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Time Courses of Equilibration for Ammonium Sulfate, Sodium Chloride and Magnesium Sulfate Heptahydrate in the Z/3 Crystallization Plate

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Abstract

Time courses of equilibration for three salts, sodium chloride, ammonium sulfate and magnesium sulfate heptahydrate have been measured in the Z/3 crystallization plate. It is shown that by varying both the diffusant and the reservoir depth the time taken to equilibrate can be as short as 200 or as long as 1400 h. Thus, the present design of the plate should accommodate a wide variety of desired crystallization kinetics.

Introduction

Macromolecular crystallization is a complex process in which thermodynamic and kinetic factors play important roles (Wilson, Bray & Suddath, 1991). We recently described a protocol and designed a crystallization plate (Z/3) that allows the investigator to tailor the time course of equilibration between a macromolecular droplet and a reservoir (Luft, Arakali, Kirisits, Kalenik, Wawrzak, Cody, Pangborn & DeTitta, 1994). The plate employs diffusion cells of varying depths as reservoirs. The depths are chosen to fit a smooth progression in d^2 to exploit the d^2 dependence of diffusion. The plate can be used in crystallization experiments employing three common techniques: hanging-drop vapor diffusion, sitting-drop vapor diffusion and microdialysis. In each of the techniques a droplet that contains the macromolecule of interest and a crystallizing agent equilibrates with the surface conditions of the reservoir, which are themselves evolving in time. We wish to report time courses of equilibration for three diffusants – sodium chloride, ammonium sulfate and magnesium sulfate heptahydrate – in the Z/3 plate. It will be shown that, for an initial surface concentration of ~0 m† and a final equilibrium concentration of ~1 m, the time to equilibration can be varied from ~200 h, for NaCl in the shallowest reservoir, to ~1400 h, for $MgSO_4.7H_2O$ in the deepest reservoir. Thus, a wide range of equilibration rates can be sampled in a plate of fixed geometry by changing the nature of the diffusant.

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† 1 m ≡ 1 molal = 1 mole of solute plus 1 kg solvent.

Z/3 plate design

The plate is modularly constructed in six distinct sub-assemblies. In each sub-assembly there are four reservoirs of uniform depth; in the distinct sub-assemblies only the reservoir depths are varied. The shallowest reservoir sub-assemblies were designed to have a well depth (51 mm) approximately three times that of a Linbro plate (17 mm). The deepest reservoir sub-assemblies were designed to maximize the well depth (95 mm) while holding the overall height of the plate to a size that would fit under a moderate-power microscope. With the microscopes at hand in the laboratory it proved possible to accommodate an ~100 mm tall plate and still have enough working distance to focus on the plate surface. A 5 mm thickness in the bottom of the deepest wells was sufficient for mechanical stability.

The well depths of the intermediate reservoirs were chosen as follows. A d^2 dependence of the diffusion process is anticipated, and a uniform vapor chamber for a hanging-drop experiment is desired. Leaving room for a 5 mm tall vapor chamber in every reser-

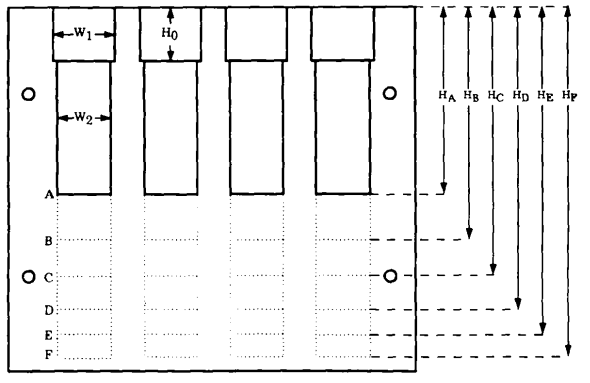

Fig. 1. A Z/3 sub-assembly. Reservoirs were machined in imperial units (in); equivalent metric units (mm) are also given. All reservoirs have W_1 = (11/16 in, 17 mm), W_2 = (9/16 in, 14 mm) and H_0 = (0.600 in, 15 mm). Reservoir depths are H_A = (2.000 in, 51 mm), H_B = (2.500 in, 64 mm), H_C = (2.875 in, 73 mm), H_D = (3.250 in, 83 mm), H_E = (3.500 in, 89 mm) and H_F = (3.750 in, 95 mm). The four bolt holes are for subunit assembly with a threaded brass rod. The outer dimensions of the Plexiglass block are 4.5 in (114 mm) wide, 4.0 in (102 mm) high and 1.0 in (25 mm) thick.

voir, the shallowest reservoir accommodates a 46 mm head of liquid while the deepest accommodates 90 mm. A plot of d^2, where d is the distance from the reservoir bottom to the liquid surface, was constructed using the two points $d^2_{min} = (46\ mm)^2 = 2116\ mm^2$ and $d^2_{max} = (90\ mm)^2 = 8100\ mm^2$. Four evenly spaced points along this line between d^2_{min} and d^2_{max} were chosen, their d values calculated, and 5 mm added for a vapor chamber. All of the relevant dimensions are shown in Fig. 1. Note that all reservoirs are of uniform diameter and that a counter sink at the top accommodates and supports a plastic screen used in sitting-drop and microdialysis experiments. In all of the time-course and endpoint studies to be described the screen was omitted, as would be the case during hanging-drop crystallizations.

Time-course experiments

Whether the approach to crystallization in the Z/3 plate be sitting drop, hanging drop or microdialysis, the solution that contains the macromolecule equilibrates with the surface layer of the reservoir solution. Therefore, time courses, in which the concentration of a diffusant at the reservoir surface is measured, were conducted for three common salts used in crystallization: sodium chloride, ammonium sulfate and magnesium sulfate heptahydrate.

The method of monitoring the surface concentration of an equilibrating reservoir was to periodically remove a small aliquot (20–30 μl) of solution, using a Gilson Pipetman micro-pipette, directly from the surface of the reservoir. The aliquot was transferred to a Bausch and Lomb Abbé 3L refractometer where its refractive index was recorded. Calibration charts made with stock solutions of known concentration allowed a conversion of n_d values to concentration units. A difficulty with this approach was that, in order to draw up any liquid, the pipette tip had to be inserted below the liquid surface. The skill used to accurately sense the liquid surface and insert the tip the minimal distance to ensure a correct draw varied with the investigator.

The time-course experiments were designed to cover a change in surface concentration of either one or two molal units. The overlayering solution in each experiment was distilled deionized water (ddH_2O). The set-up procedures were very similar. Into a clean, dry reservoir a weighed sample of crystalline diffusant was deposited, at the reservoir bottom. Using a plastic wash bottle, ddH_2O was added to the reservoir, being careful to train the stream of water onto the inner wall, thus disturbing the solid material as little as possible. As our experience increased we noted that filling the reservoir could be most effectively accomplished in three stages. In the first, only enough liquid to wet the solid was added. If the solid would not wet thoroughly before a liquid head over the solid became apparent, a thin metal spatula was introduced down the reservoir into the solid. Once air bubbles were expressed the solid would be wetted. In the second, liquid was added to the level of the countersink position, 15 mm below the top of the reservoir, rather quickly, but again gently, by training the liquid against the inner wall. In the third, liquid was slowly added to a level 5 mm below the reservoir top using a dipstick indicator. The indicator was constructed by taping a Wiretrol capillary to a plastic straight edge, the capillary axis perpendicular to the edge, so that 5 mm protruded. With the staight edge flush with the top surface of the subassembly and the capillary hanging down into the reservoir, the liquid level was adjusted in drops until it just wicked the capillary. Using this technique, and when air pockets were avoided, the liquid volume added and solid diffusant weighed yielded consistent final concentrations to within ±1%. The formation of air pockets, which prevents the thorough wetting of solid, can cause the liquid volume to be underestimated, resulting in a final concentration higher than anticipated.

Table 1. *Diffusant quantities and experimental endpoints for time courses*

Masses are for the various reservoirs shown in Fig. 1.

Diffusant	NaCl	$(NH_4)_2SO_4$		$MgSO_4.7H_2O$	
Course	'0→1 m'	'0→25%'	'0→50%'	'0→1 m'	'0→2 m'
Endpoint	1.03 m	22%	44%	0.98 m	1.91 m
Reservoir	Mass (g)	Mass (g)	Mass (g)	Mass (g)	Mass (g)
A	0.479	0.975	1.896	1.845	3.588
B	0.602	1.225	2.382	2.318	4.056
C	0.695	1.415	2.752	2.678	5.206
D	0.777	1.582	3.075	2.993	5.819
E	0.842	1.712	3.330	3.240	6.300
F	0.900	1.831	3.561	3.465	6.738

End-point measurements

Each of the experimental set-ups for the various time courses was duplicated for a measurement of the expected endpoint concentration; *i.e.* the concentration a reservoir would reach when it was fully equilibrated. Set-up was precisely that described for the actual time courses. Disposable plastic transfer pipettes were used to rapidly and thoroughly mix the reservoirs by drawing liquid from just above the solid and expressing it at the liquid surface. Dissolution and equilibration took about 10 min per reservoir. Equilibration was judged by the absence of Schlieren lines in the reservoir with continued mixing. After mixing was completed a droplet was removed from the equilibrated reservoir and its refractive index measured. Converting from n_d values to concentration units the expected endpoints are reported in Table 1.

Results

Sodium chloride time course

A single Z/3 plate was used for two time-course experiments. Two lanes (of six reservoirs) were set up for a '0→1 m' experiment; the other two lanes were set up for a '1→2 m' experiment. Aliquots (35 μl) from alternate lanes were withdrawn on alternate days. The experiment was terminated after ~590 h (~24 d). Results for the '0→1 m' experiment are shown in Fig. 2. Results for the '1→2 m' experiment (not shown) are very similar; *i.e.* the family of graphs

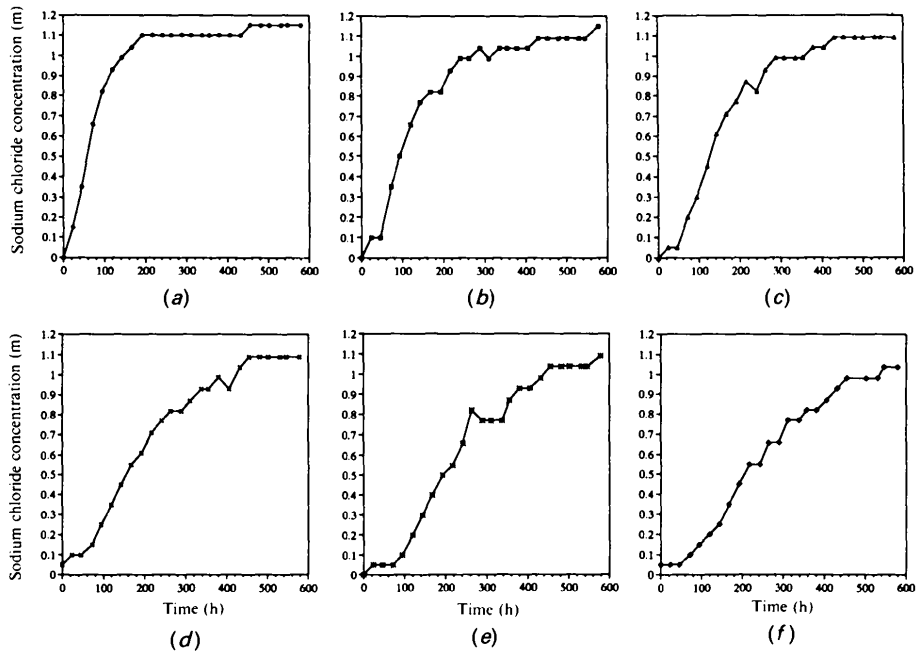

Fig. 2. The '0→1 m' NaCl time course: (*a*) reservoir *A*, (*b*) reservoir *B*, *etc.* A single Z/3 plate was employed, with repeated measurements from individual reservoirs.

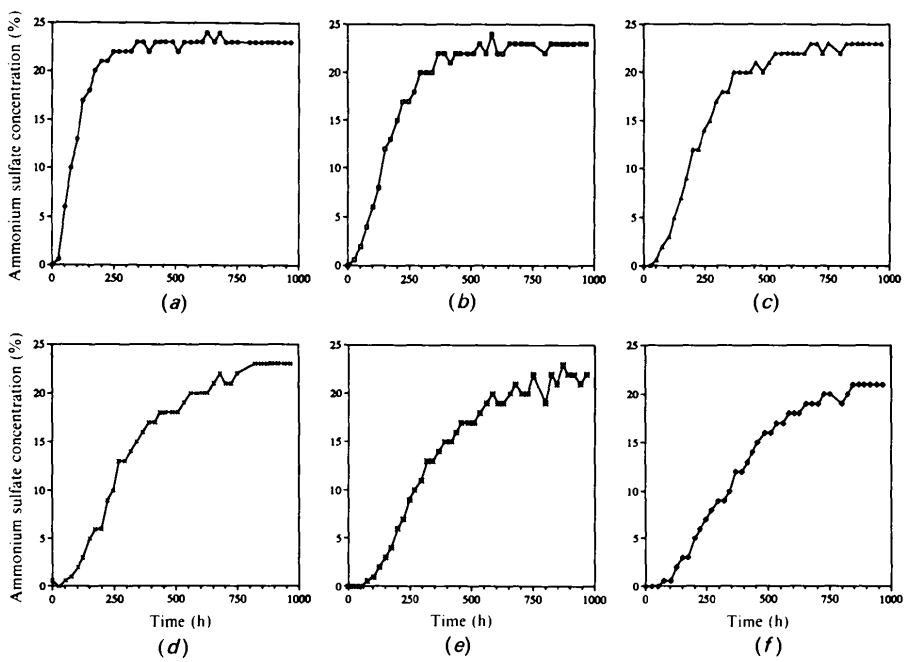

Fig. 3. The '0→25% of saturation' (~0→~1 m) $(NH_4)_2SO_4$ time course: (*a*) reservoir *A*, (*b*) reservoir *B*, *etc.* Each measurement represents a separate reservoir.

was simply shifted along the ordinate by 1 molal unit. In the '0→1 m' experiment the expected endpoint was 1.03 m. The time to reach 75% of completion (0.75 m NaCl) ranged from ~90 h for the shallowest reservoir to ~340 h for the deepest reservoir.

Ammonium sulfate time course

Ten Z/3 plates were used for two time-course experiments. One experiment sampled the time course '0→25% of saturation', Fig. 3; the other sampled the course '0→50% of saturation'

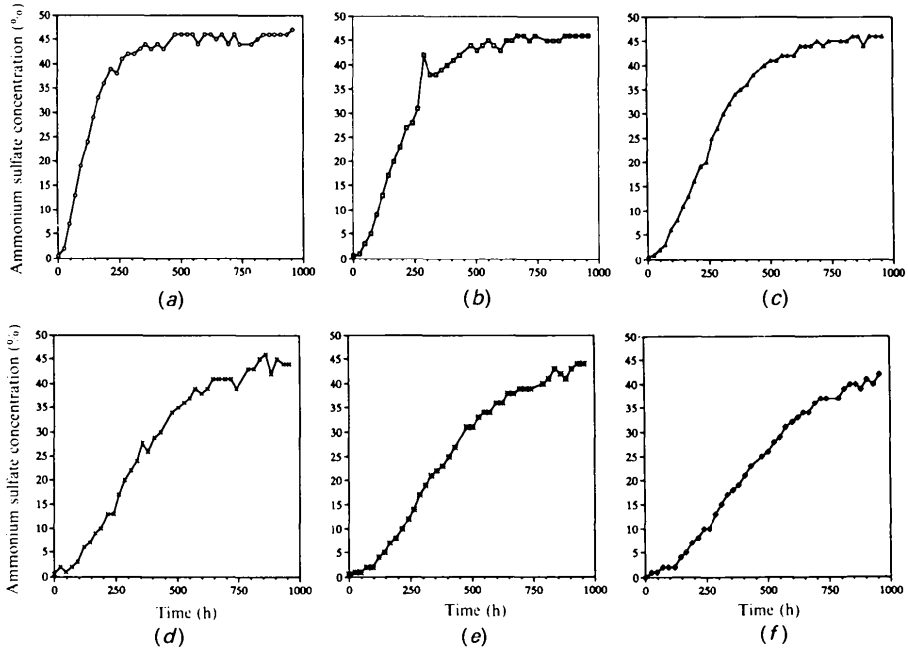

Fig. 4. The '0→50% of saturation' (~0→~2 m) $(NH_4)_2SO_4$ time course: (*a*) reservoir *A*, (*b*) reservoir *B*, *etc*. Each measurement represents a separate reservoir.

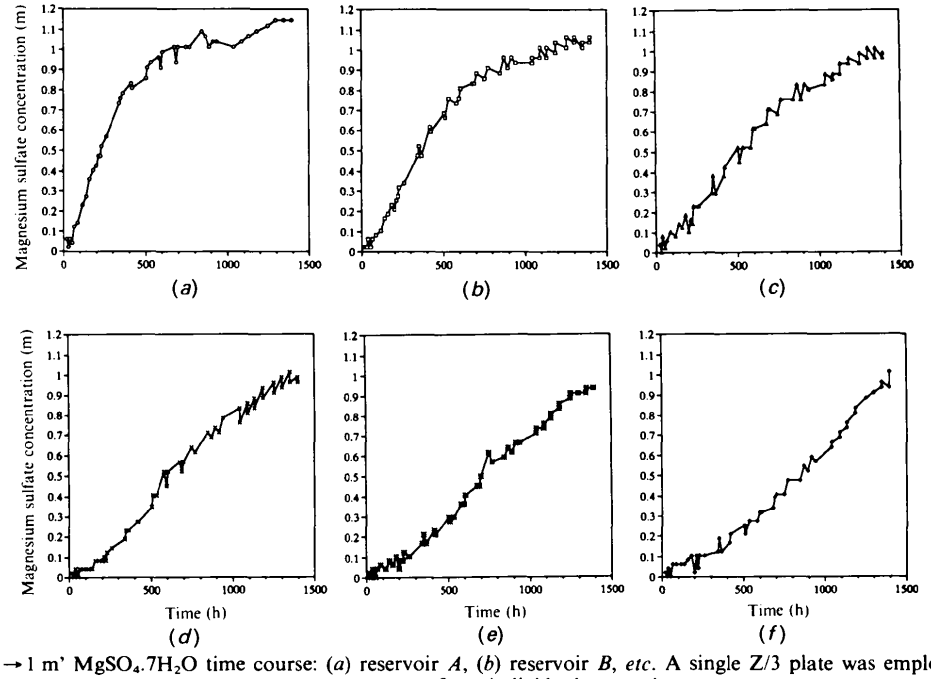

Fig. 5. The '0→1 m' $MgSO_4.7H_2O$ time course: (*a*) reservoir *A*, (*b*) reservoir *B*, *etc*. A single Z/3 plate was employed, with repeated measurements from individual reservoirs.

ammonium sulfate, Fig. 4. Units of concentration of percentage saturation are employed for ammonium sulfate because of their ubiquity in the macromolecular literature; 25% of saturation corresponds to ~1 molal or ~1 molar. With ten plates a rotation schedule could be constructed to measure the surface concentration using a separate reservoir per measurement; once opened the reservoir was discarded. The experiments covered approximately 1000 h (42 d). The endpoint of the '0→25% of saturation' experiment was 22% of saturation; that of the '0→50% of saturation' was 44% of saturation. Time to 75% of completion for the former (~16% of saturation) varied from ~120 h for the shallowest reservoir to ~490 h for the deepest reservoir. Corresponding times for the latter (33% of saturation) range from ~175 to ~600 h.

Magnesium sulfate heptahydrate time courses

One Z/3 plate was used for two time courses. Two lanes were set up for a '0→1 m' experiment, Fig. 5; the other two lanes were set up for a '0→2 m' experiment, Fig. 6. As in the sodium chloride experiment, alternate lanes were read on alternate days. The experiment was terminated after 1400 h (58 d). The endpoints were 0.98 m for the '0→1 m' experiment and 1.91 m for the '0→2 m' experiment. Time to 75% of completion for the former (0.74 m) ranged from ~350 to ~1125 h, and for the latter (1.43 m) from ~580 to ~1375 h. In the $MgSO_4.7H_2O$ experiments the formation of air pockets in the solid charge, and the necessity of their removal, was heightened by the length of the column of solid material. In particular, solid $MgSO_4.7H_2O$ occupied the bottom 37 mm of the deepest reservoir in the '0→2 m' experiment.

Discussion

A wide range of equilibration rates can be achieved in the Z/3 plate as currently designed. By simply changing diffusants the times to effect an ~0.7 molal change in surface concentration vary from less than 100 to more than 1000 h. Such flexibility in a single embodiment of the principle allows a search over a wide range of kinetics for optimal nucleation and growth rates. Although the graphs of surface concentration shown are somewhat jagged, this is primarily because of small variations in preparing the reservoirs or in reading their surface refractive indices. It is anticipated that in a sealed, undisturbed reservoir, as when a crystallization experiment is underway, the change in surface concentration will be a very smooth function of time. Also, the sodium chloride and magnesium sulfate experiments, conducted with a single Z/3 plate each, show the effects of repeated removal of aliquots for measurement from the same reservoir. These effects slowly accumulate and explain why these time courses tend to overshoot their expected endpoints. In actual crystallization experiments, where the reservoirs are not sampled, the actual and expected endpoints are very close, as

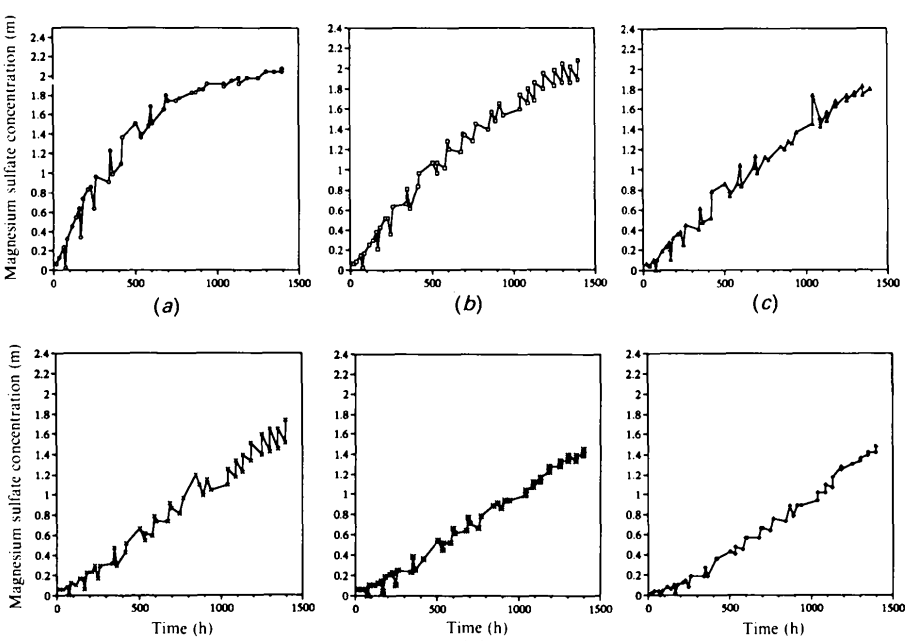

Fig. 6. The '0→2 m' $MgSO_4.7H_2O$ time course: (*a*) reservoir *A*, (*b*) reservoir *B*, *etc.* A single Z/3 plate was employed, with repeated measurements from individual reservoirs.

evidenced by the ammonium sulfate time courses where individual reservoirs were measured only once.

In a vapor-equilibration approach to crystallization the reservoir is segregated from the sitting or hanging drop that contains the macromolecule and crystallizing agent. Contact is only through the vapor phase, confined to the vapor chamber, and is principally mediated by water, although whenever ammonium sulfate is present in either the drop or the reservoir there is the additional possibility of ammonia transfer (Rodeau, Mokol, Giegé & Lutin, 1991). The role of the reservoir is, in general, to dehydrate the drop. Although there are certain conveniences realized when the diffusant in the reservoir and crystallizing agent in the drop are the same chemical species, it is by no means necessary that they be so. For example, it might be known that the optimal crystallizing agent for a particular protein is sodium chloride, yet the desire is to equilibrate at a very slow rate, suggesting that the diffusant should be magnesium sulfate. The question arises as to how to tailor the initial conditions of drop and reservoir so as to take advantage of the optimal choices for both. As an example, consider a protein known to exhibit no sign of microcrystallinity at 0.5 molal NaCl, microcrystallinity at 1.0 molal and rapid precipitation at 2.0 molal NaCl. We desire a time course that will take a drop from ~0.5 to ~1.5 molal NaCl over the course of ~1000 h. The diffusant is magnesium sulfate; the amounts are approximately those reported for the '0→1 m' $MgSO_4.7H_2O$ time course. The macromolecule is dissolved in an appropriate buffer that is 0.5 molal in sodium chloride. The magnesium sulfate charges are placed in the reservoirs. As an overlayering solution we choose 0.5 molal sodium chloride solution. This insures that the drop and reservoir surface are initially in equilibrium. As the diffusant reaches the reservoir surface the vapor pressure of water over the reservoir lowers, causing a mass flux of water out of the drop and into the reservoir. Depending on the drop size and a number of other critical factors (Mikol, Rodeau & Giegé, 1990), the kinetics of equilibration can be controlled by the diffusional flux in the reservoir. An analogous situation to the one just described is of interest when working with PEG.

In microdialysis experiments it is necessarily the case that the diffusant and crystallizing agent be one and the same. Therefore it is critical to ensure chemical compatibility of the diffusant and the system in crystallization. In particular, we mention that magnesium sulfate is not suitable as a diffusant if the macromolecule must be maintained in a phosphate buffer. It is also unlikely that PEG would be useful, inasmuch as it will not readily cross the dialysis membrane. Under these conditions, if slower equilibrations than possible with sodium chloride or ammonium sulfate are desired, it is always possible to use deeper reservoirs or to search for slower diffusing species.

The plates were designed to incorpoate the d^2 dependence of diffusion directly into the hardware. However, the process of dissolution and diffusion in the Z/3 plate is not an idealized one, in which infinitesimal changes in surface concentration are considered. In order to crystallize macromolecules we expect to have to change surface concentrations by large amounts in order to propel the droplet conditions from those of undersaturation to saturation to nucleation. Therefore, the amounts of diffusants are quite large, and the volumes occupied by the solid diffusants are such that an appreciable fraction of the reservoir is occluded by solid. The question arises as to what constitutes the working 'depth' of the reservoir when so much of the bottom part is filled with solid. We propose that the depth should be measured from the liquid surface to the top of the solid. As the top of the solid dissolves and diffuses into the liquid the interface height decreases and, therefore, the reservoir depth increases. This continues until all of the solid dissolves, at which point the d^2 dependence built into the hardware should be exact (Fig. 7). The effect of the decreasing reservoir depth is apparent when we compare the '0→25%' and '0→50%' of saturation ammonium sulfate time courses, Figs. 3 and 4. Focusing on the shallowest wells in both experiments it is clear that, in the ascending limbs of the graphs, the equilibration rate for the '0→25%' experiment is actually lower than the rate for the '0→50%' experiment.

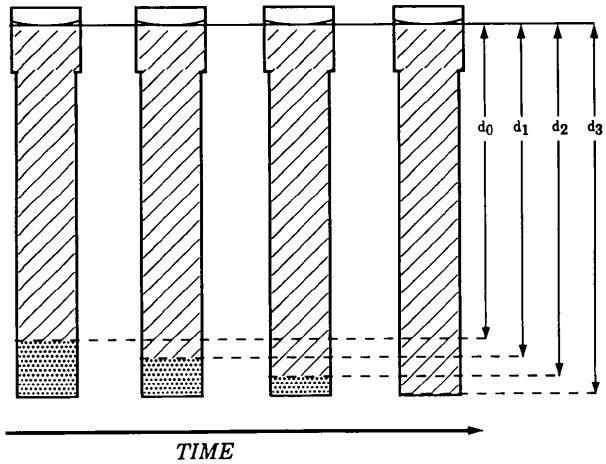

Fig. 7. Time evolution of the effective depth of the reservoir. At t_0, when the overlayering solution is just added, the solid in the reservoir bottom decreases the effective depth, as defined by the distance from the liquid surface to the very top of the solid–liquid interface, d_0. As the diffusant dissolves and diffuses, the level of the solid drops and the effective depth increases ($d_0 \rightarrow d_1 \rightarrow d_2$). At d_3 all of the solid has dissolved and the effective depth, d_3, is the actual machined depth shown in Fig. 1.

(Note that, as drawn, the ordinates of both families of graphs are the same height, but represent ammonium sulfate concentrations that differ by a factor of two.) This is because in the former the initial reservoir depth is ~40 mm while in the latter it is ~35 mm. The ratio of initial d^2 values is 1.0/0.75.

We have shown that a wide range of equilibration rates can be realized in the Z/3 crystallization plate using three common diffusants. A benefit of using sodium chloride or magnesium sulfate heptahydrate in vapor-diffusion crystallization experiments is that each is available commercially at very low cost in the form of common, uniodized table salt and Epsom's salts, respectively. When the approach to crystallization is microdialysis, where the diffusant is in intimate contact with the macromolecule, it makes sense to use a good chemical grade of these salts.

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