Chapter 1
Important and Essential Theoretical Aspects of AUC

Susumu Uchiyama and Fumio Arisaka

Abstract Analytical ultracentrifugation (AUC) is a powerful method to reveal biophysical behavior of solute in solution. AUC has a long history and is based on well-established and concrete thermodynamic and hydrodynamic theory. AUC provides valuable parameters such as the sedimentation and diffusion coefficients, from which molar mass and information on hydrodynamic shape and solvation of the solute can be derived. Here, important and essential theoretical aspects of AUC are described.

Keywords Analytical ultracentrifugation • Sedimentation equilibrium • Sedimentation velocity • Theory

1.1 Introduction

This chapter is devoted to the basics of analytical ultracentrifugation. Efforts have been made to cover the minimum essentials and to make it qualitatively but precisely understandable. Two fundamentally important concepts in analytical ultracentrifugation are sedimentation and diffusion. Detailed theories can be found in the published literature (Fujita 1962; Cantor and Schimmel 1980; van Holde et al. 2005).

There are two modes of experiments, namely, sedimentation velocity (SV) and sedimentation equilibrium (SE). In SV experiments, both sedimentation and diffusion take place simultaneously. In SE experiments, sedimentation and diffusion are balanced and reach equilibrium.

S. Uchiyama (✉)
Department of Biotechnology, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan

Department of Bioorganization Research, Okazaki Institute for Integrative Bioscience, 5-1 Higashiyama, Myodaiji-cho, Okazaki, Aichi 444-8787, Japan
e-mail: suchi@bio.eng.osaka-u.ac.jp

F. Arisaka
Life Science Center, College of Bioresource Science, Nihon University, 1866 Kameino, Fujisawa, Kanagawa 252-0880, Japan

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1.2 Principle for Sedimentation and Diffusion

1.2.1 Sedimentation

Suppose we suspend some homogeneous fine grains of sand in water and stir and leave it still (Fig. 1.1). The boundary between the air and the solution is called "meniscus." The sand will leave the meniscus and slowly sediment and the moving boundary will appear. Above the moving boundary, sand grains have sedimented down and are already gone. Below the boundary, the same concentration or the same number of grains per unit volume of sand is still there sedimenting with the same velocity. The speed of the moving boundary, v, is proportional to the acceleration of gravity, and the proportionality constant, s, will define the sedimentation coefficient of the sand particle.

$$v = sg \tag{1.1}$$

If all the sand particles have the same s-value, they sediment with the same velocity toward the bottom and the concentration at plateau region is kept constant. The particles that have reached at the bottom accumulate there.

Now, s is related to the mass, m, and specific volume, $\overline{v}$, of the particle and the density of water, ρ:

$$s = \frac{m(1 - \overline{v}\rho)}{f}, \tag{1.2}$$

where f is the frictional coefficient of the particle. The term $\overline{v}\rho$ is the excluded volume of the particle and $m\overline{v}\rho$ is the mass of the excluded water, leading to the

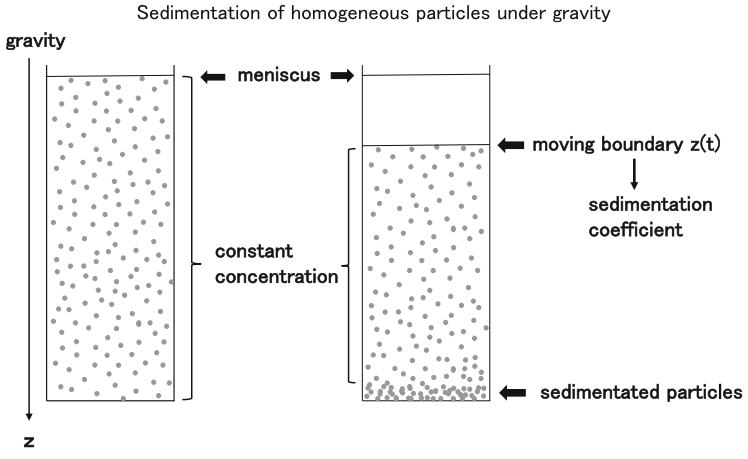


Fig. 1.1 Sedimentation of homogeneous grains in a cylinder under gravity

buoyancy. The term $m(1-\overline{v}\rho)$ is called the buoyant mass. If the sand particles are not homogeneous, each size of the sand grain sediments with its own sedimentation coefficient, and we may observe the distribution of sedimentation coefficients.

Before invention of the analytical ultracentrifuge, Thé Svedberg measured the size distribution of colloidal gold which he was studying by the method as described above. In order to measure the position of the moving boundary precisely, he observed the boundary using a microscope. Eventually, Svedberg got interested in measuring the size of proteins which had been recognized to be very important in biological organisms. However, s-values of proteins are too small and do not sediment at all under the normal gravity due to the overwhelming diffusion. In order to let them sediment in spite of diffusion, the gravity has to be much increased, and he decided to utilize the centrifugal force of a centrifugation. Special devices, then, had to be developed in order to observe the moving boundary or the concentration gradient in the cell in a revolving rotor. Thus, Svedberg designed and constructed a prototype of analytical ultracentrifuge.

The velocity, v, of sedimentation of the moving boundary, r_b, is proportional to the centrifugal force, and the sedimentation coefficient is defined as the proportionality constant:

$$v = sr_b\omega^2, \tag{1.3}$$

where r_b is the position of the moving boundary from the center of revolution and ω is the angular velocity. It is noted that g in Eq. (1.1) is now replaced by $r\omega^2$, the acceleration of centrifugal force. The sedimentation coefficient is related to the mass of the particle, m, and the frictional coefficient, f:

$$s = \frac{m(1-\overline{v}\rho)}{f} = \frac{M(1-\overline{v}\rho)}{N_A f}, \tag{1.2a}$$

where N_A is the Avogadro's number, ρ is the density of the solvent, and M is the molar mass. s has the dimension of time and commonly used with the unit of S, where 1 S is 10^{-13}s. Simple classical mechanics treatment shows $\overline{v}$ to be a specific volume, but more rigorous treatment of the transport process by nonequilibrium thermodynamics shows that it is a partial specific volume or

$$\overline{v} = \left(\frac{\partial V}{\partial m}\right)_{T,p}, \tag{1.4}$$

where $\overline{v}$ is a volume increase of a solution of a large volume when 1 g of lyophilized protein or other solute molecules had been dissolved. If the concentration of the solute is low enough, it is close to the specific volume. From Eq. (1.3) or $(\mathrm{d}r_b/\mathrm{d}t) = sr_b\omega^2$,

$$\ln(r_b) = \ln(r_m) + s\omega^2 t \tag{1.5}$$

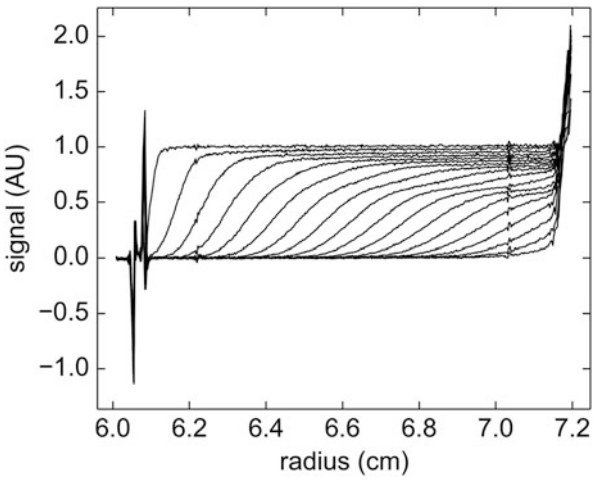


Fig. 1.2 Typical data of sedimenting boundary in sedimentation velocity experiment

so that

$$s = \ln\left(r_b/r_m\right) / \left(\omega^2 t\right),$$

where r_m denotes the position of the meniscus.

Svedberg noticed at the early stage of the development of his analytical ultracentrifuge (AUC) that the cell has to be sector shaped instead of rectangular, because the particle sediments in the direction of radius and if the cell is rectangular some particles will collide with the wall and the moving boundary will be distorted. The consequence of the sector-shaped cell is that the concentration of the solute at the plateau region, c_p, will gradually decrease due to the fact that the cross section of the flow of the solute will expand proportionally to the distance, r, from the center of revolution (Fig. 1.2):

$$C_p = C_0 e^{-2s\omega^2 t} \tag{1.6}$$

The analysis of moving boundary will be described more in detail when we introduce the Lamm equation which describes the time course of the concentration gradient in sedimentation velocity. r_b is then defined as the position of r, where the concentration is half that of the plateau region.

The value of sedimentation coefficient thus determined depends on the buffer conditions and temperature. The buffer condition changes the density and viscosity and the temperature mainly affecting the viscosity of water. In order to obtain the intrinsic physical parameter of the solute, the density and viscosity have to be corrected to reflect the experimental conditions.

It may be noted that in the above discussion, we are not looking at the behavior of each sedimenting molecule in solution but looking at the moving boundary or the concentration gradient at the boundary. All the information we extract concerning

the characteristics of the solute molecules are contained in the shape and its change during time course of the moving boundary. In fact, the moving boundary contains abundant information concerning homogeneity/heterogeneity, sedimentation coefficients and their distribution, diffusion coefficient and interactions between solute molecule, non-ideality, etc. Measurement of moving boundary is similar to the frontal analysis of the size exclusion chromatography.

1.2.2 Diffusion

Sedimentation coefficient and frictional coefficient are related through Eq. (1.2a). Now, there is a simple relation between diffusion coefficient and frictional coefficient for ideal solution which is called the Einstein-Sutherland equation:

$$D = \frac{RT}{N_A f} \tag{1.7}$$

Replacing f in Eq. (1.2a) with f in Eq. (1.7) will give the Svedberg equation:

$$\frac{s}{D} = \frac{M(1 - \overline{v}\rho)}{RT} \tag{1.8}$$

This equation indicates that if we have the values for s and D, we can determine the molar mass, M, with the advance knowledge of $\overline{v}$ and ρ. The molar mass thus determined does not depend on the shape of the molecule as the equation implies.

In the case of non-ideality, D can be expressed as

$$D = \frac{RT}{N_A f}\left\{1 + C\frac{\partial \ln \gamma}{\partial C}\right\} \tag{1.9}$$

and the corresponding Svedberg equation is

$$\frac{s}{D} = \frac{M(1 - \overline{v}\rho)}{RT\left\{1 + C\frac{\partial \ln \gamma}{\partial C}\right\}}, \tag{1.10}$$

where γ is the activity coefficient of the solute.

Now, assume a cell of uniform cross section with infinite length and that a sharp concentration gradient or boundary is present at $x = 0$ at time $t = 0$ (Fig. 1.3). The time course of the change of concentration gradient can be predicted by the Fick's second law (diffusion equation):

$$\left(\frac{\partial C(x,t)}{\partial t}\right)_x = D\left(\frac{\partial^2 C(x,t)}{\partial x^2}\right)_t, \tag{1.11}$$

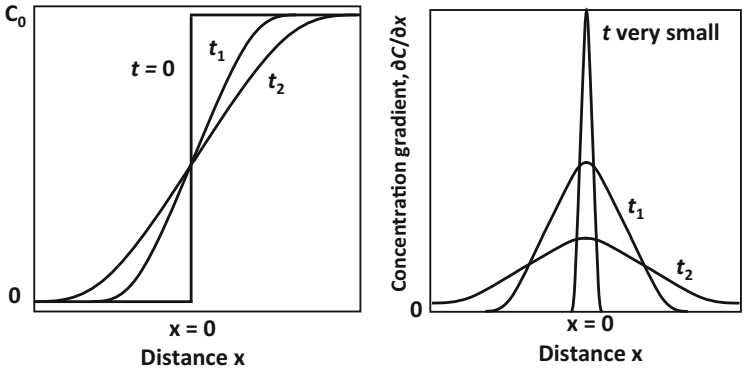


Fig. 1.3 Change of concentration gradient of solute due to diffusion

where D is the translational diffusion coefficient. Equation (1.6) can be solved with the initial condition and boundary condition. Under these conditions of free diffusion, the solution of Eq. (1.6) is

$$C(x,t) = \frac{C_0}{2}\left\{1 - \frac{2}{\sqrt{\pi}}\int_0^{x/2\sqrt{Dt}} e^{-y^2 dy}\right\} \tag{1.12}$$

and the derivative of $C(x,t)$ with respect to r is simply a Gaussian "error" curve:

$$\left(\frac{\partial C(x,t)}{\partial x}\right)_x = \frac{C_0}{2\sqrt{\pi Dt}} e^{-x^2/4Dt}, \tag{1.13}$$

where c_0 is the concentration of the solute at $t = 0$. We can get the gradient either by calculating from the experimental concentration gradient data or measure the gradient by traditional so-called schlieren optical system. The height of the bell-shaped curve, H, is given by

$$\left(\frac{\partial C(x,t)}{\partial x}\right)_{x=0} = \frac{C_0}{2\sqrt{\pi Dt}} = H \tag{1.14}$$

and

$$\left(\frac{C_0}{H}\right)^2 = 4\pi Dt \tag{1.15}$$

We can thus determine D by plotting $(C_0/H)^2$ with respect to t. There is a special cell, synthetic boundary cell, which can be used to form a sharp boundary to determine the diffusion coefficient by the method as described above. In current practice, both

sedimentation coefficient and diffusion coefficient are determined by direct curve fitting to the Lamm equation solution to the raw data of sedimentation velocity.

1.3 Sedimentation Velocity

1.3.1 Lamm Equation

As soon as the solute molecules leave the meniscus and start to sediment, a concentration gradient will appear. In the concentration gradient (moving boundary), sedimentation and diffusion take place simultaneously. It was Ole Lamm, a Ph.D. student of Thé Svedberg, who reported a partial differential equation which describes the time course of the concentration gradient of the solute in sedimentation velocity (Lamm 1929):

$$\left(\frac{\partial C}{\partial t}\right)_r = -\frac{1}{r}\left\{\frac{\partial}{\partial r}\left[s\omega^2 r^2 C - Dr\left(\frac{\partial C}{\partial r}\right)_t\right]\right\} \tag{1.16}$$

This equation, called the Lamm equation after Ole Lamm, precisely describes the time course of the sedimentation together with diffusion. Detailed derivation of the Lamm equation is described elsewhere. Due to the consideration of the sector-shaped cell, the right-hand side of the equation is somewhat complicated, but, basically, it consists of two terms in the brackets [ ]. The first term has the coefficient s, and the second term contains D. The former describes sedimentation and the latter diffusion. In current methods of SV analysis, s and D are determined by nonlinear least squares curve fitting to the raw data.

Equation (1.16) assumes that s and D are constants (i.e., there is no hydrodynamic non-ideality) under the same conditions. The measured s- and D-value are usually corrected to a standard condition which is traditionally in water at 20 °C, $s_{20,w}$, $D_{20,w}$. If the actual measurements were made in a buffer solution at temperature T, $s_{T,b}$, (1.2a) and (1.7) contain the frictional coefficient, which is related to the viscosity of the solvent by the Stokes law:

$$f = 6\pi\eta R_s, \tag{1.17}$$

where R_s is the Stokes radius and has been worked out for particles of various shapes. In any event, it is directly proportional to the viscosity of the solvent. The measured $s_{T,b}$ and $D_{T,b}$ are, therefore, corrected for viscosity and the density of the solvent:

$$s_{20,w} = \frac{(1-\overline{\nu}\rho)_{20,w}}{(1-\overline{\nu}\rho)_{T,b}}\frac{\eta_{T,b}}{\eta_{20,w}}s_{T,b} \tag{1.18}$$

$$D_{20,w} = \frac{293.1}{T}\frac{\eta_{T,b}}{\eta_{20,w}}D_{T,b} \tag{1.19}$$

Correction for viscosity is mainly due to the temperature dependence of that of water. For example, the viscosity of water at 4°C is about 1.3 times larger than that of water at 20°C. The *s*- and *D*-values thus corrected are known to depend on the concentration of the solute and need to be extrapolated to zero concentration in order to obtain the real intrinsic physical parameter of the solute $s^0_{20,w}$. For common spherical soluble proteins, extrapolation to zero concentration may not be necessary if the concentration is below 1 mg/mL or so. However, care has to be taken for extremely elongated proteins such as triple-helical collagen or highly negatively or positively charged molecules such as nucleic acids. In fact, the s-value of nucleic acids has a much higher concentration dependence than proteins and has to be measured at very low concentration. Fortunately, nucleic acids, DNA and RNA, have much higher extinction coefficients than proteins, and, as a result, they can be measured at much lower concentration. Extinction coefficient of nucleic acids is about 20 times larger than those of proteins. Much less frequently, sedimentation coefficients may decrease as the concentration decreases. In such a case, subunit dissociation may be anticipated.

1.3.2 Relationship Between s and M

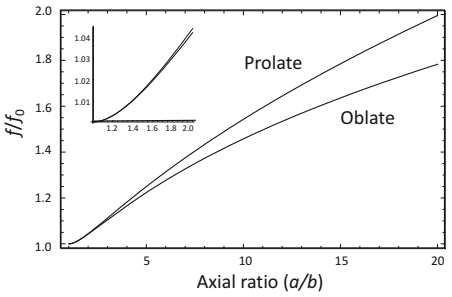
The molar mass, *M*, of the sedimenting particle can be estimated by Svedberg Eq. (1.18), which requires the *s*- and *D*-values. The *s*-value can be determined rather accurately with the error of one or two percent. On the contrary, *D*-values are more difficult to evaluate precisely, and they have been frequently obtained from DLS measurement and combined with the *s*-value from SV experiments. However, recent software, such as *c*(*s*) analysis in SEDANAL, SEDFIT, or ULTRASCAN, utilizes the relationship of *D* and f/f_0, called the scaling law:

$$D(s) = \frac{\sqrt{2}}{18\pi} kTs^{-1/2} (\eta (f/f_0)_w)^{-3/2} ((1 - \overline{v}\rho) / \overline{v})^{1/2} \tag{1.20}$$

The rationale of using this equation for analysis is discussed by Peter Schuck. In *c*(*s*) analysis, a common value for f/f_0 value is assumed, but there is another mode of analysis *c*(*s*, f/f_0) in SEDFIT or 2DSA in ULTRASCAN, where *s* and f/f_0 are independently fitted for each molecular species. SEDANAL fits directly for *s* and *D* for each species with or without constraints relating the frictional coefficients of each component.

1.3.3 Molecular Shape and f/f_0

Molecular shape may be discussed based on the frictional ratio, f/f_0. The frictional ratio, obtained from the SV analysis, may be related to the molecular shape of the



$$\text{Prolate} \quad f/f_0 = \frac{P^{-1/3}(P^2-1)^{1/2}}{\ln\left[P+(P^2-1)^{1/2}\right]}$$

$$\text{Oblate} \quad f/f_0 = \frac{(P^2-1)^{1/2}}{P^{2/3}\tan^{-1}\left[(P^2-1)^{1/2}\right]}$$

$$P = a/b \qquad (1.21)$$

Fig. 1.4 Relationship between axial ratio and frictional ratio

assumed prolate, oblate, or rod, through Perrin's equations (Fig. 1.4). As shown in Fig. 1.4, a small increase of f/f_0 increases the axial ratio quite a bit, but a large change in the axial ratio does not affect the f/f_0 very much, which is the rationale of using Eq.(1.21) independent of the molecular species. Note that spherical protein will give the f/f_0 of about 1.2 instead of 1.0, which is because f_0 is estimated for a protein without solvation, whereas the experimentally determined f includes solvation. We cannot discuss the molecular shape in detail but can decide if the molecule is close to sphere or elongated (or flattened). For example, native triple-helical collagen has a large f/f_0 value, larger than 2.

1.3.4 Sedimentation Coefficients Estimated from the X-ray Structure of the Proteins

If the atomic structure of a protein is known, one can estimate the hydrodynamic values including the sedimentation coefficient and diffusion constant. Although it is not so simple to predict the structure from the sedimentation coefficient, if a number of possible model structures are available, one could assess each structure by estimating each s-value and judge which model would fit the measured s-value (Rocco and Byron 2015).

1.4 Sedimentation Equilibrium

When solution is centrifuged at a relatively low speed as compared with that of the SV experiments, both sedimentation and diffusion contribute significantly to the concentration distribution. As a result, the moving boundary which we see in SV experiments will not be seen. Instead the concentration of the solute at the meniscus will decrease and that at the bottom will increase and eventually reach the equilibrium, when sedimentation and diffusion are balanced. The resultant

concentration gradient will be used to estimate the molar mass:

$$\frac{d\ln C}{d\left(r^2\right)} = \frac{M\left(1-\overline{\nu}\rho\right)\omega^2}{2RT} \tag{1.22}$$

Equation (1.22) can be derived by assuming that the total chemical potential including centrifugal potential is common everywhere in the cell. It can be also derived from Lamm Eq. (1.16) by setting $\partial C/\partial t = 0$.

Integration of Eq. (1.22) gives

$$C(r) = C\left(r_a\right)\exp\left(M_{w,app}\left(1-\overline{\nu}\rho\right)\left(r^2 - {r_a}^2\right)\right) + \text{baseline} \tag{1.23}$$

$$c(r) = \sum_{i=1}^{n} c_i\left(r_a\right)\exp\left(M_i\left(1-\overline{\nu}_i\rho\right)\left(r^2 - r_a^2\right)/2RT\right) \tag{1.24}$$

In signal units:

$$S(r) = S_{\text{baseline}}(r) + \sum_{i=1}^{n}\epsilon_i c_i\left(r_a\right)\exp\left(M_i\left(1-\overline{\nu}_i\rho\right)\left(r^2 - r_a^2\right)/2RT\right) \tag{1.25}$$

Currently, most programs utilize nonlinear fitting of the raw data to Eq. (1.25) to obtain the molar masses, M_i. Here, it should be noted that the fitting data from a mixture of molecules using the model of a single ideal component results in an "average" M that is closer to Mz (z-average molecular weight) than Mw but falls between these and is not equal to either. As in the case of SV experiment to obtain the molar mass, which utilizes Svedberg Eq. (1.8), the values of $\overline{\nu}$ and ρ are required. The partial specific volume $\overline{\nu}$ can be measured by precision densitometry using a densitometer, e.g., Anton Paar DMA5000, but in many cases the requirement of the amount of proteins, on the order of 10 mg, may not be realistic and the values are commonly calculated based on the weighted average of the values for amino acid residues as listed in Cohn and Edsall (1943). The protocol for calculation is implemented in SEDNTERP.

At early times, sedimentation equilibrium was thought to be unrealistic due to the prolonged time required for equilibration. It was van Holde and Baldwin (1958) who showed that the required time to reach equilibrium is proportional to the square of the solution column length and is inversely proportional to the diffusion constant:

$$t \approx 0.7\frac{\left(r_b - r_a\right)^2}{D}, \tag{1.26}$$

where r_b and r_a are, respectively, the distance of meniscus and the bottom from the center of rotation and D is the diffusion coefficient of the solvent. Since then, shorter columns have been used for SE experiments, and the time of measurement became more realistic. Experiment at lower temperature requires longer time to reach equilibrium. Traditionally, judgment of equilibration was made if three or four scans are superimposed. There are some programs with which one could decide if the system reached equilibrium, like HeteroAnalysis, which includes the program Match that uses a least squares fitting procedure to determine whether or not equilibrium has been achieved, available from Jim Cole. Also a calculator is available to predict the time to attain equilibration in ULTRASCAN and SEDFIT. It should be noted that the time to equilibrium may be seriously underestimated for reversibly associating systems.

It is the usual case that the mass average molar mass, $M_{w,app}$, has concentration dependence due to nonideal behavior of the solute and thus can be expressed using second virial coefficients, B_2, and third virial coefficients, D_2, as

$$\frac{1}{M_{w,app}} = \frac{1}{M_w}\left(1 + B_2C + D_2C^2 + \cdots\right) \tag{1.19}$$

At concentrations below 10 mg/mL, usually $M_{w,app}$ is linearly dependent on the concentration; thus molar mass at infinite dilution, M_w and B_2, can be estimated by fitting of $M_{w,app}$ at different concentrations.

When the solute is not homogeneous, it is shown that the obtained molar mass is that of the weight average:

$$M_w = \sum_i^n C_i M_i / \sum_i^n C_i \tag{1.20}$$

In the case of interacting systems, the association-dissociation equilibrium between or among species is achieved at any position, r; nonlinear fitting of the $C_{\text{total}}(r)$ gives equilibrium dissociation constants between or among species.

1.5 Conclusions

In this chapter, the basics of analytical ultracentrifugation including theoretical background and essential mathematical expression used for understanding SV-AUC and SE-AUC were introduced. More details will be described in the following chapters dealing different topics.

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