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Chaperone Salts, Polyethylene Glycol and Rates of Equilibration in Vapor-Diffusion Crystallization

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Abstract

The kinetics of water-vapor equilibration in macromolecular crystallization were investigated for sitting droplets of aqueous polyethylene glycol (PEG) 8000 as a function of concentration. Equilibrations, set up with initial concentrations of PEG in the droplet at half those in the reservoir, were very slow for concentrations of relevance to the macromolecular crystal growth problem. At 301 K, 24 μl droplets at initial concentrations of 2.5, 5.0 and 7.5%(*w*/*v*) PEG require 12, 5, and 3 weeks to reach equilibrium, respectively. On the other hand, the addition of modest quantities of sodium chloride to both droplet and reservoir increases the rate of equilibration for aqueous PEG sitting droplets significantly. At 293 K, droplets with initial volumes of 24 μl and PEG concentrations of 5%(*w*/*v*) require 12 weeks to reach equilibrium, while droplets of the same volume and initial concentrations of 5%(*w*/*v*) PEG and 200 m*M* NaCl require less than two weeks to reach equilibrium. The slow vapor-diffusion equilibrations of pure PEG solutions, and the subsequent increase in these rates with colligative agents such as salt, are a consequence of the non-ideality of aqueous PEG solutions. These results are of interest both from a practical and a theoretical viewpoint. They underscore the importance of kinetic factors in macromolecular crystal growth, help to explain apparent inconsistencies of outcome in PEG-mediated crystallizations, and yield another methodology for the optimization of crystal growth conditions, namely the control of the kinetics of equilibration using colligative agents.

Introduction

Since its adaptation to the microscale by Hampel *et al.* (1968), the vapor-diffusion method has become an important tool for macromolecular crystal growth (Ducruix & Giegé, 1992). In the method a droplet containing the macromolecule and a crystallizing agent is allowed to equilibrate with a reservoir containing a dehydrating agent. Equilibrium is achieved as water, in the form of vapor, leaves the droplet, traverses the vapor space, and enters the reservoir. As water leaves, the concentrations of both the macromolecule and the crystallizing agent increase. In the favorable case conditions evolve within the droplet such that nucleation and crystal growth ensue.

Various classes of chemicals have been used successfully as crystallizing agents. Salts such as ammonium sulfate and sodium chloride are quite commonly used, as are the polyethylene glycols (PEG). The advantages of PEG have been discussed by McPherson (1985). We have recently described experiments that measured concentrations of PEG and of salts that are in vapor-pressure equilibrium in a sitting-drop arrangement (Arakali, Luft & DeTitta, 1995). We found that, in concentrations useful for crystallization experiments, PEG has a very modest colligative effect on the vapor pressure of water. We also found that graphs of [PEG] *versus* [salt] that are in vapor-pressure equilibrium are highly non-linear over the entire range of concentrations examined. Those observations suggested important kinetic consequences in vapor-diffusion experiments utilizing PEG as the crystallizing agent. In particular, we anticipated interesting concentration-dependent effects on the rates of water equilibration when PEG was involved. Here we report rates of water equilibration in a sitting-drop arrangement as a function of PEG concentration in the reservoir. We will show that at concentrations relevant to the crystallization problem water equilibrations involving PEG are very slow. We will then go on to show that the rates of equilibration can be greatly increased by the inclusion of salts, at very modest concentrations, in both droplet and reservoir. The salts, by virtue of their enhanced osmotic properties, guide the course of the equilibrations and act as chaperones to insure that PEG concentrations in the droplet reach desired values at specified times. Even when salts are present in concentrations too low to have a significant effect on macromolecular solubility *per se*, they can have an important kinetic effect on the vapor-diffusion process.

Experiments

All of the experiments were carried out in a sitting-drop geometry using Linbro plates and microbridges from Hampton Research. Solutions were prepared using PEG

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8000 from Fluka and sodium chloride from Sigma. Each reservoir solution was prepared by weighing out solute and diluting to an appropriate volume with distilled deionized (Barnstead NANOpure II; $>17\ M\Omega$ cm) but not degassed water. Each droplet solution was either similarly prepared (experiment I) or prepared by one-to-one dilution (experiments II and III) of 50 ml quantities of the corresponding reservoir solution. Droplet volumes were uniformly 24 µl. In particular, droplets were not constituted by adding 12 µl aliquots of water to 12 µl aliquots of reservoir solution, as is common practice in setting up crystallization experiments. This was to minimize the uncertainty concerning the concentration of solutes at the start of the experiments. PEG concentrations are expressed as %(*w/v*), a unit related to molarity; NaCl concentrations are in m*M*. In their analysis of the hanging-drop experiment Mikol, Rodeau & Giegé (1990) observed that the presence of protein had no measurable effect on the rate of water equilibration. Therefore, in our experiments we have restricted the colligative agents present in droplet and reservoir to PEG and salt.

Experiments were set up by introducing a 1 ml aliquot of reservoir solution, inserting a microbridge, depositing a droplet in the microbridge depression, and sealing the well with clear plastic label tape using a HANGMAN jig (Luft & DeTitta, 1992). Typically two rows of wells, with six wells per row, were set up at a time. All of the reservoir solutions were added first, then the microbridges, then the droplets.

Experiments were read on a regular basis. Droplets were retrieved by making a small incision in the label tape directly over the microbridge depression and inserting a micropipette tip. The droplet was immediately transferred to the prism of a Bausch and Lomb Abbé 3L refractometer where its refractive index was recorded. Calibration charts were used to convert refractive index values into PEG or PEG/salt concentrations. Calibrations using known concentrations of solutes were made for each reservoir solution.

The experiments were conducted either at 301 K in a warm-temperature room (experiment I) or at 293 K in a constant-temperature incubator with a nominal tolerance of 0.1 K. Originally the plan was to conduct all the experiments at 293 K but preliminary studies with pure PEG solutions at low concentrations, for the purposes of establishing an optimal sampling rate, suggested that more reliable measurements would be made at elevated temperatures. In part this was because equilibrations with pure PEG were very slow, and it would be easier to demonstrate the concentration dependence more clearly if the equilibration rates could be measured more accurately. But more particularly we encountered difficulties at 293 K with water-vapor condensation in wells containing pure PEG at low concentration. Apparently the constant temperature incubator introduces cooled air into the top of its chamber, causing a very slight undercooling of the tops of the Linbro plates with respect to their bottoms. Because PEG has such a modest colligative effect on aqueous solutions even the slightest thermal gradient causes distillation of water. Although the droplet and reservoir solutions themselves were likely in very good thermal contact, as the legs of the microbridge that supports the droplet are immersed directly in the reservoir solutions, the presence of visible water droplets on the underside of the tape seal was considered unacceptable. We later learned that, even at low PEG concentrations, condensation problems could be mitigated by simply stacking a few blank plates over the tops of the equilibration plates. It also became clear that temperature stability could be improved by insuring that most of the incubator volume was filled with experiments. Although there was a bit of fogging in the reservoir containing pure PEG in experiment III*a*, the condition was temporary, and in those experiments there were never any visible droplets of water on the tape surfaces.

Experiment I

Vapor-diffusion equilibrations with pure PEG solutions in droplet and reservoir were conducted at 301 K. Reservoir concentrations were 5, 10 and 15%(*w/v*) PEG; initial droplet concentrations were 2.5, 5.0 and 7.5%(*w/v*) PEG, respectively. The reservoir concentrations were chosen as representative of conditions most frequently reported as leading to successful crystal growth outcomes in the NIST/CARB/NASA Biological Macromolecule Crystallization Database (Gilliland & Bickham, 1990), as surveyed by Cudney (1994). Individual droplets were harvested daily for 12 d and thereafter less frequently for an additional 13 d. Concentrations in the droplets as a function of time are shown in Fig. 1.

Experiment II

Vapor-diffusion equilibrations with fixed initial concentrations of PEG and varying initial concentrations of NaCl, in droplet and reservoir, were conducted at 293 K. Reservoir concentrations were uniformly 20%(*w/v*) PEG and initial droplet concentrations were uniformly 10%(*w/v*) PEG. Four conditions with varying concentrations of NaCl were tested. Reservoir concentrations were 0, 100, 200, and 400 m*M* NaCl; initial droplet concentrations were 0, 50, 100, and 200 m*M* NaCl, respectively. Six droplets for each of the four conditions were harvested approximately every two days. Concentrations in the droplets, averaged over the six replicates, as a function of time are shown in Fig. 2. Although the concentrations are expressed as a PEG concentration it should be clear that there are varying amounts of NaCl in the droplets as well. As the

concentrations of the cosolutes vary in a one-to-one ratio the corresponding NaCl concentrations as a function of time can be calculated in a straightforward fashion.

Experiment III

As in the previous experiment, fixed initial concentrations of PEG and varying initial concentrations of NaCl, in droplet and reservoir, were conducted at 293 K. Reservoir concentrations were uniformly 10%(*w/v*) PEG and initial droplet concentrations were 5%(*w/v*) PEG. Four conditions, with the same varying concentrations of NaCl in droplet and reservoir as employed in the previous experiment, were tested. Again, six droplets for each of the four conditions were harvested approximately every two days and averaged. Concentrations in the droplet as a function of time are shown in Fig. 3. Again, they are expressed as a PEG concentration but there are varying concentrations of NaCl in the droplet as well.

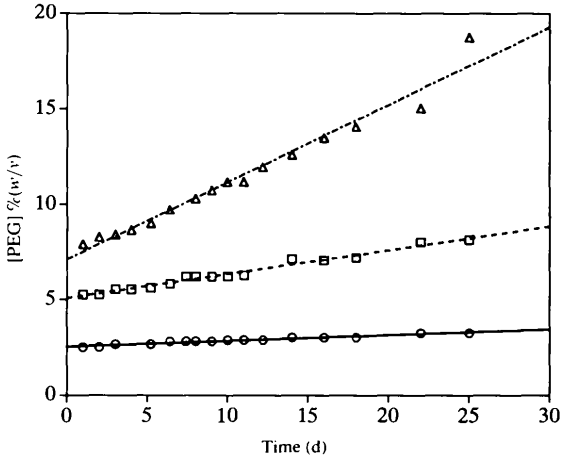

Fig. 1. Concentration of PEG in the droplet as a function of time for equilibrations involving pure aqueous PEG 8000 solutions, experiment I. The equilibrations were carried out at 301 K using 24 µl sitting droplets and Linbro plate reservoirs. Reservoir concentrations were (○) 5%(*w/v*), (□) 10%(*w/v*), and (△) 15%(*w/v*) PEG. Initial droplet concentrations were half those of their respective reservoirs. Each point represents a single observation.

Results

Each time course, Figs. 1–3, was analyzed to estimate an initial rate of equilibration and a time to equilibrium, Table 1. Rates of equilibration were estimated visually from the linearly ascending portions of the time-course curves. They are expressed as a change in PEG concentration, in %(*w/v*) PEG, per day. Times to equilibrium could be estimated visually for those experiments that had continued to completion. For those that had not, it was estimated from the expected change in concentration at equilibrium and the rate of equilibration assuming a linear time course. Times to equilibrium are measured in days.

Discussion

It is not unusual to find salts present in vapor-diffusion crystal growth experiments that employ PEG as a crys-

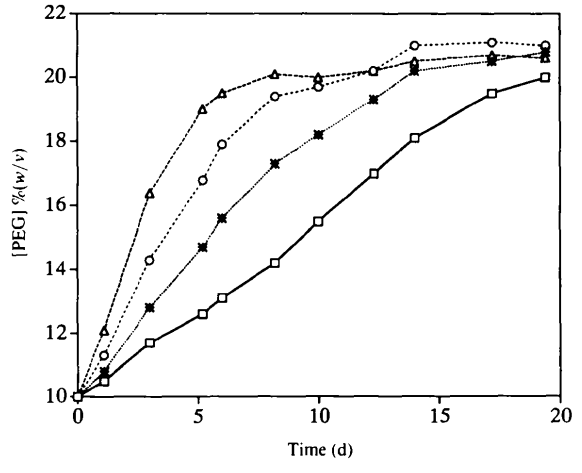

Fig. 2. Concentration of PEG in the droplet as a function of time for equilibrations involving PEG solutions with varying quantities of sodium chloride, experiment II. The equilibrations were carried out at 293 K using 24 µl sitting droplets and Linbro plate reservoirs. All reservoir concentrations were 20%(*w/v*) PEG 8000, plus (□) 0 m*M*, (*) 100 m*M*, (○) 200 m*M*, and (△) 400 m*M* NaCl. Initial droplet concentrations were half those of their respective reservoirs; *e.g.* (*) 10%(*w/v*) PEG, 50 m*M* NaCl. Each point is the average of six observations.

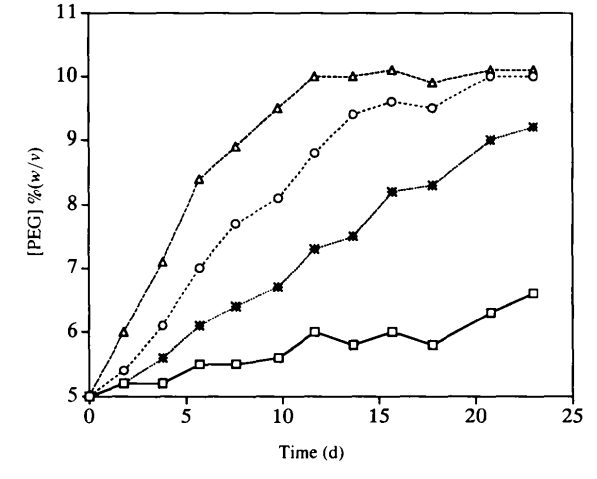

Fig. 3. Concentration of PEG in the droplet as a function of time for equilibrations involving PEG solutions with varying quantities of sodium chloride, experiment III. The equilibrations were carried out at 293 K using 24 µl sitting droplets and Linbro plate reservoirs. All reservoir concentrations were 10%(*w/v*) PEG 8000, plus (□) 0 m*M*, (p) 100 m*M*, (○) 200 m*M*, and (△)400 m*M* NaCl. Initial droplet concentrations were half those of their respective reservoirs; *e.g.* (△) 5%(*w/v*) PEG, 200 m*M* NaCl. Each point is the average of six observations. Figs. 2 and 3 differ in the concentrations of PEG but represent common NaCl concentrations.

Table 1. *Initial conditions and results for experiments* I, II *and* III

Experiments with 24 µl sitting drops and 1 ml reservoirs. Experiment I at 301 K, experiments II and III at 293 K. [PEG] in %(*w*/*v*) PEG 8000; [NaCl] in m*M*. Droplet concentrations are at the beginning of the equilibrations. Δ[PEG]/Δt is the rate of equilibration expressed in %(*w*/*v*) PEG 8000 per day. t_{eq} is the observed time in days to equilibration for experiments that went to completion and is the estimated time to completion for experiments that had not gone to completion by the end of the time course. In the latter case t_{eq} is calculated from $\{[PEG]_{reservoir}-[PEG]_{droplet}\}/\{\Delta[PEG]/\Delta t\}$.

	Droplet		Reservoir			
	[PEG] [%(*w*/*v*)]	[NaCl] (m*M*)	[PEG] [%(*w*/*v*)]	[NaCl] (m*M*)	Δ[PEG]/Δt	t_{eq} (d)
I*a*	2.5	—	5.0	—	−0.030	83
I*b*	5.0	—	10.0	—	−0.135	37
I*c*	7.5	—	15.0	—	−0.360	21
II*a*	10	—	20	—	−0.60	20
II*b*	10	50	20	100	−1.00	14
II*c*	10	100	20	200	−1.38	10
II*d*	10	200	20	400	−2.13	8
III*a*	5	—	10	—	−0.06	83
III*b*	5	50	10	100	−0.19	26
III*c*	5	100	10	200	−0.35	21
III*d*	5	200	10	400	−0.58	12

tallizing agent. Occasionally they are there to maintain the stability or biological activity of a macromolecule. Frequently they are integral components of buffers. Sometimes they are present in concentrations high enough that they must be regarded as cocrystallizing agents. Quite apart from any intended effect they may have on the crystallization process, it should be clear that salts will have a collateral effect on the kinetics of vapor-diffusion experiments.

Lengthy vapor-diffusion equilibrations are the rule when PEG is the only colligative agent in both droplet and reservoir, Fig. 1. Even at 301 K we predict that 24 µl sitting droplets of 2.5%(*w*/v) PEG will take 12 weeks to fully equilibrate with 5.0%(*w*/*v*) PEG reservoirs. Lengthy equilibrations are a direct consequence of the non-ideality of aqueous PEG solutions (Michel & Kaufmann, 1973). According to the model developed by Fowlis *et al.* (1988), the molar current of water leaving a droplet in a vapor-diffusion experiment is proportional to the difference in the vapor pressure of water directly over the droplet and over the reservoir. Our isopiestic measurements (Arakali *et al.*, 1995) allow us to estimate that difference for PEG droplets equilibrating with PEG reservoirs. The measurements yield concentrations of PEG in a sitting droplet and NaCl in a reservoir that are in water vapor-pressure equilibrium. For example, 10%(*w*/*v*) PEG droplets are in equilibrium with 13 m*M* NaCl reservoirs, as are 20%(*w*/*v*) PEG droplets with 125 m*M* NaCl reservoirs. Sodium chloride is an isopiestic standard; absolute values of the vapor pressure of water are known as a function of the concentration of NaCl (Robinson & Stokes, 1959). We estimate the vapor-pressure difference for a 10%(*w*/*v*) PEG droplet over a 20%(*w*/*v*) PEG reservoir as 0.070 mm Hg at 293 K. In contrast, the vapor-pressure difference between a 1.0 *M* NaCl droplet and 2.0 *M* NaCl reservoir is ~0.72 mm Hg at 293 K, an order of magnitude greater. Thus, the driving force for vapor-diffusion equilibration, which is a maximum at the start of the experiment and which diminishes as the equilibration progresses, is quite small for pure PEG equilibrations.

These considerations also suggest a rationale for the common observation of condensation in hanging- and sitting-drop vapor-diffusion crystallizations involving PEG. Normally the droplet acts as a source and the reservoir as a sink for water vapor. However, when a container surface is undercooled sufficiently that the vapor pressure over it is lower than the vapor pressure over the reservoir, water will condense upon it. For example, in an equilibration involving a 20%(*w*/*v*) PEG reservoir, undercooling of the tape seal by as little as 0.06 K relative to the reservoir at 293 K will cause water vapor to condense on the tape. On the other hand condensation is rarely seen in vapor-diffusion experiments employing salts as the crystallizing and dehydrating agents. For example, in an experiment involving a 2.0 *M* NaCl reservoir the vapor pressure of water over the reservoir is 1.33 mm Hg lower than that over pure water. This would require an undercooling of the sealing tape by more than 1.2 K relative to the reservoir at 293 K before condensation would be evident.

While equilibrations involving pure PEG are slow, the addition of salts, even in modest concentrations, can greatly speed the diffusion process. Thus, at 293 K we observe that a 24 µl sitting droplet of 10%(*w*/*v*) PEG equilibrates with a 20%(*w*/*v*) PEG reservoir in about three weeks, Fig. 2. The presence of 50 m*M* NaCl in the droplet and 100 m*M* NaCl in the reservoir reduces the equilibration time to two weeks, and of 200 m*M* NaCl in the droplet and 400 m*M* NaCl in the reservoir to about one week. The speed-up is even more dramatic for lower PEG concentrations. Thus, we estimate that 24 µl sitting droplets of 5%(*w*/*v*) PEG will equilibrate with 10%(*w*/*v*) PEG reservoirs in 12 weeks, Fig. 3, but the presence of 200 m*M* NaCl in the droplet and 400 m*M* NaCl in the reservoir reduces that time to less than two weeks. Evidently, at low PEG concentrations it is the colligative effect of the salt that primarily determines the rate of water-vapor equilibration. In essence, the PEG in the droplet plays a minor role in the equilibration kinetics, even if it plays the major roll in reducing the solubility of the macromolecule (Atha & Ingham, 1981).

As expected, the concentration dependence of the rate of equilibration, either when PEG is the sole colligative agent or when it appears in combination with NaCl, is a complicated one. Qualitatively, we observe that increasing the concentrations of solutes in the reservoirs while maintaining a constant dilution ratio (the ratio of

the initial concentrations of solutes in the droplets to their concentrations in the reservoirs) increases the rate of equilibration, but the increase is non-linear. Thus, in experiment I the reservoir concentrations are in a ratio of 1.0 (*a*) to 2.0 (*b*) to 3.0 (*c*), but the rates of equilibration are in a ratio of 1.0 (*a*) to 4.5 (*b*) to 12.0 (*c*). Again, this is in line with our observation of the non-linear relationship between concentrations of PEG and concentrations of salt that are in vapor-pressure equilibrium (Arakali *et al.*, 1995). In addition, experiments (II*a*, III*a*), (II*c*, III*b*) and (II*d*, III*c*) represent three pairings of conditions that differ in initial droplet and reservoir concentrations by a factor of two. The ratios of their rates of equilibration are 10.0, 7.2, and 6.1 to 1.0, respectively.

Mikol, Rodeau & Giegé (1990) have thoroughly analyzed the factors that affect the kinetics of water equilibration in a hanging-drop geometry. They suggest that the most important factors are temperature, the droplet volume, the dilution factor, and the nature of the crystallizing agent. In experiment II*a* we duplicated the temperature (293 K), droplet volume (24 µl) and dilution factor (0.5) for experiment E27 of Mikol *et al.* While they employed 20%(*w*/*v*) PEG 6000, mildly buffered with 20 m*M* Bistris/HCl (pH 7.0), as the reservoir solution we employed pure, unbuffered 20%(*w*/*v*) PEG 8000. The presence of buffer in the hanging-drop experiment increased that rate of equilibration slightly. The difference in molecular weight for the two PEG's employed is considered insignificant, as preliminary experiments carried out here with PEG 4000 showed no significant kinetic differences with comparable PEG 8000 experiments when both experiments are carried out with the same weight/volume percentages of PEG. Money (1989) has shown that PEG's over the molecular weight range 1500–10 000 Da have very similar osmotic properties, so our observations of very similar kinetics are not surprising. We calculate, from the analytic function Mikol *et al.* fitted to their kinetic data, a rate of equilibration of –1.25%(*w*/*v*) PEG per day for the hanging-drop experiment, which is about twice the rate we observe for the comparable sitting-drop experiment. Thus, it appears that sitting-drop equilibrations are inherently slower than comparable hanging-drop equilibrations.

These results may help to explain any seeming variability of outcome when PEG is employed as the crystallizing agent in vapor-diffusion experiments. What may be considered insignificant variations in, for example, the buffer capacity in the droplet, or presence of buffer in the reservoir, can now be seen to exert a considerable effect on the kinetics of equilibration. That kinetics can have a major influence on the outcome of a crystal growth experiment has been demonstrated by Feher & Kam (1985). Recently two new protocols for crystal growth that actively control the kinetics of equilibration have been described. In the gel acupuncture method (García-Ruiz & Moreno, 1994) crystals are grown directly in capillaries that have been punctuated into a silica gel bed. Crystallizing agent is layered over the gel bed, through which it diffuses and enters the capillary. In the *Z*/3 crystallization plate (Luft *et al.*, 1994; Arakali, Easley, Luft & DeTitta, 1994) reservoirs of varying depths chosen in a progression in d^2 are employed as diffusion cells. The diffusion of dehydrating agent from the reservoir bottom to its top causes the reservoir surface conditions to evolve in a controlled manner.

In his analysis of the advantages of PEG as a crystallizing agent McPherson (1985) observed that, compared to salts, PEG crystallizations succeed over a narrower concentration range (4–18%), are quicker, and are less demanding with regard to locating an optimal concentration of crystallizing agent. He noted that often crystals of macromolecules appear within hours or days of setup, and that PEG concentrations within 2 or 3%(*w*/*v*) of optimal value were frequently successful. These observations are seemingly at odds with ours concerning the lengthy equilibrations for PEG in the useful concentration range, and with those of Atha & Ingham (1981) who found that the solubility of proteins is quite sensitive to small changes in PEG concentrations. For example, the solubility of human serum albumin in PEG 4000 solutions is reduced by an order of magnitude when the PEG concentration is increased by 4%(*w*/*v*). Together, those observations suggest that successful crystallization experiments employing PEG in low concentrations as the crystallizing agent and the hanging- or sitting-drop vapor-diffusion method as the technique are, appearances notwithstanding, 'microbatch' in nature rather than 'vapor-diffusion' experiments in the accepted sense of the term.

The presence of salts in PEG-mediated vapor-diffusion crystallizations has been shown to exert a strong effect on the kinetics of water-vapor equilibration. From a practical point of view, this information can be used in a variety of ways. If nothing else, it should serve as a warning that even minor changes in the experimental conditions can have major effects on the outcome of an experiment. It may also clarify seeming inconsistencies in optimal 'vapor-diffusion' and 'microbatch' conditions for crystallizations. Finally, it offers the crystal grower another active strategy for optimizing crystallizations *via* kinetic control of nucleation and growth processes.

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