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Rate of Water Equilibration in Vapor-Diffusion Crystallization: Dependence on the Residual Pressure of Air in the Vapor Space

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Abstract

The kinetics of water equilibration in vapor-diffusion crystallization experiments are sensitive to the residual pressure of air in the vapor chamber. Experiments with sitting droplets of 10%(*w*/*v*) PEG, allowed to equilibrate with reservoirs of 20%(*w*/*v*) PEG, were conducted at pressures ranging from 80 to 760 mm Hg. Equilibrations were interrupted after one, four, five and seven days to assess their progress. Even down to the lowest pressures examined it was found that a decrease in pressure leads to an increase in the rate of equilibration. The residual pressure of air in the vapor chamber can be varied to tailor the time course of equilibration in macromolecular crystal growth experiments.

Introduction

The vapor-diffusion method, as adapted to the microscale by Hampel *et al.* (1968), has become a principal tool for the crystallization of macromolecules (McPherson, 1982; Ducruix & Giegé, 1992). In the method, a droplet containing the macromolecule and a crystallizing agent is allowed to equilibrate in a closed system with a reservoir containing a dehydrating agent. Equilibration is effected as water, in the form of vapor, leaves the droplet, traverses the vapor space and enters the reservoir. In the process the concentrations of macromolecule and crystallizing agent increase, and in the favorable case, conditions evolve in the droplet toward those that promote nucleation and crystal growth.

The kinetics of water equilibration in a hanging-drop arrangement have been studied experimentally by Mikol, Rodeau & Giegé (1990) and developed into a mathematical model by Fowlis *et al.* (1988). In the Fowlis *et al.* model the process of equilibration is considered to occur in five distinct steps (Fig. 1). They are as follows.

(I) Water from within the droplet diffuses to the droplet surface.

(II) Water evaporates from the surface of the droplet and enters the vapor space.

(III) Water traverses the vapor space by diffusional processes.

(IV) Water condenses on the surface of the reservoir.

(V) Water diffuses from the surface layer into the bulk of the reservoir.

Fowlis *et al.* argued that steps II and IV are instantaneous. They then undertook order-of-magnitude calculations to identify the rate-limiting step among the remaining three. The calculations suggested that step I, diffusion within the droplet, was by far the slowest step in the equilibration process. However, Fowlis *et al.* further argued that evaporation of water creates solute-rich dense layers on the droplet surface which, in a gravitational field, lead to convective mixing. Mixing would relax the requirement that water reaches the surface of the droplet by diffusional processes, and make step I more rapid. From these considerations Fowlis *et al.* concluded that step III, diffusion of water vapor in air across the vapor chamber, was rate limiting, and went on to elaborate a model of the equilibration process that is in qualitative agreement with observation. As the

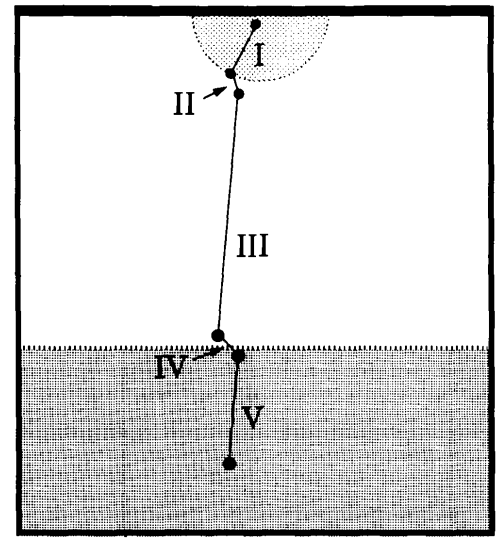

Fig. 1. Stages in the equilibration of a hanging droplet, after Fowlis *et al.* (1988). A water molecule (•) traverses the droplet (I), leaves the droplet surface (II), traverses the vapor space (III), is adsorbed on the surface of the reservoir (IV), and diffuses into the bulk (V). In the Fowlis *et al.* model, step III, diffusion in the vapor space, is rate limiting.

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model was developed from first principles, the agreement supports the argument concerning convective mixing and the proposition that diffusion across the vapor space is rate limiting.

Indeed, there have been a number of reports concerning solutal convection currents in hanging-drop crystallizations, such as those of Pusey, Witherow & Naumann (1988). These currents arise as a result of density gradients formed in the near vicinity of a growing crystal, as the macromolecule is sequestered from solution into the crystal lattice. However, these currents are not the ones that Fowlis *et al.* invoke when they argue that step III, not step I, is rate limiting. Their model does not require the presence of the macromolecule or the formation of crystals. It only requires the presence of crystallizing agent in the droplet. The particular agent they studied was ammonium sulfate but their argument works equally well for any agent that forms aqueous solutions of increasing density with increasing concentration. Sibille, Clunie & Baird (1991) directly addressed the question of density gradient-induced convective mixing in Plaas-Link capillaries. Two closed-end capillaries are joined by Tygon tubing to form a closed system in which the evaporating capillary plays the role of the droplet and the condensing capillary plays the role of the reservoir. When the inner diameters of the capillaries are narrow enough, surface tension allows them to be oriented vertically. When oriented vertically with the condenser above the evaporator, a dense solute-rich layer should accumulate on the evaporator surface and a less dense water-rich layer should accumulate on the condenser surface. Neither should be gravitationally stable. On the other hand, when oriented vertically with the evaporator above the condenser, the boundary regions should be stable in a gravitational field. Yet Sibille *et al.* find virtually identical kinetics of equilibration for the two orientations, suggesting that convective mixing has a minor role in determining the rates of water equilibration, at least in Plaas-Link capillaries.

It is conceivable, though highly improbable, that step III is not the rate-limiting step in hanging-drop equilibrations, and that agreement between theory and observation is fortuitous. However, the model developed by Fowlis *et al.* admits of a number of direct experimental verifications. Their fundamental equation (2.5.12) expresses t/τ, where t is the time and τ is a time constant associated with the diffusion process in step III, as a rather complicated function of a dimensionless quantity related to the geometry of the drop and the distance from the drop to the surface of the reservoir. The time constant τ is inversely proportional to D_1, the diffusion coefficient of water vapor in air. At room temperature and atmospheric pressure $D_1 = 0.26 \times 10^{-4}\ m^2\ s^{-1}$ (Cussler, 1984). According to the kinetic molecular theory of gases (Atkins, 1978) D_1 is itself inversely proportional to p, the pressure of air through which the water vapor must diffuse. A reduction in p should lead to a proportional reduction in τ, and thence in t, for any particular value of the dimensionless geometrical factor. Put more simply, a reduction in the air pressure in the vapor space should speed up vapor-diffusion experiments in a predictable way, provided that step III is rate limiting. Pressure dependence of the equilibration process would be unlikely if either step I or step V, both of which are diffusion processes within the liquid phase, were rate limiting. Assuming for the moment that steps II and IV are instantaneous, as argued by Fowlis *et al.*, step III should be the only step sensitive to the atmospheric pressure in the vapor chamber.

We expect time courses of equilibration for sitting drops to differ from those for hanging drops (Luft & DeTitta, 1995). However, the models developed by Fowlis *et al.* for the hanging drop and by Sibille *et al.* for the Plaas-Link capillary both involve expressions for t/τ that depend similarly on D_1. We expect the pressure dependence of equilibration rates to be similar for vapor-diffusion arrangements of virtually any kind. Here we present the results of equilibrations of sitting drops as a function of the residual pressure p in the vapor chamber. We will show that, over the pressure range 80–760 mm Hg, a reduction in p leads to an increase in the rate of water-vapor equilibration. We will also show that the increase is sufficiently significant at low pressures that equilibrations involving polyethylene glycol solutions become practicable over relatively short time intervals. Finally, we will propose a device for vapor-diffusion experiments that permits the crystal grower to use pressure to tailor the time course of water-vapor equilibrations.

Experimental

Six identical low-pressure crystallization vessels (LPCV's) were constructed in the laboratory, Fig. 2. Each consists of three Plexiglas plates, approximately 127 × 127 mm in cross section and 18 mm thick. A 76 mm diameter hole is drilled through one of the plates which is then permanently bonded, face to face, with another of the plates using an adhesive specially formulated for Plexiglas. Together these form the base and the chamber of the vessel. Plumbing hardware is fitted into the remaining plate, which serves as the removable lid of the vessel. The vessel is sealed as two O-rings, straddling six machine bolts, are compressed by the manual tightening of the bolts. The inner O-ring, diameter 93 mm, actually makes the vacuum seal while the outer O-ring, diameter 125 mm, serves as a strain relief for the Plexiglas as the machine bolts are tightened. The plumbing consists of a needle valve (*A*) in line with a vacuum gauge in line with a second needle valve (*B*). A standard welder's fitting allows the LPCV to be connected to a ballast tank for evacuation.

When empty, the vacuum chamber is nominally 100 cm^3 in volume, taking into account the thickness of the O-rings. In use, the chamber is filled with 20 ml of reservoir solution and with a boat, Fig. 3, that accommodates a dozen plastic microbridges (Hampton Research). The volume of the boat, which measures 39 × 39 × 15 mm, is about 23 cm^3. In its original use, the boat housed Millipore filters. As the filters are round there is a short raised circular ridge (~1.4 mm high, 1.2 mm thick, 29.2 mm diameter) on the inner surface of the boat. The ridge causes the microbridges at the outer corners to tip slightly.

Rates of water equilibration were determined in a standard manner. The LPCV reservoir was filled with 20.0 ml of reservoir solution. Microbridges were inserted in a regular pattern into the boat and 24 µl droplets were placed in each of the dozen microbridges. The top plate was immediately placed to cover the droplets and the six bolts tightened until the O-rings were visibly flattened. The LPCV was connected to a ballast tank (~13 300 cm^3) by a vacuum hose. The tank was previously evacuated with a roughing pump and air had been bled back until the pressure in the tank had reached a desired value as measured on the vacuum gauge. The LPCV was evacuated by opening valve *B* completely and valve *A* just slightly. Evacuation to the ballast tank took a few minutes, at which point valve *A* was fully opened to insure that the tank and LPCV were at the same pressure. The LPCV was closed to the ballast tank in a particular order. First, valve *A* was fully closed, allowing the gauge to be read as a residual pressure in the LPCV. Then valve *B* was closed, both to lessen the exposure of the gauge to water vapor and to sequester the vapor equilibration to the known dimensions of the vessel.

Equilibrations were allowed to proceed at room temperature for a predetermined time. Before the LPCV was opened the vacuum seal was checked by opening valve *B* fully and re-reading the gauge. If there was a significant change in residual pressure from the starting value the experiment was discarded and repeated. The vessel was brought back to atmospheric pressure by opening valve *A* slightly and watching the gauge. Typically, repressurization took a few minutes. Once atmospheric pressure was achieved the machine bolts were loosened and the top plate was removed.

Immediately upon removal, the top of the chamber was exposed to the atmosphere. To reduce evaporative losses in the droplets the chamber opening was covered with an inverted beaker fitted with two or three layers of Parafilm. Individual droplets were recovered in a set order from the microbridges with a micropipette and transferred to the prism of a Bausch and Lomb Abbé 3L refractometer. Immediately upon recovery of a droplet the Parafilmed beaker was replaced over the chamber opening. The refractive index of the droplet was recorded, the prism was cleaned and dried, and the next droplet was retrieved. After all 12 droplets were retrieved the refractive index of the reservoir solution was recorded, the old boat was removed, a fresh boat was inserted, droplets were deposited, and the chamber was sealed for the next experiment.

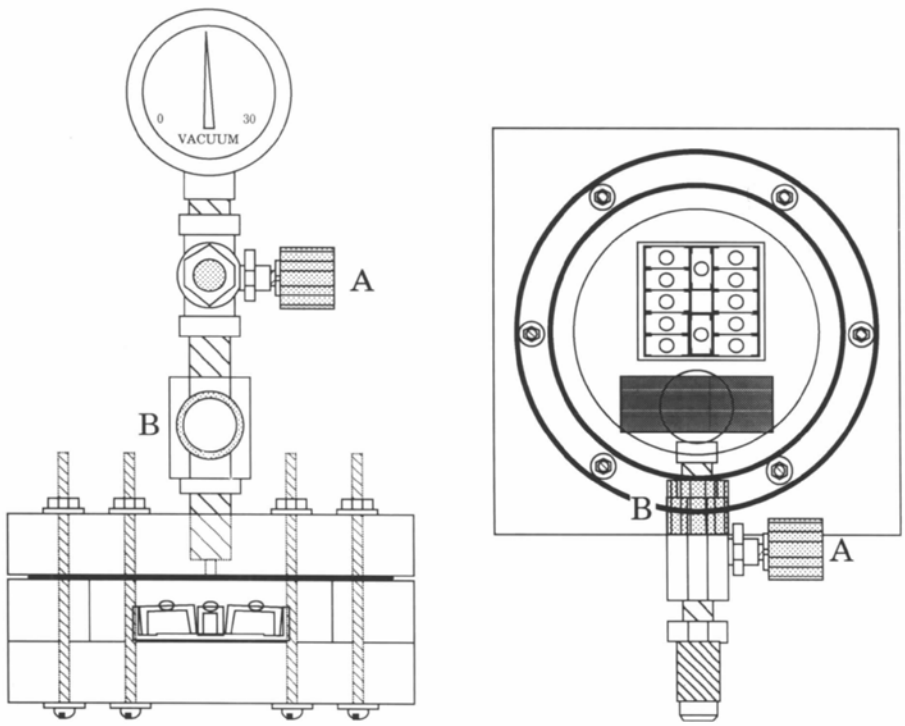

Fig. 2. The low-pressure crystallization vessels (LPCV's) used in these studies. On the left is a side view showing the in-line needle valves *A* and *B* and the vacuum gauge between them. Machine bolts and O-rings secure the top plate to the base housing the vapor chamber. On the right is a top view showing the hexagonal array of machine bolts and the dual O-ring seal. Also shown is the plastic boat that holds a dozen microbridges and, to the front, the welder's fitting through which the vacuum chamber is evacuated.

Droplet and reservoir solutions of 10 and 20%(*w/v*) PEG 8000, respectively, were made in 200 ml quantities to insure that starting concentrations were accurately known. In particular, droplets were not constituted in the manner typically employed in crystal growth experiments; that is, they were not formed by diluting 12 µl aliquots of the reservoir solution with equal volume aliquots of distilled water. The refractive indices of the droplet and reservoir solutions agreed with those found in earlier studies (Arakali, Luft & DeTitta, 1995), allowing us to use our previously determined calibration chart, relating the concentration of PEG to the refractive index, in these studies as well. Solutions were made using PEG 8000 from Fluka and distilled deionized (Barnstead NANOpure II, $>17\,M\Omega\,cm$) but not degassed water. Experiments with 24 µl droplets were set up and allowed to equilibrate for one, four, five or seven days at pressures ranging from ~80 to ~760 mm Hg.

Results

A pattern emerged as the refractive indices of the 12 droplets were recorded in a standard order indicated by the numbers assigned to the microbridges, Fig. 3. The agreement among the refractive indices for the droplets in bridges 2, 3, 4, 6, 7, 9, 10 and 11 was typically very good, usually to 0.0003 units on a refractometer with a scale marked off in 0.0005 increments. However, readings for droplets in bridges 1, 5, 8 and 12 were often quite a bit higher, indicating that the droplets at the four corners were equilibrating more quickly than the droplets in the remaining bridges. The effect was sizable, translating into a difference in concentration of up to 1%(*w/v*). We traced the effect to the ridges present in the bottoms of the boats. Apparently, tipping the microbridges that supported the droplets affected the evaporation kinetics in some fashion, either by the reorientation outwards of the cone of evaporation (the cone of exit routes for a molecule of water at the surface of the droplet) or by the formation of a very thin layer of droplet solution close to the edge of the depression in the microbridge. In any case, we chose to defer further investigations into this effect and report the kinetics of water-vapor equilibration based on the results from the eight droplets not located at the corners of the boat. It is, however, sobering to note that such seemingly minor variations in the set up of a crystal growth experiment can have discernible kinetic consequences. The refractive indices of the droplets from microbridges 2–4, 6, 7, 9–11 were averaged and the calibration chart of Arakali *et al.* (1995) was used to convert the average refractive index to an average PEG concentration. The average values of the PEG concentration in a droplet for the four time intervals and the seven pressures examined are given in Table 1 and are shown in Fig. 4. It is immediately obvious that, over the entire range of pressures examined, a reduction in residual pressure led to an increase in the rate of water-vapor equilibration. It should also be clear that, for the most part, the equilibrations were incomplete, even after seven days. The only droplets to reach full equilibration $\{\langle[PEG]^{drop}\rangle = 20.2\%(w/v)$; $[PEG]^{reservoir} = 20.0\%(w/v)\}$ were those allowed to equilibrate at 80 mm Hg for seven days.

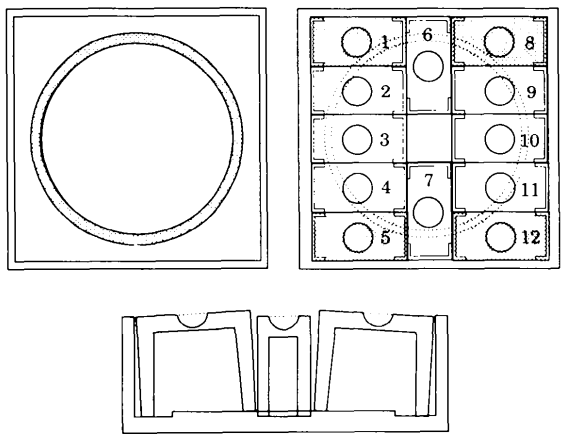

Fig. 3. The microbridge boat. On the top left is the empty boat showing the raised, circular ridge. On the top right is the boat filled with a dozen microbridges. Bridges at the four corners (shaded) held droplets that equilibrated more quickly than droplets in the remaining bridges. On the bottom middle is the boat viewed from the side, showing the bridges at the corners tipped outwards.

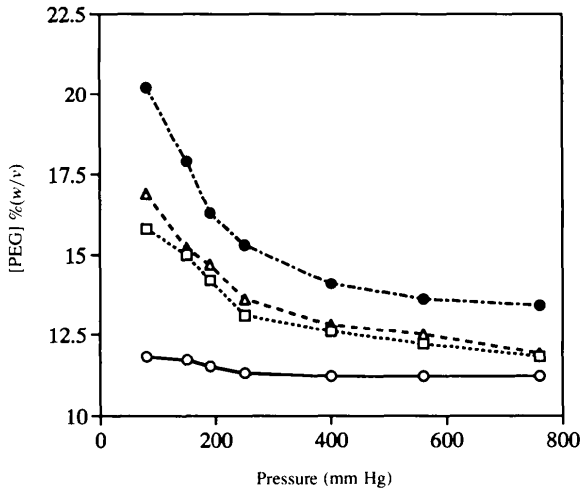

Fig. 4. Time courses of equilibration as a function of residual pressure in the vapor chamber of the LPCV's. Sitting droplets (24 µl) of 10%(*w/v*) PEG 8000 were allowed to equilibrate with reservoirs (20 ml) of 20%(*w/v*) PEG 8000 at room temperature (~298 K). Equilibrations were halted after (○) 1, (□) 4, (△) 5 and (●) 7 days. Droplets were retrieved and their refractive indices were recorded. The average refractive index for the eight droplets not at the corners of the boat, Fig. 3, was converted to an average PEG concentration using the calibration chart of Arakali *et al.* (1995). Shown is the average concentration of PEG in the droplet as a function of pressure for the four time intervals. Data used to compose the figure are given in Table 1.

Table 1. *Concentrations of PEG 8000 in the droplet as a function of time and residual pressure in the LPCV*

Concentration of PEG 8000 in %(*w*/*v*). Initial droplet volume and concentration are 25 µl 10%(*w*/*v*), respectively. Reservoir volume and concentration are 20 ml and 20%(*w*/*v*), respectively. Equilibrations in a sitting-drop arrangement were conducted at room temperature (~ 298 K).

	Pressure (mm Hg)						
Time (d)	80	150	190	250	400	560	760
1	11.8	11.7	11.5	11.3	11.2	11.2	11.2
4	15.8	15.0	14.2	13.1	12.6	12.2	11.8
5	16.9	15.2	14.7	13.6	12.8	12.5	11.9
7	20.2	17.9	16.3	15.3	14.1	13.6	13.4

Discussion

The kinetics of water-vapor equilibration in a sitting-drop experiment are clearly sensitive to the residual pressure of air in the vapor space. Even to ~80 mm Hg a reduction in the residual pressure leads to an increase in the rate of equilibration, Fig. 4. These results are consistent with the fundamental assumption of Fowlis *et al.* that step III is rate limiting and we go on to analyze our results based on that assumption. Conceivably, step III could have been rate limiting at atmospheric pressure but, as the pressure was reduced, some other process insensitive to pressure could have become rate limiting. There is, however, no evidence of a plateau in the rate of equilibration as the pressure is reduced, which would have indicated that another kinetic process had become dominant. On the contrary, it appears that there is an acceleration in the equilibration process at the lowest pressures examined. This was at first unexpected since the diffusion coefficient D is inversely (and linearly) proportional to the residual pressure p. However, the disagreement with predictions based on the kinetic molecular theory of gases is only an apparent one. In the Chapman–Enskog theory of gaseous diffusion, as discussed by Cussler (1984), the diffusion coefficient for a binary system is given by,

$$D = [K(T)^{3/2}(1/M_1 + 1/M_2)^{1/2}]/(p\sigma_{12}^2\Omega_{12}),$$

where T is the temperature, M_1 and M_2 are the molar masses of the two constituents, p is the residual pressure, σ_{12} is the collision diameter for the two constituents, and Ω_{12} is the so-called collision integral, a dimensionless quantity that describes the energetics of interaction between the two constituents. K is a constant of proportionality that takes on the value 1.86×10^{-3} when T is in K, p is in atmospheres, σ_{12} is in Å, and D is in $cm^2\ s^{-1}$. The collision diameter is taken as the average of the individual molecular diameters, $\sigma_{12} = (\sigma_1 + \sigma_2)/2$. Cussler (1984) gives values of σ for air, 3.711 Å, and water, 2.641 Å, and molar masses of ~29 Da for air (79% N_2, 21% O_2) and ~18 Da for water. As the residual pressure is reduced in the vapor chamber of the LPCV the absolute contribution of water vapor to the total pressure remains constant, at approximately the vapor pressure of pure water, ~25 mm Hg at 298 K. On the other hand, its relative contribution to the total pressure increases dramatically. At ~760 mm Hg residual pressure the contribution of water vapor represents only about 3% of the total; at ~80 mm Hg that contribution is more than 30% of the total. The diffusion process changes from one in which collisions of water molecules from the droplet with air molecules are all important to one in which collisions of water molecules from the droplet with other water molecules becomes an important process. We should expect the diffusion coefficient to reflect the varying nature of the collision processes. In the expression for D let M_1 and σ_1 represent water vapor from the droplet and let M_2 and σ_2 represent the vapor in the vapor chamber. Clearly M_2 and σ_2 should take on values close to those for air at atmospheric pressures but should take on values reflective of a much more water-laden atmosphere at low pressures. The trends would be for M_2 to go from ~29 Da towards ~18 Da and σ_2 to go from 3.711 Å towards 2.641 Å. Thus, the term $1/M_2$ in the numerator and the term σ_{12} in the denominator should become larger and smaller, respectively, leading to an increase in D because of the enrichment of the vapor mixture with water. This is an increase in D over and above that due to the reduction in the pressure *per se*, and qualitatively explains the acceleration of the equilibration process we observe at low pressures.

These experiments were undertaken with pure PEG solutions in both the droplet and reservoir. We recently demonstrated that, at atmospheric pressures and room temperatures, vapor equilibrations involving pure PEG solutions at concentrations of relevance to the macromolecular crystallization problem are very slow (Luft & DeTitta, 1995). In a traditional sitting-drop arrangement employing Linbro plate reservoirs and microbridges, a 24 µl droplet of 10%(*w*/*v*) PEG 8000 takes three weeks to equilibrate with a 20%(*w*/*v*) PEG 8000 reservoir, at 293 K and atmospheric pressure. Equilibrations in the LPCV using the same drop volumes and PEG concentrations, although at slightly higher temperatures, are complete at ~80 mm Hg after one week. Thus, equilibrations at low pressure make crystallization experiments that must be concluded within a short time frame, such as when protein stability is an issue or on microgravity missions, feasible even with pure PEG solutions.

The rate of equilibration in a vapor-diffusion crystallization experiment can have a significant effect on its outcome. Various methodologies have been described over the last few years that are specifically directed at control of the kinetic aspects of the equilibration process. At one extreme are simple, passive devices such as the *Z*/3 crystallization plate (Luft *et al.*, 1994; Arakali, Easley, Luft & DeTitta, 1994) and gel acupuncture

(García-Ruiz & Moreno, 1994). These allow the crystal grower to sample various rates of equilibration, ranging from slow to fast, in order to find an optimal one, but courses of equilibration are restricted to ones that are monotonically increasing. At the other extreme are complex, active devices such as the 'pseudo-reservoir' apparatus of Wilson, Bray & Suddath (1991). These allow the crystal grower complete freedom in the design of a course of equilibration, including the ability to halt or even reverse the equilibration process if so desired. Our results with the LPCV suggest an intermediate approach, one designed to incorporate much of the simplicity of a passive device and much of the flexibility of an active device. By fitting out a vapor chamber with plumbing for two ballast tanks, one at low pressure and the other at high pressure, it should be possible to design a course of equilibration that includes periods where the equilibration is fast or is slow, or is even effectively halted. Although such a device would not allow the crystal grower to reverse the equilibration process, its added flexibility would be at the cost of a pair of valves and a pair of ballast tanks.

Our results are fully in line with the fundamental assumption of Fowlis *et al.* that transit across the vapor space constitutes the rate-limiting step in vapor-diffusion experiments. However, this assumption can be further tested because it demands that the rate of equilibration depend on the distance from the droplet to the reservoir surface. In an accompanying paper (Luft, Albright, Baird & DeTitta, 1996) we will describe the relationship between the rate of water-vapor equilibration, in hanging-drop experiments, and the distance from drop to reservoir.

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