Vapor diffusion, nucleation rates and the reservoir to crystallization volume ratio

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In a classical vapor diffusion crystallization, the protein solution is mixed in a 1:1 ratio with the reservoir solution, containing one or more precipitant species, after which the two are placed in an enclosed chamber. As the vapor pressure is lower for the reservoir solution, due to its higher solute concentration, there is a net transfer of water through the vapor phase from the protein droplet to the reservoir. In theory, the initial conditions in the droplet are such that the protein is in either a metastable or undersaturated state with respect to crystal nucleation. The loss of water serves to both concentrate the protein and the precipitant concentrations within the drop, bringing the protein past the metastable point to nucleation. The equilibration rate is a function of the precipitant(s) used, their concentration, the temperature, the distance between the two surfaces, and the droplet to reservoir volume ratio. For a given reservoir volume smaller droplets equilibrate faster, the rate being inversely linear with the droplet volume. In attempts to maximize the number of crystallization trials, and as crystals in the 100 – 200 μm size range are sufficient, it has currently become standard practice to use starting droplet volumes of 2 - 4 μl, with reservoir volumes typically in the 200 to 500 μl range. The equilibration rates are maximized, and for most common salt concentrations and higher concentrations of polyethylene glycol (PEG) and 2-methyl-2,4-pentanediol (MPD) one can reasonably estimate that equilibration has occurred within 3 to 6 days at room temperature. Crystals appearing after this time are essentially grown under batch conditions. We experimentally find that altering the reservoir to droplet volume ratio, by changing the reservoir volume, from 50:1 (high ratio) to 5:1 (low ratio), on average increases the equilibration time by approximately 50 % when tested with solutions of 50% MPD, 1.5 M NaCl, or 30 % PEG400. However, experiments with two proteins, chicken egg white lysozyme and concanavalin a, showed an unexpected trend of slightly faster nucleation and larger crystals in the lowest ratio experiments.

Keywords: vapor diffusion; nucleation kinetics; lysozyme; concanavalin a; equilibration rate

1. Introduction

Vapor diffusion is the most commonly employed method for growing crystals of macromolecules for X-ray structure determination (Hampel *et al.*, 1968; McPherson, 1982; Ducruix & Giegé, 1992). The basic experimental procedure is to mix equal volume aliquots of the macromolecule solution with a reservoir solution that is at the condition (pH, precipitant, etc.) of interest. The mixed solution is then either placed on a pedestal or suspended from a coverslip or other surface in an enclosed volume with the reservoir of precipitant solution. The reservoir solution, having a higher solute concentration, has a lower vapor pressure than the macromolecule solution droplet. Because of this, water vapor leaving the crystallization droplet will be preferentially readsorbed by the reservoir solution, resulting in a net transfer of water to the reservoir until the two liquid bodies have equivalent vapor pressures. This process results in a progressive increase in the precipitant and solute concentrations in the crystallization drop, hopefully leading to the desolubilization of the macromolecule in a crystalline form.

Fowlis *et al.* (1988) modeled the vapor diffusion process and determined that the rate limiting step was the diffusion of water vapor from the crystallization droplet to the reservoir surface. They recognized that gradients within the droplet and reservoir, generated by water leaving and adding on respectively, could also affect the net equilibration rates. However, they suggested that the droplet convective flows would serve to mix the solution and minimize these gradients. Sibille *et al.* (1991) subsequently showed that convective mixing had little effect on the net equilibration rate.

The vapor diffusion equilibration rate is dependent upon a number of factors, the most important of these being the nature and concentration(s) of the solutes present (Mikol *et al.*, 1990), the distance from the droplet to the reservoir (Luft *et al.*, 1996), the droplet size (Mikol *et al.*, 1990), and the experimental geometry, for example whether a sitting or hanging drop (Luft & DeTitta, 1995). The air pressure within the vapor space has also been shown to affect the equilibration rate (DeTitta & Luft, 1995). All of these, save possibly the last, are experimentally optimized (solution composition) or controllable parameters. Diller & Hol (1999) developed a numerical model for the vapor diffusion process, the results of which are in close agreement with the available experimental data to that time. The model showed that there was an approximately linear dependence of the equilibration time with the droplet to reservoir distance and upon the initial droplet size.

Herein, we have returned to the vapor diffusion equilibration process. Initially this was in an effort to understand the effects in comparison to the vapor diffusion process within current and proposed future microgravity flight hardware, such as a capillary tube. Geometric constraints with this approach naturally lead to rather low reservoir to droplet volume ratios. Subsequently, we focused on the effects of the crystallization drop to reservoir volume ratio in a standard sitting drop system, the results of which are reported herein.

2. Materials and methods

Chicken egg white lysozyme was obtained from Sigma Chemical Co. (St. Louis, MO) and repurified by cation exchange chromatography as previously described (Ewing *et al.*, 1996). Concanavalin A was purified from Jack Bean (Agrawal & Goldstein, 1967) and demetallized (Olson & Liener, 1967) as previously described (Cacioppo & Pusey, 1992). Ammonium sulfate (AmS), 2-methyl-2,4- pentanediol (MPD), polyethylene glycol's (PEG) 400, 1000, and 8000, and sodium chloride were all obtained from Sigma Chemical Co. (St. Louis, MO) as reagent grade or better. 4-nitroaniline (pna), a non-reactive chromophore, was obtained from Aldrich Chemical Co. and recrystallized from ethanol prior to use.

Stock precipitant solutions, without added buffer or azide, were made up in bulk for each precipitant concentration. Equilibration rate determinations were set up by premixing equal volumes of the stock precipitant solution and pna (3.6 mM), then dispensing the precipitant alone into the wells and the mixed solution into the sitting drop pedestal. The measurements were made in Cryschem 24-well sitting drop plates, with 12 duplicate wells for each unique precipitant concentration. In all cases the initial drop size was 20 μl. The plates were sealed with clear tape and stored in an incubator maintained at 20 °C. At periodic intervals, assay of the equilibration rate was performed by opening one well and carefully pipetting aliquots of the pedestal solution into 500 μl of distilled water.

Table 1 Estimated equilibration times for several commonly employed precipitants at 20°C. These times are estimated from linear fits to the initial equilibration rate data.

Precipitant	Concentration	Days to 90% of Initial Reservoir Concentration	Precipitant	Concentration	Days to 90% of Initial Reservoir Concentration
MPD	80% (v:v)	0.9	PEG 8000	25% (w:v)	12
	70% (v:v)	2.0		20% (w:v)	18
	60% (v:v)	2.1		15% (w:v)	52
	50% (v:v)	2.4		10% (w:v)	167
	40% (v:v)	2.6	PEG 1000	40% (w:v)	2.2
	30% (v:v)	3.1		30% (w:v)	3.9
	20% (v:v)	5.9		20% (w:v)	12
NaCl	2.0M	2.5		10% (w:v)	80
	1.5M	3.8	PEG 400	40% (v:v)	1.2
	1.0M	6.2		30% (v:v)	2.7
	0.5M	16		20% (v:v)	7.7
AmS	1.5M	4.1		10% (v:v)	33
	1.0M	5.7			
	0.5M	15			

Typically, three aliquots were made from each pedestal solution assayed. The dye concentration was measured by absorbance at 381 nm after correcting for dilution. Increasing intensity of the dye absorbance corresponded to shrinkage of the drop due to equilibration with the reservoir.

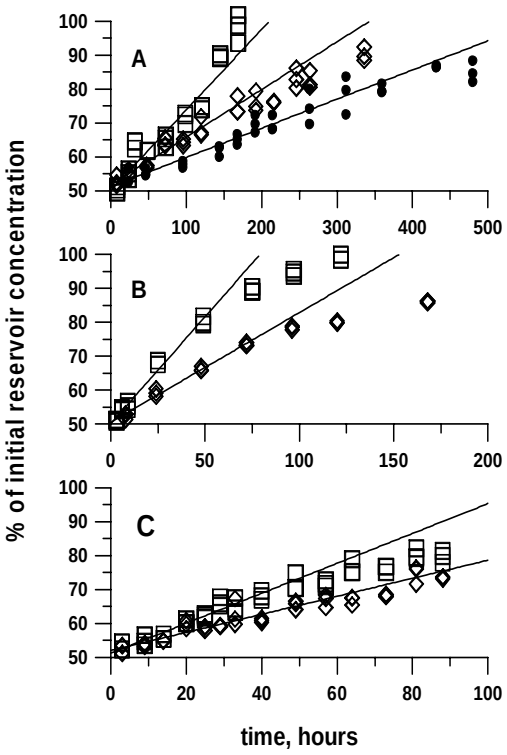

Figure 1
Equilibration data for monocomponent solutions of several macromolecule precipitants. Linear fits for each set are determined from the initial data and not the entire set. Panel A: □, 20% PEG 400; ◊, 20% PEG 1000; •, 20% PEG 8000. Panel B: □, 40% MPD; ◊, 20% MPD. Panel C: □, 1.5 M ammonium sulfate; ◊, 1.0 M ammonium sulfate.

Lysozyme sitting drop crystallizations were set up with 0.1 M sodium acetate, 4% or 5% NaCl (w:v), pH 4.6, using protein concentrations of 50 and 40 mg/ml. Demetallized concanavalin A

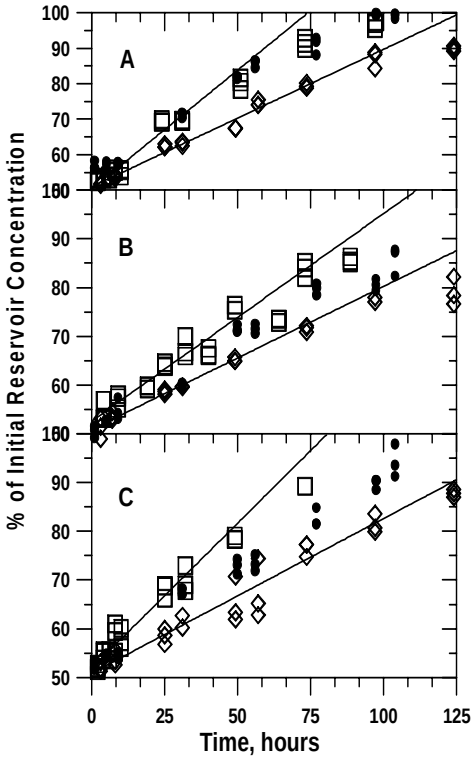

Figure 2
The effect of varying the reservoir to droplet volume ratio by holding the droplet volume constant and changing the reservoir volume on the equilibration rates of monocomponent precipitant solutions. Legend: volume ratios, reservoir to droplet, for all panels, □, 50:1 ratio; •, 25:1 ratio; ◊, 5:1 ratio. Panel A, 50 % MPD in reservoir; Panel B, 1.5M NaCl in reservoir; Panel C, 30 % PEG400 in reservoir. All experiments were at 20 °C. Linear fits are only drawn for the 50:1 and 5:1 ratio data for all panels.

Table 2 Summary of lysozyme hanging drop experiments with variable reservoir:drop volume ratio's. Drops were made from a 1:1 mixture of protein and reservoir solutions. Concentrations are of the stock protein and precipitant solutions.

	Reservoir:Drop Volume Ratio			
Conditions	**100:1**	**50:1**	**20:1**	**5:1**
50 mg/ml lysozyme				
5% NaCl				
Time to 1'st crystals	22 hr.	22 hr.	22 hr.	22 hr.
Number of crystals	23(1)	24(1)	23(1)	27(1)
Average size	400 x 255 μm	390 x 250 μm	345 x 235 μm	360 x 245 μm
50 mg/ml lysozyme				
4% NaCl				
Time to 1'st crystals	74 hr.	74 hr.	74 hr.	50 hr.
Number of crystals	18(1)	16(1)	18(1)	21(1)
Average size	420 x 260μm	440 x 270 μm	420 x 240 μm	415 x 265 μm
40 mg/ml ysozyme				
4% NaCl				
Time to 1'st crystals		---------- approx. 96 hr., no difference ----------		
Number of crystals	1(2)	5(2)	9(2)	11(2)
Average size	920 x 560 μm	790 x 630 μm	685 x 485 μm	1050 x 880 μm

(1) Average number of crystals/drop.
(2) Total number of crystals in 12 drops

crystallizations were set up at 1.4 M AmS, 0.05 M tris-acetate, pH 7.0, 1 mM $MnCl_2$, 1 mM $CaCl_2$, 0.02% sodium azide (w:v), using protein concentrations of 25 and 18 mg/ml. Also, using the same buffer make-up, 0.8 M and 1.0 M AmS trays were set up for 18.7 mg/ml protein. Three Cryschem 24-well plates were used with each set of conditions, except for the last two conditions with 0.8 and 1.0 M AmS, where only one plate was set up for each. The plates were stored at 20 ^{o}C and sealed with clear tape. The drop size was 10 μl, using the standard technique of mixing 5 μl of protein with 5 μl of precipitant solution from the reservoir. The first two trays were divided into 2 halves and each half consisted of 12 identical conditions for averaging. Rows A and B of the first tray consisted of a 100:1 ratio of reservoir volume to drop volume with rows C and D consisting of a 50:1 ratio. The second tray followed with the top two rows containing 20:1 and the bottom two with a 5:1 ratio. The third tray was set up with all 4 ratios in 6 duplicate wells. The first two trays were used to evaluate crystal number and size for each ratio after 10 days, while the third tray was evaluated twice a day for the appearance of crystal nuclei by visually inspecting the drops with a microscope.

3. Results

Equilibration rate studies were carried out using solutions given in Table 1. At least three separate rate studies were made for each solution. Typical equilibration rate curves for representative concentrations of AmS, 20% PEGs (PEG400 v:v, PEG 1000 and 8000 w:v), and MPD (v:v), are shown in Figure 1. A linear extrapolation of the initial rates was made to 90% equilibration for each precipitant and these calculated data are given in Table 1. The linear extrapolations are based upon the first half of the data, or until addition of subsequent data points resulted in a reduced correlation coefficient. We also tried fitting the data with polynomials to better follow all the curvature of the data. However, in many instances (for example, see Figure 1, panel B, 20% MPD) the data fell off, and either did not reach or could not be extrapolated to 90% equilibration. The use of linear fits kept our analysis methods in line with those used previously, and the data obtained gave comparable results (Mikol *et al*., 1990; Luft & DeTitta, 1995).

Experiments to determine the effects of varying the reservoir to droplet volume ratios were made with 30 % PEG400 (v:v), 1.5 M NaCl, and 50 % MPD (v:v). These were set up at reservoir to droplet volume ratios of 50:1, 25:1, and 5:1, keeping the droplet volumes at 20 μl and varying the reservoir volumes from 1000 to 100 μl. Reservoir volumes of 100 μl or less did not cover the bottom of the Cryschem plates, resulting in variable surface areas for the reservoir. The data are shown in Figure 2, and in all cases the equilibration rate was slower the lower the reservoir:droplet volume ratio. Note that the equilibration rates at reservoir:volume ratio. Note that the equilibration rates at best are only approximately double for an order of magnitude change in the volume ratio. From the model of Diller & Hol (1999), a 10 fold increase in droplet volume results in an approximately 5 fold increase in the equilibration time. B decreasing the reservoir volume we effectively move it further from the droplet, which again would increase the equilibration time. Thus, while the observed prolonging of the equilibration time is expected, that it only at most is doubled was an unexpected result.

Additional experiments were set up to evaluate the equilibration results using two proteins, lysozyme and concanavalin a. Each experiment had two components, one to follow the initial appearance of nuclei and a second for measuring the numbers and sizes of crystals obtained. In the first, 6 wells each of reservoir to droplet volumes of 100:1, 50:1, 20:1, and 5:1 were set up, the starting drop volumes being 10μl. In the second, 12 duplicate wells of each reservoir to droplet volume ratio were set up, 2 conditions per plate. The first plates were examined at periodic intervals with the goal of determining the time when half of the wells at any one condition had visible crystals under microscopic examination. The second set of plates were not disturbed for a fixed period, then removed, and the numbers and sizes of the crystals obtained were counted and measured respectively.

Table 2 summarizes the results from the lysozyme experiments. At high protein and precipitant concentrations (50 mg/ml protein, 5 % NaCl) nucleation was too rapid in all cases. As the protein and precipitant concentrations were reduced a trend emerged of more rapid nucleation at the lower volume ratios, counter to what would be expected from the monocomponent equilibration rate experiments. At 50 mg/ml protein and 4 % NaCl this is observed by crystals consistently first appearing in the 5:1 ratio wells (three separate experiments). At 40 mg/ml protein and 4 % NaCl progressively more crystals appear as the volume ratios are lowered, indicative of more rapid nucleation.

The concanavalin a trays were more difficult to quantitate. The first conditions were using 1.4 M AmS and 25 mg/ml protein. The same protocol as above was used with the 3 plates. After 15 hours, almond-shaped crystals had appeared in all ratios. After 9 days, the plates were removed from the incubator and examined for crystal number and size. Crystal showers were present in all wells and were

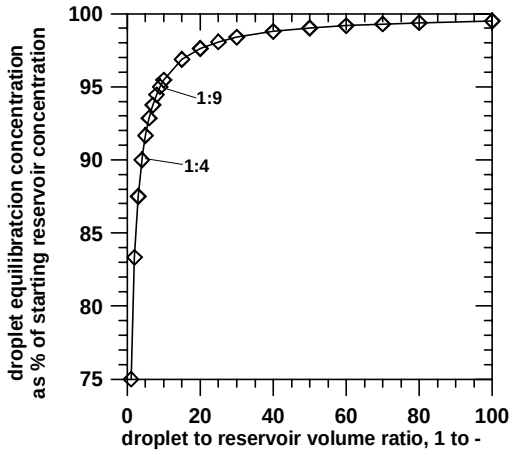

Figure 3
Calculated final droplet equilibration concentrations as a percentage of the starting reservoir concentration for varying reservoir:droplet volume ratios.

too small to count or measure. There were large and medium single crystals mixed throughout the crystal showers but no obvious differences. The procedure was repeated with a solution of 18 mg/ml con a in order to slow down the nucleation rate. After 19 hours, again all ratios had crystal showers. The same results were obtained for the longer duration plates after 11 days. These conditions were repeated a third time with protein at 17.8 mg/ml and the same results were produced. At this point, two plates were set up to monitor the nucleation rate while reducing the AmS concentration. Crystal showers appeared in all wells after 16 hours for the 0.8 M and 1.0 M conditions for all ratios.

4. Discussion

Previous researchers studied the effects of varying the crystallization droplet size while keeping the reservoir solution volume constant. It was experimentally and theoretically shown that equilibration rates decrease approximately linearly with a decrease in the drop volume (Mikol *et al*., 1990; Diller & Hol, 1999). A qualitative survey of recent crystallization reports shows that most initial crystallization droplet volumes are in the 2 – 4 µl range, being composed of 50 % each of macromolecule and reservoir solutions. However, the reservoir volumes are typically 250 to 500 µl, giving reservoir to droplet volume ratio's ranging from about 60 to 250 to 1. Luft & DeTitta (1997) showed that the rate of equilibration significantly affects the apparent crystal quality. Small droplets with large well volumes dramatically decreases the time for vapor equilibration (Diller & Hol, 1999), and it may be counter productive to use such large volume ratios. However, a converse claim is made that use of nl size drops offer a significant reduction in the crystallization time for some proteins, due to more rapid equilibration (Stevens, 2000), which is useful for initial screening experiments.

One can readily calculate the final equilibrium concentration of a droplet, relative to the starting reservoir concentration, for a given volume ratio. This is done in Figure 3 for ratios from 1:1 to 100:1. A ratio of 4:1 results in a droplet that goes to 90% of the starting reservoir concentration, while 9:1 is required to obtain 95% of the starting reservoir concentration. Thus the bulk of the reservoir solution volume is present to obtain the last 5 % of the equilibration process, and one can reasonably ask if this is critical or necessary.

The vapor diffusion process is supposed to gradually bring the macromolecule solution into a supersaturated state, from whence crystals nucleate and grow. Ideally nucleation events should occur in the latter stages of the equilibration process. Nucleation occurs when the solution has passed the metastable boundary, and continued driving of the solution conditions beyond this point will only increase the nucleation events and/or drive crystal growth at a higher rate. This condition may occur with nucleation in the early stages of the vapor equilibration process. Here nucleation is a "bulk" process, one that could as easily be obtained in the absence of any vapor phase equilibration. The crystals that appear must maintain equilibrium with the changing bulk crystallization solution, with failure to do so subjecting them to osmotic stress that may affect their overall diffraction quality. Nucleation events past the point where vapor phase equilibration has occurred are also essentially a bulk process, although in these cases in the absence of the agitation from the initial mixing and from a solution that is stable in composition. The question of osmotic stress effects has not been experimentally addressed to date. We propose that vapor diffusion experiments where protein concentrations are progressively changed to adjust the nucleation time, or bulk and vapor diffusion crystallization experiments having the same starting solution conditions, can be used together with crystal quality measurements (Bellamy *et al*., 2000; Boggon *et al*., 2000) to address this issue.

Previous investigations had shown that decreasing the droplet size proportionately decreased the equilibration time, potentially enabling much more rapid screening for crystallization conditions (Diller & Hol, 1999; Stevens, 2000). Conversely, we were interested in the effects of decreasing the reservoir to droplet volume ratio by decreasing the reservoir volume, not increasing the droplet size. Based upon previous data one would expect this to result in longer equilibration rates (Mikol *et al*., 1990; Diller & Hol, 1999), and these results are in qualitative agreement. However, whereas Diller & Hol (1999) show an estimated five fold increase in equilibration time for a ten fold decrease in reservoir:drop volume ratio, our data, with considerably smaller solution volumes, shows a less than two fold increase for a commensurate volume ratio change.

A major difference between these and previous experiments is the amount of water vapor to be transported. In previous studies the droplet volume was a variable, and decreasing volume ratios meant progressively more water had to be transported for equilibration (Mikol *et al*., 1990; Diller & Hol, 1999). At a fixed evaporation rate this means longer equilibration times. In this work the amount of water to be transported for equilibration is constant. Thus changes in equilibration times more strongly reflect changes in reservoir to drop distance, in the reservoir surface area, and due to the generally smaller volumes employed experimental "noise" effects such as vapor loss.

While monocomponent solutions did show increased equilibration times, experiments with protein crystallization solutions did not. Why lower volume ratios should result in more rapid, or essentially unchanged, protein nucleation rates is not clear at this time and counter to observations based upon monocomponent solutions. When any trends were indicated by the data, they consistently indicated more rapid equilibration in the lowest volume ratio wells, either by time of appearance of the first crystal or by numbers of crystals formed. The possibility that the increased rates are due to proportionately greater water loss due to the low volume ratios is countered by the slower measured equilibration rates, as expected based upon theory and previous results (Mikol *et al.*, 1990; Diller & Hol, 1999) for monocomponent systems. At the lowest volume ratios the reservoir surface area is also reduced, which would be expected to increase the equilibration time. This may be the primary reason for the prolonged times in the monocomponent solution experiments. At this time no satisfactory explanations for these results are available.

A clear outcome is that the current practice of using large volume ratios is not necessary. In combination with the smaller droplets now routinely used this means that higher crystallization

plate densities can be achieved with a concomitant savings in materials usage. Several crystallization plate designs having 96 crystallization wells are now commercially available, generally having well volumes in the 250 µl range. With a 2 or 4 µl droplet volume reservoirs having a volume capacity of only 10 to 40 µl are more than sufficient.

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