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Simulation of the time course of macromolecular separations in an ultracentrifuge.
II. Controlling the solute concentrations

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Abstract

We describe algorithms, based on a simulation described in Part I of this series of papers, for the control of the distribution of one or more solutes in preparative or analytical ultracentrifuges equipped with programmable speed control. All of the methods involve the determination, during numerical integration of the Lamm equation, of protocols for continuously varying the rotor speed. We show one such protocol that has been used for the prevention of cesium chloride crystallization during DNA plasmid purification in the preparative ultracentrifuge. Other protocols that are described involve the *in situ* controlled mixing of two solutes. Limitations of the method owing to problems with input parameter imprecision, fundamental physical constraints, and mechanical limitations are discussed.

Keywords: Controlled sedimentation; Sedimentation simulation; Centrifuge run optimization; Macromolecular separations by ultracentrifugation; Density gradient formation

1. Introduction

In Part I of this series, Minton [1] gives the details of a numerical integration of the Lamm equation for cesium chloride (CsCl), along with experimental results that support the validity of his algorithms. In this paper Minton's work is modified and extended by deriving an algorithm that can be used in both preparative and analytical centrifuges to locally control the concentration of one or more solutes. The algorithm involves calculation of an essentially continuously varying rotor speed, and it was motivated by the following problem:

The preparation of covalently closed circular DNA plasmids is frequently accomplished by means of isopycnic density gradient centrifugation. The plasmids, along with a saturating concentration of ethidium bromide and any undesirable contaminating macrosolutes, are centrifuged in a concentrated solution of CsCl. Under the influence of a high centrifugal field, CsCl sedimentation leads to the formation of a density gradient in the tube. Concomitantly, the plasmid/ethidium bromide complexes sediment to those locations in the density gradient at which the local density of the CsCl solution equals the buoyant density of the complexes.

Isopycnic ultracentrifugation often requires very long run times (e.g. tens of hours), and it is frequently desired to shorten the run time by using a high rotor speed. One problem with high rotor speeds, though, is that the CsCl solution can eventually saturate at the bottom of the tube, and thereafter spawn CsCl crystals, if CsCl sedimentation is excessive. The formation of CsCl crystals not only distorts the gradient in an undesirable manner: The presence of dense CsCl crystals ($\rho = 3.988$ g/ml) in a high centrifugal field can result in excessive (and occasionally destructive) local stresses on the rotor. It is therefore necessary that the speed of the rotor and/or the run time be limited such that crystallization of CsCl never occurs.

Given the above constraint of the extent of CsCl sedimentation, consider the following mathematical optimization problem: What is the rotor speed vs. time protocol that *absolutely* maximizes the rotor speed at all times without allowing saturation of the CsCl solution at the bottom of the tube?

2. The algorithm

In Minton's calculation, the rotor speed is treated as an independent variable (usually, in fact, considered to be constant), and the concentration at any time and location in the centrifuge tube is a calculated quantity [1]. In the algorithm to be described, however, each iteration contains an additional step in which precisely the opposite is true: local concentrations are independent variables, and rotor speed is the derived quantity. The following paragraph contains the essence of the algorithm:

Consider a high speed run in which, at some time t_0, the concentration of CsCl at the tube bottom reaches the critical (i.e. saturation) value. We thereafter achieve absolute rotor speed maximization by continuously adjusting the rotor speed such that the flux (denoted by J in eq. (1) of Part I of this series), and hence mass transfer, of CsCl into the last compartment (i.e., the tube bottom) is identically zero. It is clear that the CsCl concentration at the tube bottom must remain fixed after the time t_0 if, for all times after t_0, the CsCl flux into the bottom compartment vanishes.

The algorithm to simulate rotor speed maximization is thus as follows:

(1) Begin the (Minton) simulation at the highest rotor speed, ω_{max}, consistent with rotor design. Include, as a stored parameter in the simulation code, the maximum allowable concentration of CsCl, w_{sat}. Initially, during *every* iteration of the simulation, compare the concentration of CsCl at the tube bottom (w_n) with w_{sat}. As long as $w_n < w_{\text{sat}}$, continue the integration with $\omega = \omega_{\text{max}}$.

(2) At the first instance, t_0, for which $w_n \geqslant w_{\text{sat}}$, and thereafter for all $t_0 + m\Delta t$ (where Δt is the time increment and $m = 0, 1, 2,\ldots$) calculate the new rotor speed for which the immediately subsequent flux of CsCl into the bottom compartment vanishes. The rotor speed calculation is directly from Minton's equation (4):

$$0 = J(n) = s[w_b(n)]w_b(n)\omega^2 r(n) - D[w_b(n)] \times([w(n) - w(n-1)]/\Delta r) \qquad (1)$$

The resulting value of ω is used for the next iteration of the flux and concentration calculations. In other words, for all $t = t_0 + m\Delta t$ ($m = 0, 1, 2,\ldots$) in the simulation, we not only solve for the fluxes and concentrations (à la Minton) but also for the rotor speed that gives, at every iteration, vanishing flux into the bottom compartment.

Figure 1 summarizes the algorithm, now referred to as the $J = 0$ algorithm, in a flowchart. Figure 2 shows the simulated rotor speed vs. time protocol that results when the $J = 0$ algorithm is applied to a Beckman Type 80 Ti rotor loaded with CsCl at a concentration of 4.4 M. The saturation concentration is taken to be 7.3 M. The rotor speed asymptotically approaches that speed for which the equilibrium concentration at the base of the tube is w_{sat}.

In the limit of $\Delta t \to 0$, the rotor speed is absolutely maximized at all times subject to the crystallization constraint. However, prior to a real centrifugation run, a more coarsely discretized version of the curve in this figure can be loaded into the computer memory of the ultracentrifuge

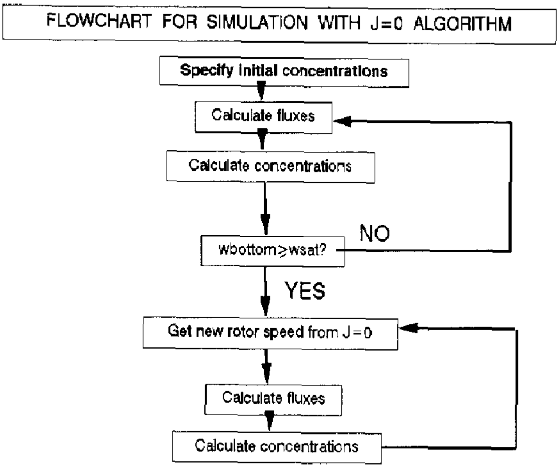

Fig. 1. Flow chart representation of the $J = 0$ algorithm. For details of the algorithm, see text. Whereas only CsCl fluxes and concentrations are calculated prior to CsCl saturation threshold at the bottom of the tube (i.e., whenever the answer is "NO" to the query $w_{\text{bottom}} \geqslant w_{\text{sat}}$?), new rotor speeds must be calculated for all iterations after the threshold is reached, i.e., after $w_{\text{bottom}} \geqslant w_{\text{sat}}$. Other than this additional rotor speed calculation, the iterations before and after the attainment of saturation threshold are identical.

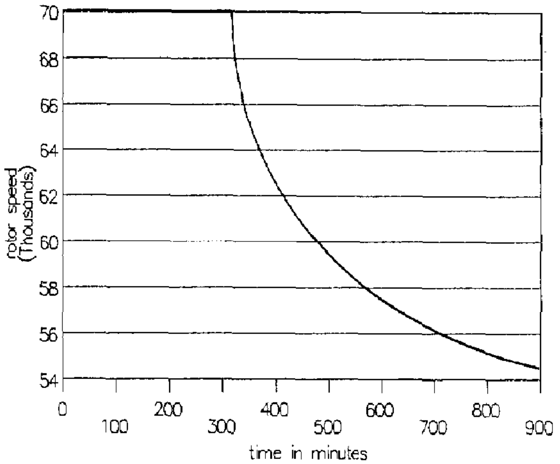

Fig. 2. Maximum rotor speed vs. time for Beckman Type 80 Ti rotor during DNA plasmid purification at 25 °C. The rotor speed is initially subjected to the constraint, commonly called the square-root reduction formula, that the hydrostatic pressure at the bottom of the tube not exceed a value set during the rotor design. Shortly after 300 minutes into the run, however, the rotor speed is further constrained by the requirement that the CsCl concentration at the bottom of the tube not be allowed to exceed the saturation threshold. At all times during the falling of the curve, the simulated CsCl concentration at the bottom of the tube is fixed precisely at its saturation threshold. At long times, the curve approaches the speed for which, at equilibrium, the CsCl concentration at the bottom of the tube equals w_{sat}.

and used to control the rotor speed. This is essentially what is done with ESP™ [2].

3. Results: Performance characteristics of $J = 0$ algorithm vs. single speed runs

The usual method for preventing CsCl crystallization in ultracentrifuge runs is to perform the entire run at that speed, ω_{eq}, for which the equilibrium CsCl concentration at the base of the tube equals w_{sat}. For the calculation of ω_{eq} it is necessary and sufficient to (1) impose $J = 0$ everywhere in the tube, and (2) require that the total mass of dissolved CsCl in the equilibrium gradient is equal to the dissolved CsCl mass that was loaded into the tube. ω_{eq} is uniquely determined by these two constraints on $w(r)$.

We have performed a comparison for several Beckman rotors between simulated DNA plasmid purification runs performed at ω_{eq} vs. simulated runs that used the $J = 0$ algorithm. Figure 3 shows

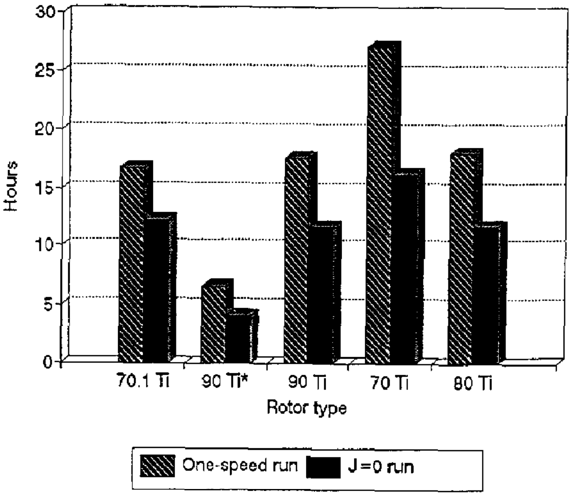

Fig. 3. Comparison of the time required for plasmid separation when using one-speed (ω_{eq}) vs. $J = 0$ algorithm for various Beckman fixed-angle rotors. The Type 90 Ti* run utilizes the g-max™ system, whereas the Type 90 Ti run does not. ω_{eq} is the speed toward which the $J = 0$ run makes an asymptotic approach (e.g., near 54,000 rpm in Fig. 2). It is also the speed for which the equilibrium concentration at the bottom of the tube is the saturation concentration, w_{sat}.

the comparison of the separation times that obtain for these two run protocols. It should be noted that the accuracy of the simulation algorithm has been verified experimentally for several rotors (ref. [3] and personal communication with Dr. Allen Furst at Beckman Instruments, Inc.).

4. Discussion: Other algorithms and applications

The potential applications that arise from the $J=0$ algorithm extend beyond the prevention of CsCl crystallization. Some of these possible applications will be discussed below.

4.1 Control of single solute concentrations

Although we applied the $J=0$ algorithm to the bottom compartment of a centrifuge tube to solve the CsCl crystallization problem, we can also utilize variations of the algorithm anywhere in the tube for other kinds of applications. For example, if we consider some compartment in the middle of the tube, say the kth, then we can keep the concentration of a solute constant in that compartment, after some time t_0, by means of rotor speeds derived from the following equation (which is quadratic in ω):

$$J_{k-1}A_{k-1}-J_kA_k=0 \tag{2}$$

Here, the subscripts $k-1$ and k refer, respectively, to the boundaries on the top and bottom of the kth compartment. The A's are the areas of the respective boundaries. If, for the times $t=t_0+m\Delta t$ $(m=0, 1, 2, 3\ldots p)$, ω is determined from eq. (2), then the net transport of solute into the kth compartment vanishes for all t such that $(t_0+p\Delta t)>t>t_0$. More generally, if we replace the zero in eq. (2) with a user-chosen function of time, $f(t)$, and if we utilize the rotor speeds that result from the solution of such a modified eq. (2), then the rate of change of concentration of the solute in the compartment will be proportional to $f(t)$, the proportionality constant being the reciprocal of the volume of the compartment.

4.2 Control of concentrations of more than one solute

We need not restrict ourselves to considerations of the concentration of a single sedimenting solute. Suppose, for example, that we denote by J the flux of one solute and J^* the flux of some other solute. Suppose further that at some time, t_0, the respective concentrations w and w^* are equal at the kth compartment and that we want to keep them equal for some period of time, $p\Delta t$, after t_0. The following equation, solved for ω at $t_0+m\Delta t$ $(m=0, 1, 2, 3\ldots p)$ yields the required rotor speed protocol:

$$J_{k-1}A_{k-1}-J_kA_k=J^*_{k-1}A_{k-1}-J^*_kA_k \tag{3}$$

Again, we can generalize this application by multiplying either side of eq. (3) by a user-chosen $f(t)$. The solution of such an equation yields rotor speeds for which the time dependence of the ratio of the mass transfers of the two solutes into the compartment goes as $f(t)$. Note that the mass transfer ratios after t_0 can be set independently of the concentrations at t_0. In particular, it is not required that $w=w^*$ before eq. (3) can be utilized.)

5. Caveats and cautions

There are three types of potential sources of problems associated with the successful implementation of these new algorithms: Physico-chemical, mathematical, and mechanical.

5.1 Physico-chemical sources of error

The precision with which solute concentrations can be reliably controlled with these methods depends on the precision with which all of the physico-chemical parameters for J (e.g., s, gradient of w, activity coefficients, etc.) are known. For example, hydrostatic pressure effects on both the solubility and the activity of CsCl in water were not included in the calculations leading to the rotor speed protocol given in Fig. 2.

It should be clear that the efficacy of all of the variations of the $J = 0$ algorithm (e.g. eq. 3) are similarly sensitive to the accuracy of the parameters that enter into the J calculations.

5.2 *Mathematical problems*

Equation (1) and its variations do not always have real solutions for the rotor speed. As an illustration, consider a situation in which the gradient of a solute at a particular location in the tube points toward the axis of rotation. In such a case, diffusion would drive the solute in the *same* direction as sedimentation, and an attempt to find a rotor speed vs. time protocol that yields constant concentration at that location (i.e., by invoking eq. 2) would necessarily fail. The possibility of obtaining a real (as distinct from imaginary) rotor speed at any particular time depends entirely on the physical situation in the tube at that time. Therefore, a judicious choice of initial conditions (i.e. loading configuration of the tube contents, relative initial concentrations in the case of more than one solute) may lead to successful rotor speed protocols where other choices may result in failure to find a protocol.

5.3 *Mechanical constraints*

There can also be cases where the derived speed protocol is mathematically permissible but utterly unrealizable by any real mechanical system. For example: Consider the case of a uniformly loaded tube with a solute having $s = 1\ S$ and a concentration of 1 mg/ml. If we want a customized initial flux of 0.1 g/s cm^2 at a radius of 5 cm, then the mathematical solution is simple but the mechanical implications are absurd: $J = 0.1 = s\omega^2 rw = (10^{-13}\ \text{s})\ (\omega^2)\ (5\ \text{cm})\ (0.001\ \text{g/ml})$, giving $\omega = 1.4 \times 10^7\ \text{s}^{-1}$, i.e. over two million revolutions per second (2 MHz)! This shows, in an admittedly exaggerated manner, that even if a choice for $f(t)$ in the algorithms (e.g. replacing the 0 in eq. (2) gives a mathematically sound solution for all times of interest, the ability to realize such a speed vs. time protocol will depend on mechanical considerations.

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