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THREE METHODS OF PROTEIN CRYSTALLIZATION IN LOW GRAVITY

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ABSTRACT

Most protein crystallizations in low gravity have been performed using the hanging-drop, vapor diffusion method. We tested the hypothesis that other methods also produce superior crystals when performed in low gravity. Lysozyme from hen egg white was crystallized in 0.1 M NaAc, pH 4.0, using NaCl as precipitant on shuttle flights STS-37 and 42 using interfacial diffusion, step diffusion and osmotic dewatering methods. These were performed in diffusion cells formed by the sliding of blocks on orbit, each block containing less than 100 hemi-cells. In step diffusion experiments salt and protein were dissolved together at various concentrations and placed in a chamber that briefly contacted a high then a low salt concentration. All methods produced high-quality crystals, 0.6 - 2.0 mm in length, depending on the method. X-ray diffraction analysis of crystals grown by the interfacial diffusion method demonstrated that they had superior Wilson statistics, rocking curves and resolution-intensity relationships. For example, rocking-curve widths were reduced from 0.75 milliradians to 0.45 milliradians. Step diffusion resulted in some crystals exceeding 2 mm in length, while the osmotic dewatering method produced results similar to those found when this method is practiced on earth.

INTRODUCTION

Knowledge of the three-dimensional structure of proteins is the foundation of protein engineering and rational drug design in biotechnology. All technologies for the rapid analysis of protein structure are in hand except reliable methods and general rules for obtaining high-quality crystals for diffraction studies /1,2/. Several methods are used to grow protein crystals, and all of them consist of bringing the protein solution above saturation with a precipitant by adding precipitant or concentrating it by water removal. These methods include dialysis, batch mixing, vapor diffusion, osmotic dewatering and double diffusion /1-5/. Various investigators have found that improved ordering occurs when the vapor diffusion or interfacial diffusion methods are used in the low-gravity environment of orbital space flight /6-8/. While data to date are firm and convincing (0.4 Å improved resolution in x-ray diffraction in many cases /8,9/), systematic studies of solution variables have not been performed, owing to the limited number of samples that have been successfully crystallized in low gravity.

Nearly all protein crystal growth in low gravity has been performed using the vapor

diffusion method /7/, so there is a lack of data on other methods and a lack of characterization of any methods with respect to controllable experimental parameters such as protein concentration, salt concentration, temperature, pH, etc. In the absence of convective currents and buoyancy-driven mixing processes, one might anticipate that high concentration gradients would be tolerated in low gravity. This hypothesis was tested by designing and performing a systematic study of the crystallization of hen egg lysozyme (E.C. 3.2.1.17), a standard protein for crystallization studies /10,11/. Crystals were grown from solutions of lysozyme from 4% to 10% (w/v) (the latter concentration is equivalent to saturation at 20°C) and NaCl concentrations from 4% to 12% (w/v). All solutions were made in 0.1 M NaAc buffer, pH 4.0.

MATERIALS AND METHODS

Space Flight Apparatus

Solutions were contacted using a multi-sample Minilab (The Materials Dispersion Apparatus /12-14/) on the April 1991 and July 1991 flights of the space shuttle Atlantis (STS-37 and STS-43). This apparatus brought about 60 protein solutions into contact with their corresponding salt solutions automatically upon the achievement of orbit. The period of solution contact included re-entry and de-integration on the second (STS-43), but not the first (STS-37) flight.

The MDA is an automated flight hardware device for contacting samples of any two, three, or four fluids in microgravity. The mass of the MDA is less than 2 kg, and it occupies about 2.8 liters of volume. It operates in the following manner: two blocks of inert material with a matching number of sample test wells are held together under pressure with a sealing mechanism in an aerospace aluminum housing. The test wells are misaligned at launch, thus separating the fluids to be mixed. After microgravity has been achieved, the blocks are moved into alignment by means of a motor-cam mechanism, allowing the fluids to contact. An option exists to contact a third fluid while in the microgravity environment or prior to or during re-entry. Mixing of fluids and solutes occurs mainly through diffusion and, depending on experiment design, other transport processes such as wetting and electrokinetic transport. Each MDA can have about 100 total test well sets. Each cavity in the "top" block was filled with 135 μl of fluid, and, upon achievement of low gravity, was slid into position so that it was continuous with a corresponding cavity containing 120 μl of another fluid in the opposite block. At the end of the low gravity period (6.6 min) the two blocks were once again misaligned, and the interaction of the two fluids was halted. Thus, the opposing wells had three specific relationships to one another: launch, coasting, and re-entry positions, as shown in Figure 1.

The following operational profile was planned and used: 1 minilab unit (70 sets of wells accommodating a variety of experiments) was held at 1 atm ambient pressure and nominal 20°C temperature (temperatures actually achieved after launch were recorded). All wells were opened 22 h into the low-gravity phase of flight and closed 24 h before onset of deceleration phase in the first (STS-37) flight.

Interfacial Diffusion

Tetragonal crystals of hen egg white lysozyme, E.C. 3.2.1.17, were grown in the laboratory and in the low gravity environment of space shuttle flights using the interfacial diffusion method. NaCl free protein and protein free NaCl solutions were brought into

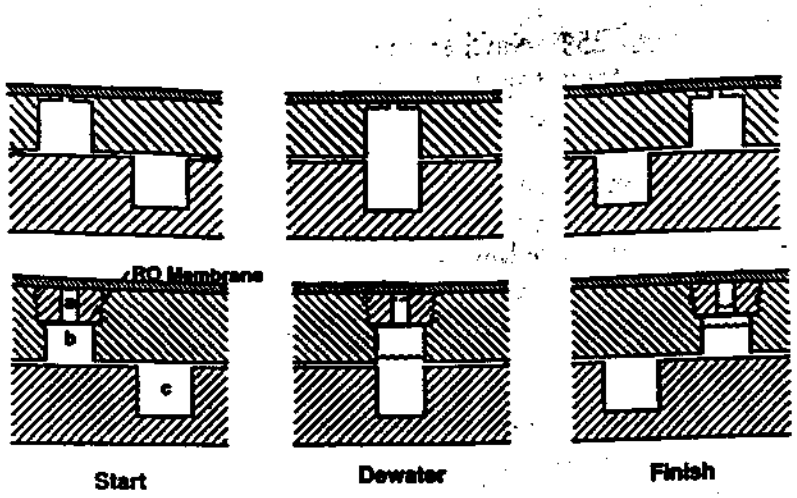

Figure 1. Operation of the Materials Dispersion Apparatus Minilab. The top row represents the sequence followed in an interfacial diffusion experiment. At launch the top well contains protein solution, and the bottom well contains salt solution. During flight (middle diagram) interfacial diffusion occurs. At the end of orbital flight (right figure) compartments are once again separated (optional). Lower row represents osmotic dewatering sequence. A subsaturated protein and salt solution is placed in compartment "a" above the reverse osmosis (RO) membrane, and a highly concentrated salt solution is placed in compartment "c", with an air gap left in compartment "b". During dewatering on orbit (middle diagram) water crosses the membrane and fills part of the air gap while the protein and salt are concentrated in compartment "a".

contact for the entire duration of the flight, which included re-entry and de-integration on the second (STS-43), but not the first (STS-37) flight. Protein concentrations were 4% - 10%(w/v) (the latter concentration is equivalent to saturation at 20°C) and NaCl concentrations from 4% to 12% (w/v). All solutions were made in 0.1 M NaAc buffer, pH 4.0.

A solution of lysozyme in 0.1 M NaAc, pH 4.0 was added to one well of a diffusion cell. Upon achievement of low gravity by the spacecraft, the other well of the diffusion cell, containing NaCl in 0.1 M acetate, pH 4.0, was brought into contact so that the salt and protein diffused freely into each other's solutions (Figure 1).

Step Diffusion

In step diffusion experiments salt and protein were dissolved together at various concentrations and placed in a chamber that briefly contacted a high then a low salt concentration. In this case, the protein-salt solution was placed in the lower chamber (see top row, Figure 1), and two chambers in the upper sliding block contained high and low salt concentrations. Initial exposure time to high salt was 10 min, which was found adequate for instantaneous nucleation.

Osmotic Dewatering

Minilab wells were adapted for the osmotic dewatering technique /5/ by filling the

bottom well completely with 25% NaCl solution in 0.1 M NaAc and placing a reverse osmosis membrane midway down the to well so that the crystallizing sample was above the membrane and an air gap was below the membrane. Salt and protein were dissolved together at various combinations of concentrations from 2 to 5% (w/v) and placed in a well with a reverse osmosis membrane at the bottom, overlying an air gap above a 25% salt solution. Thus, water was removed from the crystallizing solution through the membrane into the vapor space below it, which slowly filled with the water that was removed as the vapor condensed into the salt solution.

Crystallographic Analysis

X-ray diffraction patterns were produced at the National Synchrotron Light Source at Brookhaven National Laboratory, beam line X-12C, which is outfitted with an Enraf-Nonius FAST area-detector system.

RESULTS

Interfacial Diffusion

Concentrations of lysozyme from 4% to 10% (w/v) and of NaCl from 4% to 12% (w/v) were allowed to diffuse into one another for 4 or 10 days. Maximum crystal size increased from 0.2 to 1.2 mm with increasing diffusion time and concentration up to 10% lysozyme and 8% NaCl. Polarized light photography revealed defect-free tetragonal crystals, synchrotron x-ray diffraction patterns showed reflections to 1.47 Å and superior Wilson statistics and rocking curves when compared to earth-grown crystals. The interfacial diffusion method in therefore improved by the removal of buoyancy driven motion during crystallization.

It was found possible to effect nucleation and growth of lysozyme crystals at higher concentrations of salt and protein than are possible in the presence of gravity, where buoyancy-driven motions lead to ill-timed mixing and/or settling. Three gravity-dependent processes are absent in space flight: sedimentation of the crystals out of the supersaturated solution zone, convective mixing of the two solutions upon contact, and microconvection of the mother liquor around the growing crystal /15/. High-quality lysozyme crystals of ideal size (0.6 mm) were obtained with a starting protein concentration of 7% (w/v) and NaCl concentration of 8% (w/v), as shown in the two-parameter optimization data of Table 1.

Table 1. Concentrations of lysozyme and NaCl in wells used in partial factorial study of crystallization by double diffusion.

WELL NO.	%LYSOZYME	% NaCl	MAXIMUM CRYSTAL LENGTH, MM
1-5E	4.0	4.0	0
1-9E	4.0	5.0	0.04
1-11F	4.0	7.0	0.625
2-5E	6.0	4.0	0
2-9E	6.0	5.0	0.700
2-9F	6.0	6.0	0.270
2-13F	8.0	7.0	0.625

Polarized-light microscopy revealed that perfect crystals up to 1.2 mm were grown from higher concentrations. No monoclinic or amorphous crystallization was observed. When this method was practiced in the laboratory at 1g at the same concentrations showers of amorphous clusters of monoclinic crystals formed, and tetragonal crystals (small, <400 μm) grew only below 5% (w/v) of each solute. Higher concentrations of protein and salt can be tested in low gravity than on the ground. For example, 10% lysozyme (w/v) and 8% salt (w/v) yielded the largest and best crystals in the interfacial diffusion method, while using 12% salt resulted in shattering. These results imply that avoiding buoyancy-driven motions enhances the reliability of crystal growth from solution.

Nearly all solute transport was by diffusion. This conclusion is based on two tests of transport in the Minilabs at 1g and at low g in which convective transport was measured. As the two liquids come into contact they flow into each other at the interface if flow is not strictly laminar. The Reynolds number for this process is estimated to be about 10, well within the laminar range; however, there are discontinuities at the cavity wall, and the measured convective transport at 1g is 0.3% of the upper well volume and less at low g as determined in dye-transfer experiments /13,14/. Inertial disturbances during space flight could lead to convective flow between the cavities; this was found to be negligible on these two space shuttle flights when the transfer of suspended particles (fixed yeast cells) was measured and found not to differ from that predicted by unsteady diffusion alone. Owing to the size of these particles, it can also be concluded that sedimentation was negligible. Diffusion calculations indicated that the saturation line did not reach the end of the diffusion cell at maximum time and concentration. These results imply that diffusion is the favorable form of transport for approaching supersaturation and maintaining growth conditions in protein crystal growth.

Crystals grown at low gravity were of superior quality. All crystal samples were examined, and most were photographed at 2X, 4X or 10X magnification with unpolarized or polarized light. The better crystals were saved in vials or glass capillary tubes for subsequent evaluation by diffraction using synchrotron radiation. Polarized light photography revealed defect-free tetragonal crystals, and synchrotron x-ray diffraction of the largest crystals showed clear reflections to 1.47 Å, where, as in the case of crystals grown in the laboratory also, the diffraction patterns abruptly ended. Complete diffraction patterns were obtained to 2.4 Å resolution, and relative Wilson statistics were calculated using the published lysozyme structure factors as a standard. The relative Wilson plot is shown in Figure 1. The relative B value, ΔB, is 0.704. The superior B value indicates, as with crystals grown in low gravity by other methods /9/, that crystals grown in low gravity by interfacial diffusion are of better quality than earth-grown controls.

In a final test of crystals grown in low gravity, rocking curves were measured on several reflections with differing intensities and at different d-spacings. The results for a representative measurement are presented in Figure 2. The angle from the point of maximum spot intensity, $\Delta - \Theta$, represents, but is not equal to, the spread in diffraction angle Θ due to crystal mosaicity. The mosaic spread, η, can be derived from the expression for the sum of all causes of spread:

$$2\Delta = \delta c + \delta F + \eta + \delta\Theta$$

where 2Δ is the maximum angle through which a crystal must be rotated such that every mosaic block can diffract radiation. δc is the angle subtended by the crystal at the x-ray

of the crystal $\delta\Theta = (\delta\lambda/\lambda)\tan\Theta$ is the wavelength- dispersion spread. From the geometric and physical parameters of the synchrotron beam, $\delta c = 0.001°$, $\delta F = 0.01°$, and $\delta\lambda/\lambda = 0.0003$. Since the vertical divergence of the synchrotron beam is similar in magnitude to the rocking curve of the space-grown crystal, it was necessary to deconvolute the beam divergence from the mosaicity. Assuming a Gaussian distribution for both, the deconvoluted mosaicities of the low-g- and 1g-grown crystals become 0.0173° and 0.025°, respectively. On the basis of this data set and the published structural information on lysozyme, it is concluded that, as is also the case with vapor diffusion crystal growth /7/, the interfacial diffusion method yields more highly ordered crystals if buoyancy driven motions are avoided during crystallization.

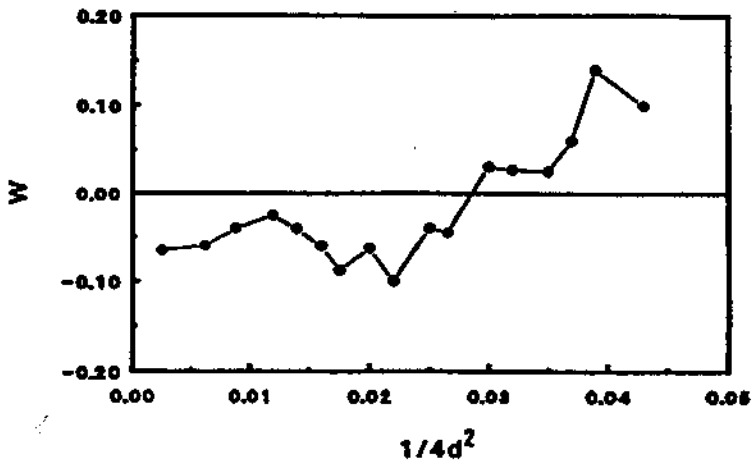

Figure 2. Relative Wilson plot for lysozyme grown in low gravity vs. lysozyme grown in the laboratory. The ordinate is the difference between the values of B (earth minus space). B is the slope of the plot of $\ln(\Sigma F_1^2/\Sigma F_L^2)$ vs. $4\sin^2\Theta/\lambda^2$, where F_1 and FL are the crystallographic structure factors for crystals grown in 1g and low g, respectively /9,18/. This relative Wilson plot reveals significant differences in B values mainly at the highest resolutions, indicating that the crystal grown in low gravity had a significantly lower (superior) overall effective B-value.

In summary, evidence is presented that gravity-dependent processes are a significant factor in the crystallization of organic macromolecules. The conclusions that can be drawn are the following: 1. Protein crystals can be grown from highly concentrated solutions, and hence to larger size in shorter times (up to 1.2 mm in 10 days), when the double-diffusion method is used in low gravity. 2. Protein crystals grown by double diffusion in low gravity, like those grown by vapor diffusion in low gravity, display a higher degree of order than their earth-grown counterparts. 3. The Minilab system provides a capability to perform factorial experiments in low gravity, an essential requirement for the future crystallization of new proteins on space flights.

Although this study was motivated as a means of evaluating a system that provides cost-effective, frequent access to low gravity, it also demonstrated that continued research in low gravity can provide interesting and important information about the physical processes of crystal growth.

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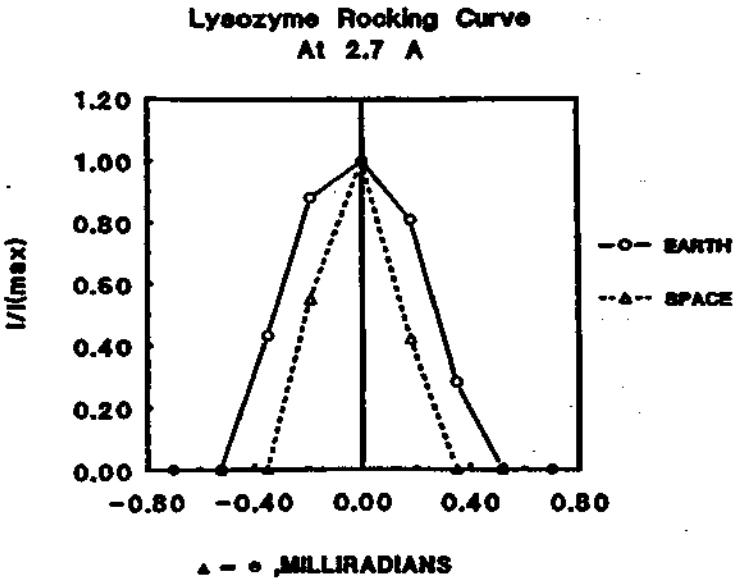

Figure 3. Rocking curves for a typical reflection from lysozyme crystals grown in low gravity (+) and in the laboratory (o). Diffraction data were obtained as in Figure 2.

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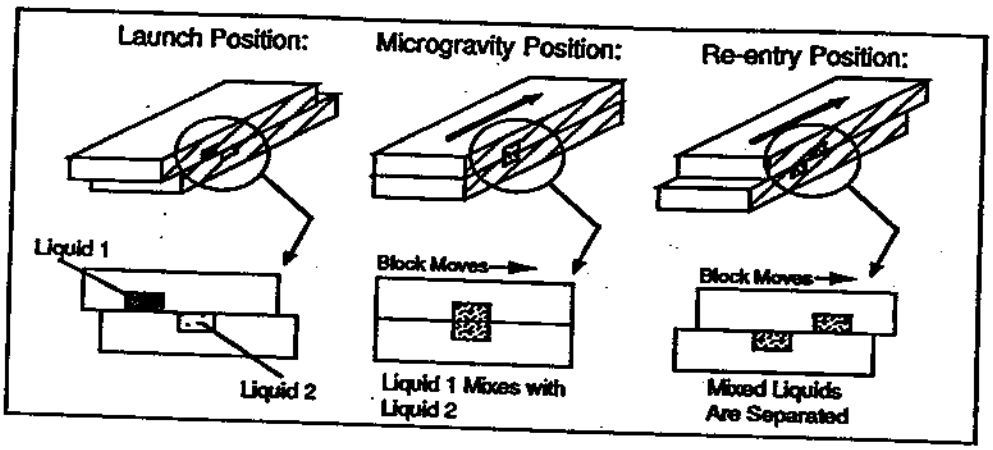
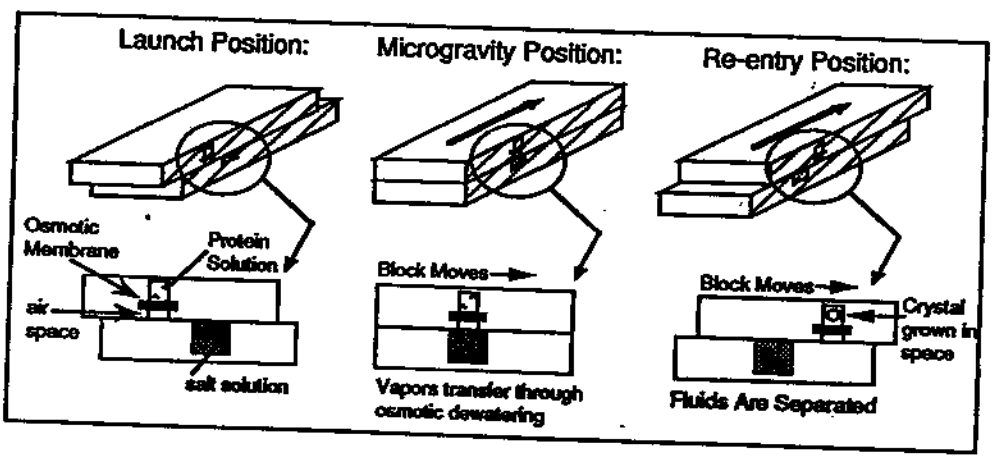
The MDA Minilab grows protein crystals using three separate techniques

LIQUID-LIQUID (DOUBLE DIFFUSION) TECHNIQUE



OSMOTIC DEWATERING (VAPOR DIFFUSION) TECHNIQUE



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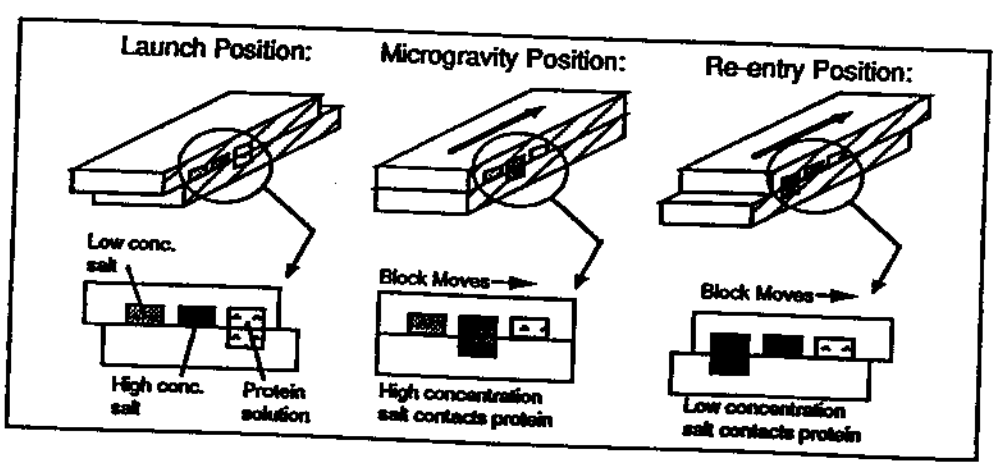

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Protein Crystal of Lysozyme Grown by Liquid-Liquid (Double) Diffusion in the MDA Minilab on STS-43

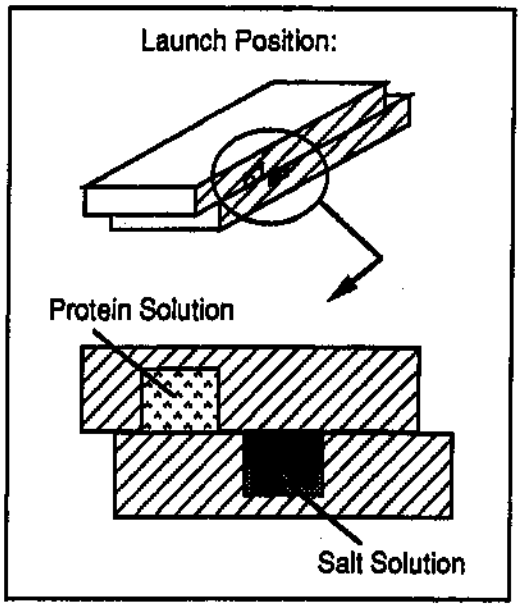

Photo courtesy of Dr. Paul Todd and Dr. Richard Korszun

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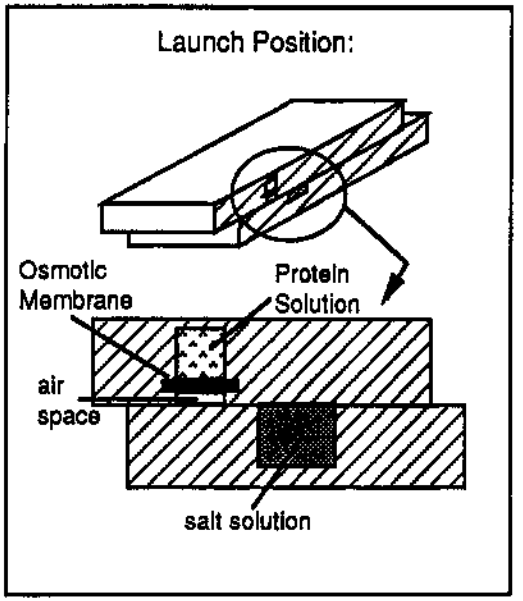
Protein Crystal of Lysozyme Grown by Vapor Diffusion (Osmotic Dewatering) in the MDA Minilab on STS-43



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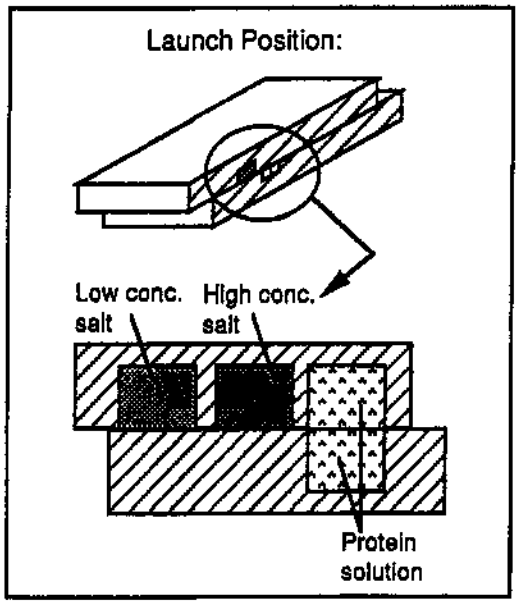
Protein Crystal of Lysozyme Grown by Gradient Diffusion in the MDA Minilab on STS-43



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