{2}} \approx 1$ for $\tau \ll 1$, it follows from Eq. (8) that $(\varepsilon \zeta)^{\frac{1}{2}} \ll 1$ and hence that $\eta/2 \ll 1$, whereupon Eq. (7) becomes

$$\frac{\partial u}{\partial \zeta} = \varepsilon \left(\frac{\partial^2 u}{\partial \eta^2} + \frac{1}{2} \frac{\partial u}{\partial \eta} \right) \quad (9)$$

Furthermore, by making the substitution

$$z = \eta + \frac{\varepsilon \zeta}{2} \quad (10)$$

Eq. (9) simplifies to

$$\frac{\partial u}{\partial \zeta} = \varepsilon \frac{\partial^2 u}{\partial z^2} \quad (11)$$

which is of the same form as the differential equation describing free diffusion described by Fick's second Law, and for which analytical solutions are readily available. A way of obtaining an analytical solution of the Lamm equation for a globular protein, under the approximations defined above, had thus been found. Indeed, by substituting a different independent variable, $w = 2[\text{yexp}(-\tau)]^{\frac{1}{2}}$, for η , Billick and Weiss (1966) have avoided the need to make the approximation that $\tau = 2\omega^2 s^0 t \ll 1$ and hence established the adequacy of the Fujita (1956, 1962) solution for all practical purposes.

Descriptions of the sedimentation velocity distributions

By means of the above approach Fujita (1956, 1962) obtained the following solution for the solute concentration throughout the centrifuge cell:

$$c = \frac{\frac{c_o \exp(-\tau)}{1-\lambda} \left[1 - \text{erf} \left\{ \frac{\xi - \gamma(\lambda)^{\frac{1}{2}}}{(1-\lambda)^{\frac{1}{2}}} \right\} \right] \exp \left\{ \frac{[\xi - \gamma(\lambda)^{\frac{1}{2}}]^2}{(1-\lambda)} \right\}}{\left[1 - \text{erf} \left\{ \frac{\xi - \gamma(\lambda)^{\frac{1}{2}}}{(1-\lambda)^{\frac{1}{2}}} \right\} \right] \exp \left\{ \frac{[\xi - \gamma(\lambda)^{\frac{1}{2}}]^2}{(1-\lambda)} \right\} + (1-\lambda)^{\frac{1}{2}} [1 + \text{erf}(\xi)] \exp(\xi^2)} \quad (12a)$$

where

$$\xi = \frac{1 - \left[(r/r_o)^2 \exp(-\tau) \right]^{\frac{1}{2}}}{[\varepsilon \{1 - \exp(-\tau)\}]^{\frac{1}{2}}} \quad (12b)$$

$$\lambda = k_s c_o [1 - \exp(-\tau)] \quad (12c)$$

$$\gamma = (k_s c_o / \varepsilon)^{\frac{1}{2}} \quad (12d)$$

and where $\text{erf}(x)$ is the error function defined by

$$\text{erf}(x) = \frac{2}{\sqrt{\pi}} \int_0^x \exp(-x^2) dx \quad (12e)$$

Eq. (12) generates an experimental distribution comprising a symmetrical boundary between solvent and solution plateaux that is centered about a radial position r_b (Fig. 1) described (Fujita 1956, 1962) by

$$r_b = r_o \exp\left(\frac{\tau}{2}\right) [1 - k_s c_o \{1 - \exp(-\tau)\}]^{\frac{1}{2}} \quad (13)$$

However, prior to the introduction of Rayleigh interference and UV absorbance optics, the only optical records of sedimentation velocity runs were schlieren patterns, which depict the radial description of that concentration gradient distribution. Fujita derived the expression

$$\frac{H}{A} = \frac{\exp\left(-\frac{\tau}{2}\right) F(\xi_b)}{r_o \left[1 + (1-\lambda)^{\frac{1}{2}}\right] [\varepsilon \{1 - \exp(-\tau)\}]^{\frac{1}{2}}} \quad (14a)$$

$$F(\xi_b) = \frac{\text{erf}^{-1}(\xi_b)}{1 + \text{erf}(\xi_b)} + 2\xi_b \quad (14b)$$

for the height/area ratio of the peak (H/A), located at radial distance r_b (Fig. 1). ξ_b is the particular value of ξ [Eq. (12b)]

Fig. 1 Schematic representations of the solute distribution in a sedimentation velocity experiment showing the concentration profile arising from an initial infinitely sharp boundary located at radial distance r_o (solid line) and the corresponding concentration gradient distribution, characterized in terms of peak area (A) and peak height (H) at radial distance r_b (broken line)

with $r = r_b$, and the expression for the inverse error function,

$$\text{erf}^{-1}(\xi_b) = \frac{2}{\sqrt{\pi}} \exp(-\xi_b^2) \quad (14c)$$

follows from Eq. (12e).

Experimental accommodation of the initial boundary distribution

A potential weakness of the above description of the concentration distribution at time t (reflected as τ in the reduced coordinate system) is, of course, its reliance on an initial distribution with an infinitely sharp boundary between solvent and solution located at radial distance r_o , an initial distribution at variance with that prevailing experimentally. Instead, traditional sedimentation velocity patterns reflect an initial distribution in which the solute concentration is uniformly c_o for $r \geq r_o$, where r_o corresponds to the air–liquid meniscus position. Ultracentrifugation leads to a progressive decrease in protein concentration at the meniscus to zero. Only then do the experimental patterns match the theoretical requirement of a boundary between solvent and solution regions. Fortunately, the existence of the meniscus at time zero has been shown by numerical solutions of the Lamm equation (Dishon et al. 1967) to have minimal effect on the evaluation of D by application of the Fujita (1956, 1962) expression to sedimentation velocity distributions with a solvent plateau between the meniscus and the boundary. The next practical challenge thus becomes a search for an effective timescale whereby the assumed initial sharp boundary would have been located at r_o , now the position of the air–liquid meniscus.

Because a revolution counter provided the only quantitative information on rotor speed, an accurate estimate of ω was deduced from the number of rotor revolutions in the time elapsed between the first and last optical records of the solute distribution, the first of which was not recorded until after the attainment of constant rotor speed. The sedimentation coefficient could then be obtained from the slope ($\omega^2 s$) of the dependence of $\ln(r_b/r_o)$ upon elapsed time since the first optical record; a revised origin of the effective timescale for centrifugation at angular velocity ω could also be obtained from the extrapolated abscissa intercept of the $\ln(r_b/r_o)$ time dependence (Fig. 2).

Practical applications of the approximate analytical solution

The availability of an approximate analytical solution to the Lamm equation that incorporated allowance for boundary sharpening arising from a linear s – c dependence had at last

Fig. 2 Deduction of an absolute timescale for centrifugation at angular velocity ω from the time dependence of boundary migration. The linear time dependence of $\ln(r_b/r_o)$ based on $t=0$ for the first measurement (vertical broken line) is extrapolated to a value of zero to deduce the effective time taken for the boundary to migrate from the meniscus to the arbitrary origin used for determination of the slope ($\omega^2 s$). Data taken from Table 1 of Baldwin (1957) for the sedimentation of bovine serum albumin at 59,780 rpm

provided a meaningful basis for the detailed analysis of boundary spreading in sedimentation velocity experiments on globular proteins. However, there still remained the problem of finding ways to take advantage of this major development. Three such methods followed in relatively quick succession.

The Baldwin procedure

The first application of Eq. (14) was reported in a sedimentation velocity study of bovine serum albumin by Baldwin (1957), who rearranged the expression in the form

$$\left(\frac{AF(\xi_b)}{H}\right)^2 = \frac{2D}{\omega^2 s^0} (\exp(\tau) - 1) \left(1 + (1 - \lambda)^{\frac{1}{2}}\right)^2 \quad (15)$$

to allow the determination of D under circumstances where values of s^0 and k_s were already available from studies of the linear s - c dependence. An undesirable aspect of this approach is the need of a value for the diffusion coefficient for the calculation of ξ_b , which renders the method iterative in that a disparity between the input and returned estimates of D necessitates repetition of the analysis with revised initial estimates until the required coincidence of values is achieved. As illustrated in Fig. 3, the diffusion coefficient is obtained (Baldwin 1957) from the slope, $2D/(\omega^2 s^0)$, of the dependence of $[AF(\xi_b)/H]^2$ upon $[\exp(\tau) - 1][1 + (1 - \lambda)^{\frac{1}{2}}]$. In that regard the observation of a relatively minor but finite value for the ordinate intercept (-0.0019 ± 0.0009) instead of the theoretical prediction of zero presumably reflects a non-constant angular velocity during rotor acceleration to attain speed, as well as restricted (rather than free) diffusion on the centripetal side of an incompletely resolved boundary during the early stages of centrifugation (Baldwin 1957). The diffusion coefficient of $6.8 (\pm 0.4) \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$ that is obtained

from the results of these sedimentation velocity experiments at 59,780 rpm and 25 °C on 6.7 (Fig. 3, open diamond) and 13.5 (Fig. 3, filled diamond) mg/mL albumin solutions agrees reasonably well with those of $6.85\text{--}6.94 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$ obtained from free diffusion measurements (Creeth 1952).

Those results thus confirmed an expectation (Baldwin 1957) that the procedure should return an estimate within 5% of the true value. This situation was also realized in the only other application of this approach (Creeth and Winzor 1962), where boundary analysis of sedimentation velocity patterns for ovalbumin yielded a diffusion coefficient within 3% of the value obtained from free diffusion measurements (Creeth et al. 1958).

The Fujita procedure

The need for iteration in the above procedure was soon eliminated by Fujita (1959), who expressed Eq. (14a) in an alternative form, namely

$$G(\xi_b) = 2\alpha r_o \left(\frac{H}{A}\right) \sinh(\tau/2) \approx \alpha r_o \left(\frac{H}{A}\right) \tau \quad (16a)$$

where

$$G(\xi_b) = 2\xi_b \left[\xi_b + \frac{\exp(-\xi_b^2)}{\sqrt{\pi}[1 + \text{erf}(\xi_b)]} \right] \quad (16b)$$

The approximate form of Eq. (16a), which takes advantage of the already inherent assumption that $\tau \ll 1$ gives rise to the following expression for ξ_b ,

$$\xi_b = G^{-1} \left[\alpha r_o \left(\frac{H}{A}\right) \tau \right] = G^{-1} \left[2r_o \omega^2 s^0 k_s c_o \left(\frac{H}{A}\right) t \right] \quad (17)$$

where $G^{-1}(x)$, the inverse function of $G(x)$, is a complex function for which numerical values have been tabulated (Fujita 1959) as a function of x .

Fig. 3 Illustration of the Baldwin (1957) procedure for evaluating the diffusion coefficient for bovine serum albumin from sedimentation velocity experiments conducted at 59,780 rpm. Results for 6.7 mg/mL (open diamond) and 13.5 mg/mL (filled diamond) solutions are plotted according to Eq. (15), which signifies a slope of $2D/(\omega^2 s^0)$ for the linear dependence. Data taken from Fig. 2a, b of Baldwin (1957)

An alternative expression for ξ_b is obtained by expanding Eq. (12b) in powers of τ to give, in terms of original variables,

$$\xi_b = \frac{r_o \omega^2 s^0 k_s c_o}{2\sqrt{D}} \left[1 - \frac{1}{2} (1 - k_s c_o) \omega^2 s^0 t \right] \sqrt{t} \quad (18)$$

whereupon the combination of these two expressions for ξ_b leads to the conclusion that

$$\begin{aligned} & \left[1 - \frac{1}{2} (1 - k_s c_o) \omega^2 s^0 t \right] \sqrt{t} \\ &= \frac{2\sqrt{D}}{r_o \omega^2 s^0 k_s c_o} G^{-1} \left[2r_o \omega^2 s^0 k_s c_o \left(\frac{H}{A} \right) t \right] \end{aligned} \quad (19)$$

which signifies a slope of $2\sqrt{D} / (r_o \omega^2 s^0 k_s c_o)$ for the linear dependence of $\left[1 - \frac{1}{2} (1 - k_s c_o) \omega^2 s^0 t \right] \sqrt{t}$ upon $G^{-1}[2r_o \omega^2 s^0 k_s c_o (H/A)t]$.

Fujita (1959) employed the above results for serum albumin (Baldwin 1957) to illustrate the determination of D by this approach, which is summarized in Fig. 4. Linear least-squares analysis of results for the 6.7 mg/mL (Fig. 4, open diamond) and 13.5 mg/mL (Fig. 4, filled diamond) albumin solutions leads to respective diffusion coefficient estimates of 6.8 and $6.9 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$, which effectively duplicate the value of $6.8 (\pm 0.4) \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$ deduced above from Fig. 3. The ability of this approach to yield an acceptable estimate of D was confirmed by the earliest use of numerical simulation to solve the Lamm equation for a system with linear s - c dependence (Dishon et al. 1967).

The Van Holde adaptation of the Fujita procedure

Delineation of the experimental s - c dependence is clearly a prerequisite for applying the above two procedures because of their reliance upon knowledge of s^0 and k_s ; but the requirement for knowledge of k_s has been removed by an adaptation (Van Holde 1960) of the Fujita (1959) procedure.

The Van Holde approach retains the approximate form of Eq. (16a) as well as Eq. (18) for the quantitative description of

ξ_b , but avoids introduction of the $G^{-1}(\xi_b)$ by expanding $G(\xi_b)$ as a Maclaurin series to obtain the expression

$$G(\xi_b) = (2\sqrt{\pi})\xi_b - \left(\frac{2(\pi-2)}{\pi} \right) \xi_b^2 + \left[\left(\frac{2}{\sqrt{\pi}} \right) \left(\frac{4-\pi}{\pi} \right) \right] \xi_b^3 + \dots \quad (20)$$

in which the cubic term contributes negligibly for $\xi_b < 0.4$. Incorporation of Eq. (20) into the approximate form of Eq. (16a) then gives (Van Holde 1960)

$$\begin{aligned} & \left(\frac{H}{A} \right) \sqrt{t} \\ & \frac{1}{1 - \frac{1}{2} \omega^2 s t} = \frac{1}{\sqrt{4\pi D}} + \left[\frac{(\pi-2)r_o \omega^2 s k_s c_o}{4\pi D(1-k_s c_o)} \right] \left(1 - \frac{1}{2} \omega^2 s t \right) \sqrt{t} + \dots \end{aligned} \quad (21)$$

where s is the sedimentation coefficient associated with loaded concentration c_o of solute. The predicted linear dependence of $(H/A)\sqrt{t} / [1 - \frac{1}{2} \omega^2 s t]$ upon $[1 - \frac{1}{2} \omega^2 s t]\sqrt{t}$ is thus characterized by a slope of $[(\pi-2)r_o \omega^2 s k_s c_o / (4\pi D)] [k_s c_o / (1-k_s c_o)]$ and an ordinate intercept of $1/\sqrt{4\pi D}$. An obvious advantage of this procedure over the Fujita (1959) approach is its removal of any reliance upon the availability of a value for k_s .

These features of the Van Holde approach are evident from Fig. 5, which summarizes its application to the sedimentation velocity data on bovine serum albumin (Baldwin 1957) used in Figs. 3 and 4 to illustrate the two previous analyses. The respective estimates of 6.7 and $6.9 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$ for D that emanate from the very similar ordinate intercepts for 6.7 mg/mL (Fig. 5, open diamond) and 13.5 mg/mL (Fig. 5, filled diamond) albumin solutions clearly confirm the values obtained by the other two procedures. Furthermore, the consequent values of k_s deduced from the slopes of the two dependencies in Fig. 5, namely 5.5 and 7.3 mL/g, are certainly consistent with the more accurately determined experimental coefficient (5.98 mL/g) obtained by linear least-squares analysis of the sedimentation coefficient data reported in Fig. 6 of Baldwin (1957).

Fig. 4 Application of the Fujita (1959) procedure for evaluating the translational diffusion constant (D) from the Baldwin (1957) sedimentation velocity data on bovine serum albumin. The results for 6.7 mg/mL (open diamond) and 13.5 mg/mL (filled diamond) solutions are plotted according to Eq. (19). Data taken from Figs. 2 and 3 of Fujita (1959)

Fig. 5 Evaluation of the diffusion coefficient from the Baldwin (1957) sedimentation velocity data on serum albumin by the Van Holde (1960) procedure, which takes advantage of Eq. (21) to determine both D and k_s from the results for 6.7 mg/mL (open diamond) and 13.5 mg/mL (filled diamond) protein solutions. Data taken from Fig. 1 of Van Holde (1960)

Comparable findings have emerged from studies of chicken liver carboxylesterase (Inkerman et al. 1975) in that the estimates of 6.72 , 6.62 and $6.53 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$ obtained by Van Holde analysis of sedimentation velocity distributions for 16.3 , 10.0 and 4.0 mg/mL enzyme solutions agree well with that of $6.61 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$ deduced from a synthetic boundary experiment with 4.0 mg/mL carboxylesterase; and also in that the estimates of 7.6 , 6.3 and 7.5 mL/g for k_s are all in the vicinity of 6.3 mL/g , the value deduced from experimental studies of the s - c dependence.

Summary of practical applications

All three procedures that have been developed to take advantage of the approximate analytical solution of the Lamm equation for solutes exhibiting linear s - c dependence are seemingly valid for globular proteins. The estimates of diffusion coefficients deduced by the analysis of boundary spreading in sedimentation velocity experiments agree reasonably well with their counterparts from free diffusion measurements. Of the three procedures described for such determination of diffusion coefficients from sedimentation velocity experiments, the Van Holde (1960) adaptation of the Fujita (1959) method has the obvious advantage of avoiding the need for prior knowledge of the magnitude of k_s . The requirement of a value for s^0 is readily met in that the ordinate intercept of an s - c dependence is relatively insensitive to the selection of c_o or $\bar{c}_p$, the average of plateau concentrations in distributions used for the determination of s (Kegeles and Gutter 1951), as the abscissa parameter (Fig. 6). Although the latter concentration is the relevant parameter for the determination of k_s (Patel et al. 2017), the incorrect use of c_o still persists.

Unfortunately, a general loss of interest in analytical ultracentrifugation soon after the report (Fujita 1956, 1962) of the approximate solution of the Lamm equation has meant that its potential remains largely unexploited.

Fig. 6 Concentration dependence of the measured sedimentation coefficient ($\bar{s}$) for ovalbumin with sedimentation coefficient (c) taken as the initial value c_o (open circle) and the average of plateau concentrations for the first and last distributions used for sedimentation coefficient measurement (filled circle). Latter data taken from Fig. 5 of Creeth and Winzor (1962)

Application of the Van Holde method to simulated sedimentation velocity data

From the outset the validity of the assumptions inherent in the approximate analytical solution of the Lamm equation was regarded as a question that required closest scrutiny. The above demonstrations show reasonable agreement between the estimate of D deduced from analysis of boundary spreading in sedimentation velocity distributions and its counterpart from free diffusion measurements. This thus afforded the most stringent criterion of validity verification available at that time. However, the subsequent development of readily accessible numerical integration procedures (Claverie et al. 1975; Schuck 1998; Solovyova et al. 2001; Stafford and Sherwood 2004; Li et al. 2012) for generating sedimentation velocity distributions for systems with defined values of D , s^0 and k_s has provided an opportunity to subject the utility of the Fujita approximate solution to even closer scrutiny. Although a satisfactory outcome emanated from the only previous attempt to analyze simulated sedimentation velocity distributions (Dishon et al. 1967), it was noted that the return of an acceptable estimate of D was extremely reliant upon the accuracy of the value of k_s incorporated into the Fujita (1959) analysis. We therefore employ the Van Holde (1960) adaptation of the Fujita (1959) procedure because of its return of an estimate of k_s as well as of the translational diffusion coefficient.

Simulation of sedimentation velocity distributions

To provide a stricter test of the validity of employing Eq. (21) to evaluate D and k_s from sedimentation velocity distributions, concentration profiles were first generated for the centrifugation at 20°C of a protein with the sedimentation characteristics of serum albumin in phosphate-buffered saline [$D = 6.08 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$, $s^0 = 4.24 \text{ S}$, $k_s = 8 \text{ mL/g}$]: the diffusion coefficient and limiting sedimentation coefficient are inferred from values of $D_{25,w}$ and $s_{25,w}^0$ reported by Baldwin (1957), whereas the value of k_s is typical of globular proteins. For that purpose the SEDFIT program (Schuck 1998, 2000), a moving-hat adaptation (Schuck et al. 1998) of the finite element approach (Claverie et al. 1975) to solving the differential equations numerically, has been used to simulate distributions at 5-min intervals for a solution of solute ($c_o = 10 \text{ mg/mL}$, $J_o = 33.3$ fringes) subjected to centrifugation at $50,000 \text{ rpm}$ ($\omega = 5236 \text{ rad s}^{-1}$): a random error of 0.02 fringes was superimposed on the simulated distributions to replicate the experimental situation (Rowe 2005). Representative Rayleigh distributions are shown in Fig. 7, which summarizes distributions generated after 50–125 min of simulated centrifugation: every third distribution is presented.

Allowance for a concentration rather than a concentration gradient distribution

The form of Eq. (21) was designed for graphical evaluation of the two parameters of unknown magnitude (D and k_s) in the days when nonlinear regression analysis was not a viable option. A more appropriate rearrangement for current analysis is

$$\frac{H}{A} = \frac{1 - \frac{1}{2} \omega^2 st}{\sqrt{4\pi D}} + \left[\frac{(\pi - 2)r_o \omega^2 s k_s c_o}{4\pi D(1 - k_s c_o)} \right] \left(1 - \frac{1}{2} \omega^2 st \right)^2 + \dots \quad (22)$$

which achieves the desired separation of variables. However, this rearrangement requires adaptation to accommodate sedimentation velocity patterns generated as the radial dependence of concentration (Fig. 7) rather than a concentration gradient. For a boundary between solvent and a solution plateau with solute concentration corresponding to J Rayleigh interference fringes, ideal diffusion is described by the expression (Longworth 1953; Creeth 1955, 1958)

$$f(j) = \frac{(2j - J)}{J} = \text{erf}(x) \quad (23)$$

where j denotes a particular Rayleigh fringe (counting from the solvent plateau) in a pattern of J fringes, and where $\text{erf}(x)$ is the error function [Eq. (12e)] with the upper limit of integration defined by

$$x = \text{erf}^{-1} \left[\frac{(r_j - r_b)}{\sqrt{4\pi D t}} \right] \quad (24)$$

in which erf^{-1} again denotes the inverse error function, and r_b the center of the boundary [the position corresponding to that of the maximum height (H) in a symmetrical schlieren pattern]. This requirement for precise knowledge of r_b is avoided (Longworth 1953; Creeth

Fig. 7 Simulated concentration distributions for the migration of a 10 mg/mL solution ($J_o = 33.3$ fringes) of a globular protein ($s^0 = 4.24$ S, $k_s = 8$ mL/g, $D = 6.08 \times 10^{-7}$ cm²/s) subjected to centrifugation at 50,000 rpm for 50 to 125 min, there being a 15-min interval between distributions

1955) by defining a parameter Y_t from the displacement between two fringes (j_1 and j_2) as

$$Y_t = \frac{(r_2 - r_1)}{(x_2 - x_1)} = \sqrt{4\pi D t} \quad (25)$$

On the basis that the corresponding analysis of the schlieren recording of the same diffusing system is

$$\frac{H}{A} = \frac{1}{\sqrt{4\pi D t}} \quad (26)$$

the adaptation of the Van Holde (1960) procedure to accommodate Rayleigh interference recording of the sedimentation velocity patterns merely requires the substitution of $1/(\bar{Y}_t \sqrt{\pi})$ for (H/A) , where $\bar{Y}_t$ is the mean of measurements of Y_t for a range of fringe couplings. The counterpart of Eq. (22) then becomes

$$\frac{1}{\bar{Y}_t} = \frac{1 - \frac{1}{2} \omega^2 st}{\sqrt{4\pi D t}} + \left[\frac{(\pi - 2)r_o \omega^2 s k_s c_o}{4\sqrt{\pi D}(1 - k_s c_o)} \right] \left(1 - \frac{1}{2} \omega^2 st \right)^2 \quad (27)$$

for the evaluation of D and k_s as the two parameters of unknown magnitude to be obtained by nonlinear least-squares curve-fitting of the $[t, \bar{Y}_t]$ data set to this expression.

Specific details of the analysis

Because of the theoretical requirement for complete resolution of the boundary from the air–liquid meniscus region (r_o), the distribution for $t = 3000$ s was the first pattern to be analyzed for this system. The sedimentation coefficient evaluated as $[\ln(r_b/r_o)]/(\omega^2 t)$ from the mean boundary position (r_b) for distributions at $t = 3000 - 7500$ s was essentially constant (3.94 ± 0.01 S) for plateau concentrations c_p in the range $8.8 \leq c_p \leq 9.4$ mg/mL. This estimate compares favorably with that (3.93 S) for $\bar{c}_p = 9.1$ g/L but underestimates slightly the required value, 3.90 S, for the loading protein concentration (10 mg/mL) that is calculated from the expression $s = s^0(1 - k_s c_o)$.

In accordance with earlier practice (Creeth 1955, 1958) the fringes used for the evaluation of Y_t from the first part of Eq. (25) were in the range $0.10 J - 0.25 J$ and $0.75 J - 0.90 J$ for j_1 and j_2 , respectively. Values of $\text{erf}^{-1}(x)$ for $f(j)$ [see Eq. (23)] were calculated by means of the expression

$$\text{erf}^{-1}(x) = \left[-\frac{2}{\pi a} - \frac{\ln(1-x^2)}{2} + \sqrt{\left\{ \frac{2}{\pi a} + \frac{\ln(1-x^2)}{2} \right\}^2 - \frac{\ln(1-x^2)}{a}} \right]^{\frac{1}{2}} \quad (28)$$

with a value of 0.147 for a (Winitzki 2008) to obtain x_1, x_2 and hence Y_t via Eq. (23). $\bar{Y}_t$ was then taken as the mean of those values of Y_t for a given time (t) of centrifugation.

A plot of the time dependence of $1/\bar{Y}_t$ values (filled circle) is presented in Fig. 8, where the solid line denotes their description obtained by nonlinear least-squares curve-fitting to Eq. (27). Although the best-fit estimates [± 2 standard deviations (SD)] of $5.91 (\pm 0.18) \times 10^{-7} \text{ cm}^2/\text{s}$ for D and $7.1 (\pm 0.9) \text{ mL/g}$ for k_s underestimate slightly their input counterparts ($6.08 \times 10^{-7} \text{ cm}^2/\text{s}$ and 8 mL/g respectively), the comparison is seen to be reasonably favorable when the uncertainty envelopes are taken into account. Furthermore, the extent of agreement between the value and estimate of D (a difference of 3%) would certainly be regarded as acceptable in an experimental context.

On the grounds that the above estimates of D and k_s are interdependent because of their simultaneous evaluation as curve-fitting parameters obtained by nonlinear regression analysis of boundary spreading according to Eq. (27), we have also estimated the diffusion coefficient by the application of Eq. (19), the expression developed by Fujita (1959) to evaluate D from sedimentation velocity distributions for a solute with known sedimentation coefficient characteristics. In that regard, values of the inverse G function have been calculated from the relationship

$$G^{-1}(x) = 0.88207x - 0.45502x^2 + 0.18892x^3, \quad (29)$$

a multinomial expression that was found to provide an adequate description of the tabulated data (Fujita 1959) for $0 \leq x \leq 0.6$. Analysis of the experimental results according to Eq. (19) with the exact value of k_s yields an estimate of $6.27 (\pm 0.03) \times 10^{-7} \text{ cm}^2/\text{s}$ for D , where the uncertainty (± 2 SD) clearly refers to the precision rather than the accuracy of the estimate. Inasmuch as the disparity between input and returned values for the diffusion coefficient (3%) is the same as that for D obtained by the Van Holde (1960) analysis of the same data, it would seem that the concurrent deduction of a second parameter (k_s) in the latter approach has not prejudiced the acceptability of the diffusion coefficient estimate. In that regard

Fig. 8 Analysis of boundary spreading in the simulated sedimentation velocity experiment showing time dependence of the quantitative measure of boundary spreading ($1/\bar{Y}_t$) (filled diamond) and best-fit description obtained by nonlinear least-squares curve-fitting of those results to Eq. (27) (solid line)

we note that similar disparities between the estimates of diffusion coefficients from sedimentation velocity and free diffusion measurements have been observed in the few experimental studies (Baldwin 1957; Fujita 1959; Van Holde 1960; Creeth and Winzor 1962; Inkerman et al. 1975) where advantage has been taken of the approximate analytical solution (Fujita 1956, 1962) of the continuity equation for ultracentrifugation of a single solute (Lamm 1929).

Ramifications of the analysis of simulated sedimentation velocity distributions

This failure of the Fujita (1959) and Van Holde (1960) procedures to return exact values of D is hardly surprising on the grounds that only an approximate analytical solution (Fujita 1956, 1962) of the relevant continuity equation (Lamm 1929) has been used for their evaluation. Nevertheless, it at least provides an avenue for acceptable quantitative analysis of boundary spreading by solutes exhibiting a negative concentration dependence of the sedimentation coefficient—a situation that most users of the simulative approaches seem reluctant to handle. In that regard the existence of relatively few examples [e.g. Solovyova et al. (2001) and Li et al. (2012)] of such obvious application of the simulative procedures does raise the possibility that there may be technical difficulties associated with incorporating additional iterations into the highly iterative procedures—a stance supported by the recommendation (Schuck 2005) that analysis of boundary spreading be confined to distributions for sufficiently low protein concentrations to justify neglect of the effect of s - c dependence. Such action may well improve the accuracy with which s , D and hence molecular mass may be determined, but it minimizes any opportunity for detecting weak self-association of the species being characterized. As noted in the Introduction, the fact that many industrial samples, such as monoclonal antibodies, are used clinically at high concentration dictates a need for their characterization at high concentration to test for the presence of self-association.

Concluding remarks

This review of early endeavors to take advantage of the Lamm (1929) equation for the quantitative analysis of boundary spreading in sedimentation velocity experiments on globular proteins serves to draw the attention of current ultracentrifuge users to the vast amount of work done on boundary spreading in the middle of the last century. Solutions to the problem of allowing for boundary sharpening as the result of negative s - c dependence were available long before the availability of the current simulative procedures employing numerical solution of the Lamm equation for the determination of diffusion coefficients. This review also serves to emphasize the ingenuity

required to achieve that goal (Fujita 1956) in an era when analytical solution of differential equations provided the only means for their application. Although the present application of procedures based on that approximate analytical solution (Fujita 1959; Van Holde 1960) to simulated sedimentation velocity distributions has revealed a slight disparity between returned and input values of the diffusion coefficient, the discrepancy (about 3% in D) is within the uncertainty envelope surrounding current diffusion coefficient estimates obtained by current simulative procedures based on numerical integration of the Lamm equation. In other words, the massive technological developments in the gathering and manipulation of sedimentation velocity data for globular proteins have certainly had a dramatic effect on the manner in which boundary spreading is analyzed; but they have not led to any significant improvement in the diffusion coefficient thereby deduced.

Acknowledgements We thank Dr. Peter Schuck for helpful advice on the use of his SEDFIT program for the simulation of sedimentation velocity distributions. The financial support of this investigation by the Science and Technology Facilities Council (UK) through an award to DJS is also gratefully acknowledged, as is the travel and accommodation support provided to DJW by the University of Nottingham over many years for this collaborative project.

Compliance with ethical standards

Conflict of interest Donald J. Winzor declares that he has no conflicts of interest. David J. Scott declares that he has no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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