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The Facts and Fancy of Microgravity Protein Crystallization

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Introduction

The first step in the elucidation of the three-dimensional structure of a molecule by X-ray crystallography is the growth of diffraction quality crystals of that molecule. Many diverse techniques have been developed to obtain crystals of the requisite quality. Growth of crystals in the absence of gravity has been hypothesized to be one such method that might be of general utility for the improvement of crystal quality. Since 1983, over a dozen experiments have been conducted in low Earth orbit on a variety of platforms by several consortiums of American and European scientists in an effort to take advantage of this method. In this review, we explore the scientific basis for this hypothesis and evaluate the published results from these experiments, a particularly timely undertaking since the US Congress is currently considering spending tens of *billions* of dollars to build a space station which, among other tasks, would be used as a microgravity crystallization laboratory.

X-ray crystallography is a powerful technique for the elucidation of the three-dimensional structures of biological macromolecules, but depends upon the availability of suitable crystals. Crystallography has yielded the structures of hundreds of proteins, nucleic acids and viruses, as can be seen by a casual perusal of the literature since the first crystal structure of a protein was determined in 1960 by Kendrew *et al.* The availability of these structures has profoundly altered our understanding of the structural basis of enzymology, immunology and molecular biology. Visualization of the structures of proteins involved in disease has extended our understanding of the mechanisms

Abbreviations: BLD, boundary layer diffusion device; HEWL, hen egg white lysozyme; PMP, polymethylpentene; RNase S, ribonuclease S; 10-THFS, 10-formyl tetrahydrofolate synthetase; VD, vapour-diffusion device.

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of illness to the molecular level, and rational drug design holds the promise of creating new treatments for many of these disorders. X-ray crystallography remains a difficult and demanding technique, though several advances have made structures much more amenable to solution in the past decade. These advances include an enormous increase in the computer power affordable by the average crystallographer as well as the development of equally powerful computer software and algorithms, the development of X-ray sources and detector systems in individual laboratories and at synchrotron light source facilities, and advances in molecular biology which have allowed for the production of large quantities of pure proteins through recombinant expression systems.

However, the process of X-ray crystallography remains dependent on the availability of diffraction-quality crystals of the molecule to be studied, hence the term 'crystallography'. The physical processes underlying the crystallization of biological macromolecules remain to be determined. Consequently, the methods used by crystallographers to crystallize proteins have been determined by trial and error for the most part; rational algorithms for determining the crystallization conditions for a new protein do not exist. Often the crystals obtained of a given sample prove to be unsuitable for diffraction experiments. In these cases, it is not always apparent what to try next, or even if there is any set of conditions that will yield suitable crystals. Because of these reasons, crystallization is often described as the 'bottle neck' of X-ray crystallography, and explains the intense interest in developing techniques that might yield diffraction-quality crystals from situations where there are only poor-quality crystals or none at all.

Protein crystallization and the microgravity environment

Biological macromolecules are crystallized by creating the necessary degree of supersaturation in an appropriate chemical solution. The traditional methods of protein crystallization (see McPherson, 1982, for a thorough review) involve mixing a protein solution in an appropriate buffer (to maintain a suitable pH and ionic strength) with a precipitating agent. For biological macromolecules, these agents include salts (such as ammonium sulphate), organic solvents (such as 2-methyl-2, 4-pentanediol) and organic polymers (such as polyethylene glycol). If the conditions have been chosen correctly, the formation of suitable nuclei occurs, inducing the growth of crystals. Otherwise, the result is usually an amorphous precipitant. Nuclei form in protein and precipitant solutions at characteristic degrees of supersaturation, which can be quite high for proteins. Nucleation can occur on dust particles or on irregularities in the surface of the container. In the simplest crystallization method, known as 'batch' crystallization, the initial solutions are set up at the necessary degree of supersaturation at the beginning of the experiment. The crystallographer simply waits for the formation of nuclei and the growth of crystals once the solutions have been mixed.

The most commonly used method of crystallization, the vapour dialysis technique, involves mixing a volume of protein solution with some initial

concentration of the chosen precipitating agent. A drop of this mixture is then suspended in a sealed container together with a much larger volume, or reservoir, of a solution of the precipitating agent at the desired final concentration for the experiment. The drop then equilibrates with the reservoir through the vapour phase, resulting in a reduction in the size of the drop with a concurrent increase in the concentrations of the protein and the precipitant. If the conditions have been chosen correctly, the necessary degree of supersaturation will be reached and crystals will be obtained. The advantages of vapour diffusion experiments include the ability of the vapour diffusion experiment to be conducted on smaller quantities of protein than are usually used in batch experiments, an important point when one considers the arduous task of obtaining proteins which are difficult to purify. The vapour dialysis experiment also gives the crystallographer some control over the approach to nucleation, since unlike the batch methods, the experiment does not involve static concentrations.

In the microdialysis or free-interface diffusion experiment, a variation on the vapour dialysis method, the drop of protein or precipitant solution is separated from the precipitant solution reservoir not by an air space but either by a dialysis membrane (microdialysis) or by layering the two solutions on top of each other and depending on the difference in density of the two solutions to keep them separate (free-interface diffusion). In this technique, the protein concentration does not change, as the volumes remain constant; the diffusion of the protein is either restrained by the dialysis membrane or by the greater mass of the protein relative to the precipitating agent. Only the precipitant concentration varies, giving the crystallographer yet another means to approach nucleation. (*see Figure 1* for a graphical description of these techniques).

The elimination of gravity from crystallization experiments may reduce the detrimental effects of several processes and create conditions highly conducive to the growth of high-quality crystals. In the Earth's gravity well, concentration gradients in solutions and air spaces lead to the formation of convection currents through differences in density; everyday examples of this phenomenon include hot air balloons and oil rising to the top of salad dressing. Many consequences of this process occur in crystallization experiments. (1) Protein crystals are usually denser than the solutions they grow from, and therefore crystals not attached to a surface tend to collect in the bottom of hanging drops or crystallization vials. They bump into each other and clearly interact during their growth (Koszelak and McPherson, 1988). Crystals often grow fused together, limiting their utility in diffraction experiments, and the close proximity of growing crystals may hinder or limit growth. (2) Convection currents in sealed crystallization chambers lead to the formation of convection cells in the air spaces of vapour diffusion experiments, which hastens the approach to equilibrium, possibly to the detriment of the resultant crystals. (3) It is often impossible to set up free-interface diffusion experiments in the laboratory because the density differences between the different solutions are insufficient to establish stable solution layers. (4) Perhaps the most detrimental effect occurs at the surface of the

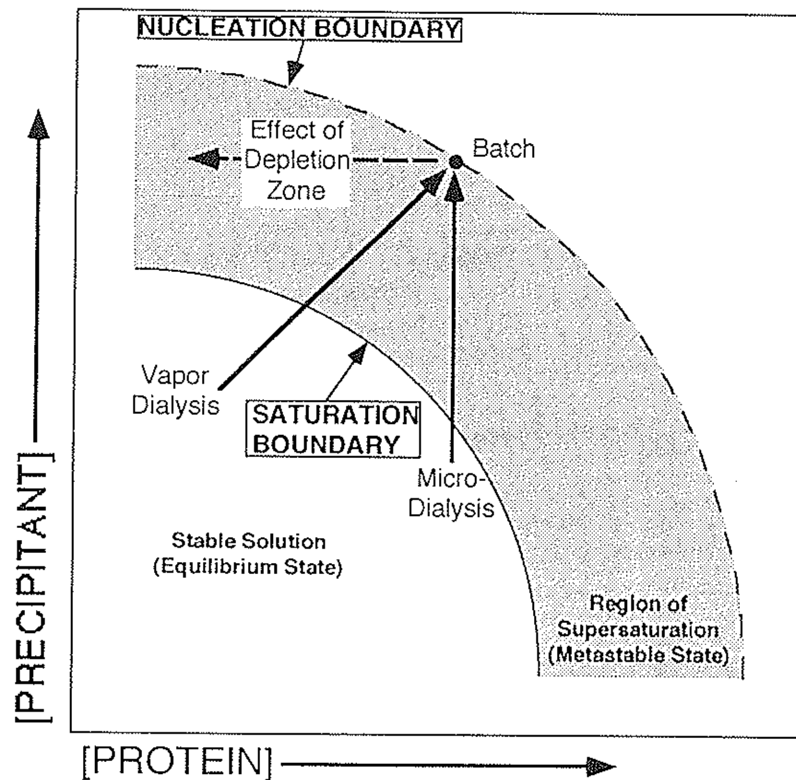

Figure 1. Phase diagram for protein crystallization. The growth of crystals involves the creation of a supersaturated solution of protein, usually at a high concentration, and a chemical precipitating agent. This concentration region is shown as the grey shaded area of protein *vs* precipitant concentration. Batch methods of crystallization are initially mixed at a concentration condition where supersaturation exists at time = 0, and nucleation then occurs without any subsequent equilibration effects. This method often works when nucleation is a very slow, kinetically rate-limited process. Vapour dialysis provides a method of concentration equilibration in which the concentrations of both protein and precipitant increase over time; often, pH gradients can also be created through the use of volatile acidic or basic agents in the protein drop. Concentration equilibration also occurs in microdialysis (liquid diffusion) experiments, but in this case only the concentration of precipitant increases. The initial concentration of protein and precipitant can represent a sub- or supersaturated solution.

growing crystal. As the crystal grows, protein is abstracted from the solution adjacent to the surface of the crystal resulting in a 'depletion zone' around an actively growing crystal. This zone is less dense than the bulk growth solution and rises in a stream from the crystal, which can be visualized photographically (DeLucas and Bugg, 1991; Shlichta, 1986). This results in turbulence near the growing surface, potentially to its detriment by encouraging the introduction of dislocations or other imperfections into the growing crystal.

None of these drawbacks would exist in a crystallization experiment conducted in the microgravity environment found in low Earth orbit. In addition, there may be some unique advantages to such an experiment. In particular, the 'depletion zone' now stably formed around a growing crystal, hanging suspended in the middle of the growth solution, undisturbed by close neighbours, would act to lower the degree of supersaturation locally around the crystal. This results in slower, potentially more controlled, growth of a

crystal after nucleation (*see Figure 1*). In ground-based experiments, the crystal grows at the degree of supersaturation that is necessary for the formation of nuclei, which may be considerably higher than that necessary to sustain growth. The result is rapid growth that may limit the order, and therefore the quality of the resultant crystals. The combination of these potential benefits has been the driving force behind 10 years of microgravity crystallization experiments.

Microgravity-based macromolecular crystallization: History and results

In the past 10 years, over a dozen experiments have been conducted by several consortiums of American and European scientists on the US Space Shuttle, the Russian *Mir* Space Station and various unmanned platforms using a variety of crystallization hardware. These experiments are listed in *Table 1*, and cover a wide range of crystallization hardware, methodology and macromolecules. The majority of the experiments have been conducted by three consortiums: the University of Alabama at Birmingham group (the Center for Microgravity Research), headed by Lawrence DeLucas and Charlie Bugg, which conducts experiments on the US Shuttle; a group of primarily Western European crystallographers, which conducts experiments on unmanned, retrievable orbiters, organized by the company INTOSPACE (the COSIMA missions); and the experiments organized by Payload Systems, Inc. which fly on the Russian space station *Mir*, for which the authors of this review act as the Science Advisory Board (the PSI missions). Table 1 does not include recently performed microgravity experiments (such as USML-1 on the US Shuttle), planned experiments (such as Spacelab D-2, the PSI-3 mission or the EURECA free-flyer experiments), or failed experiments (such as the COSIMA-3 and COSIMA-4 missions), for which there are no published results. In addition, the Russian crystallographic community and a small number of collaborators have been performing microgravity crystallization experiments for many years, both on *Mir* and on the *Photon* orbiter, but few of the results of these experiments have been published. The results of these experiments must be interpreted in ways that are mindful of the difficulties inherent in conducting any experiment on an orbital platform, while acknowledging the subjective nature of the concept of crystal quality.

A number of different crystallization geometries and designs have been utilized over the past 10 years in microgravity. As discussed above, there are four basic methods for producing equilibration of protein solutions to a supersaturated state: bulk, batch, liquid-interface diffusion (which is usually performed by the use of a dialysis membrane barrier between protein and precipitant in the Earth-bound laboratory) and vapour-diffusion (using hanging or sitting drop geometries). In microgravity, virtually all reported experiments have used slow-diffusion equilibration methods, using either free liquid-interface diffusion or vapour diffusion. Due to the lack of gravity-dependent density forces, different solutions of varying density can equilibrate directly in microgravity without the use of dialysis membranes. Also, vapour dialysis can be performed without the consideration of hanging versus

Table 1. Previously reported microgravity-based crystallizations

	Platform	Date	Experiments	Duration	Comments
PSI-2	*Mir* (Russia)	Jan-March 1992	19 proteins	57 days	Vapour-diffusion experiments
IML-1	US Shuttle/ Spacelab	Jan 1992	>2 proteins	8 days	Free-interface diffusion experiments
MASER3	Sounding rocket	1991	RNase S	7 min	Short-duration microgravity
STS-31	US Shuttle	April 1990	?	?	Vapour-diffusion experiments
PSI-1	*Mir* (USSR)	Dec 1989 to Feb 1990	HEWL and Datase	56 days	Free-interface and vapour-diffusion experiments
STS-32	US Shuttle	Jan 1990	?	?	Vapour-diffusion experiments
COSIMA-2	*Photon* (USSR)	Sept 1989	>12 proteins	10 days	Vapour-diffusion experiments
STS-29	US Shuttle	March 1989	15 proteins	?	Vapour-diffusion experiments
STS-26	US Shuttle	Nov 1988	8 proteins	3 days	Vapour-diffusion experiments
COSIMA-1	*Long March* (China)	1988	>12 proteins	8 days	Vapour-diffusion experiments
Russian	*Photon* (USSR)	April 1988	5 proteins	14 days	Free-interface diffusion experiments
STS-61C	US Shuttle	Jan 1986			While many crystallization experiments were conducted, these flights were primarily for hardware development
STS-61B	US Shuttle	Nov 1985	12 proteins/ 3 nucleic acids	4–10 days	
STS-51F	US Shuttle	July 1985			
STS-51D	US Shuttle	April 1985			
STS-61A Spacelab D-1	US Shuttle/ Spacelab	Oct 1985	β-gal and HEWL	?	Failure of automated hardware
STS-9 Spacelab-1	US Shuttle/ Spacelab	Nov 1983		β-gal and HEWL	Free-interface diffusion experiments

References: Day and McPherson (1992), DeLucas and Bugg (1991), DeLucas *et al.* (1986, 1991), Littke and John (1984), Stoddard *et al.* (1992), Strong *et al.* (1992), Trakhanov *et al.* (1991).

sitting drop geometries. Below, we summarize the hardware design considerations used during protein crystallization experiments on *Mir* (Strong *et al.*, 1992; Stoddard *et al.*, 1992) as being representative of one particular hardware design used in these experiments. For a good overview of the hardware design used on the US Space Shuttle, see DeLucas and Bugg (1990, 1991) and DeLucas *et al.* (1991).

VAPOUR DIFFUSION CRYSTALLIZATIONS

The vapour-diffusion device, or VD (*Figure 2*), was designed to reproduce hanging or sitting drop techniques in a microgravity environment. Equilibration in this experiment occurs through differences in osmotic pressure between the protein solution (initially at low ionic strength) and a reservoir solution (initially at high ionic strength), which leads to bulk transfer of water through the vapour phase and an increase in protein and precipitant concentration to supersaturation. The device is designed to maintain physical separation of the two solutions during flight and recovery, and to allow activation and creation of a vapour pathway between protein and reservoir on orbit. The bodies of the devices were originally constructed of polymethylpentene (PMP), a light (0.83 g cm^{-3}), strong (430 kg cm^{-2} bending strength) and inert plastic with a high melting point (235°C), which is hydrophobic and non-interactive with macromolecules. The devices are now constructed from a variety of other materials, such as polycarbonate and cel-cast acrylic plastic, depending upon the preferences of the investigator. Protein solutions, mixed with a low initial concentration of precipitant are placed in cups of 10, 25 or 50 µl (*Figure 2a*), which are surrounded by moats of 500–750 µl of precipitant at the final concentration (*Figure 2B*). The precipitant in each moat is immobilized by a coil of filter paper. A screw-plunger in the top plate of the device is closed down on the protein cup. When screwed down, the plunger presses a silicone rubber septum against the edges of the protein-filled cup, thus sealing the protein solution away from the precipitant. To allow vapour-phase equilibration (activation), the septum is raised, establishing an air path between the solutions. To deactivate the experiment, the cup is resealed with the septum. The device has a template attached to the bottom plate for photographing the protein wells. The surfaces of the optical pathway are polished to photography-grade flatness and quality.

BOUNDARY LAYER CRYSTALLIZATIONS

The basic principle of equilibration to supersaturation using boundary layer crystallization methods is similar to vapour-phase diffusion, although in this case the process of diffusion occurs across a direct solution interface. A stable interface can be created in microgravity due to the lack of density differences. The boundary layer diffusion device, or BLD (*Figure 3*), was derived from a proposal of Littke and John. Protein and precipitant solutions are held in chambers separated by a rotating shaft with connecting chambers filled with 'neutral' buffer (no salt or protein).

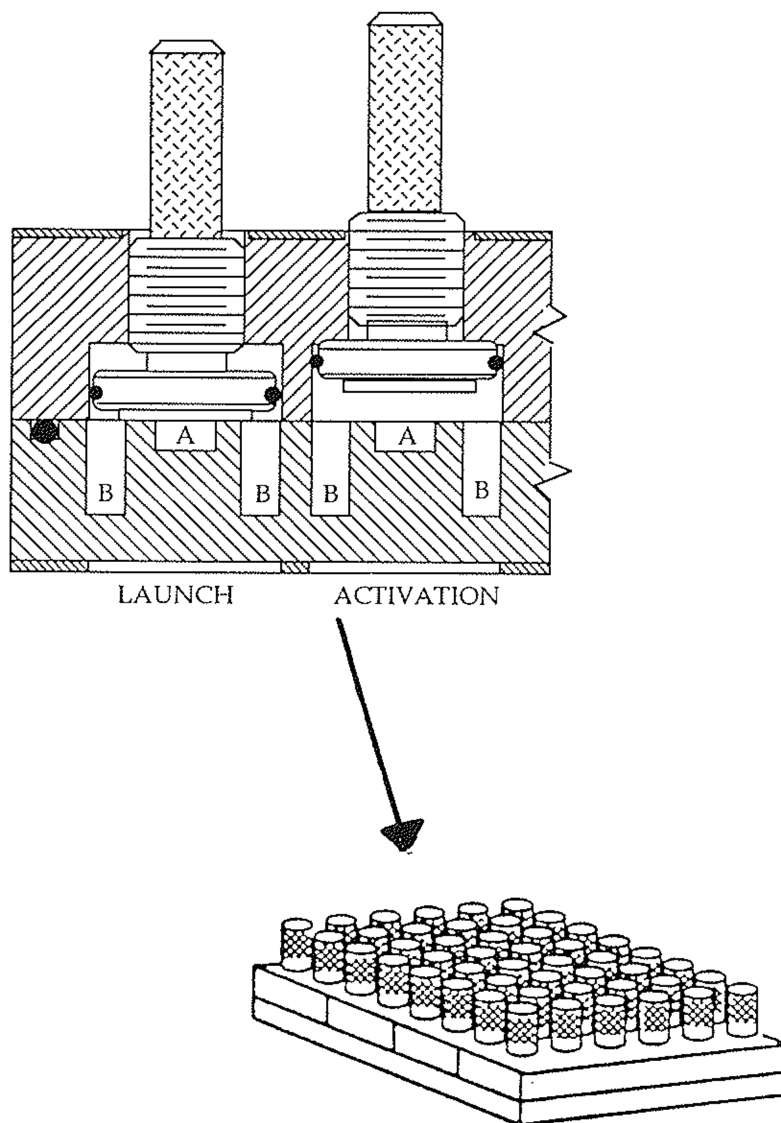

Figure 2. Vapour-diffusion crystallization device (Strong *et al.*, 1992). The device contains a protein well of variable volume (A) and a reservoir containing a high initial concentration of precipitating agent (B). A silicon rubber septa seals the protein well during launch, and is raised through the use of a manually operated thumbscrew. Variations in this design allow on-orbit mixing of protein and precipitant. Each device currently contains 48 individual crystallization wells.

The BLD device is constructed of the same materials as the vapour-diffusion device. The teflon screws sealing the individual chambers incorporate a teflon-coated silicone rubber septum. As each device is sealed, trapped air is removed by inserting needles through the septa and into the chambers. For the first flight, each chamber accommodated 500 μl of protein solution,

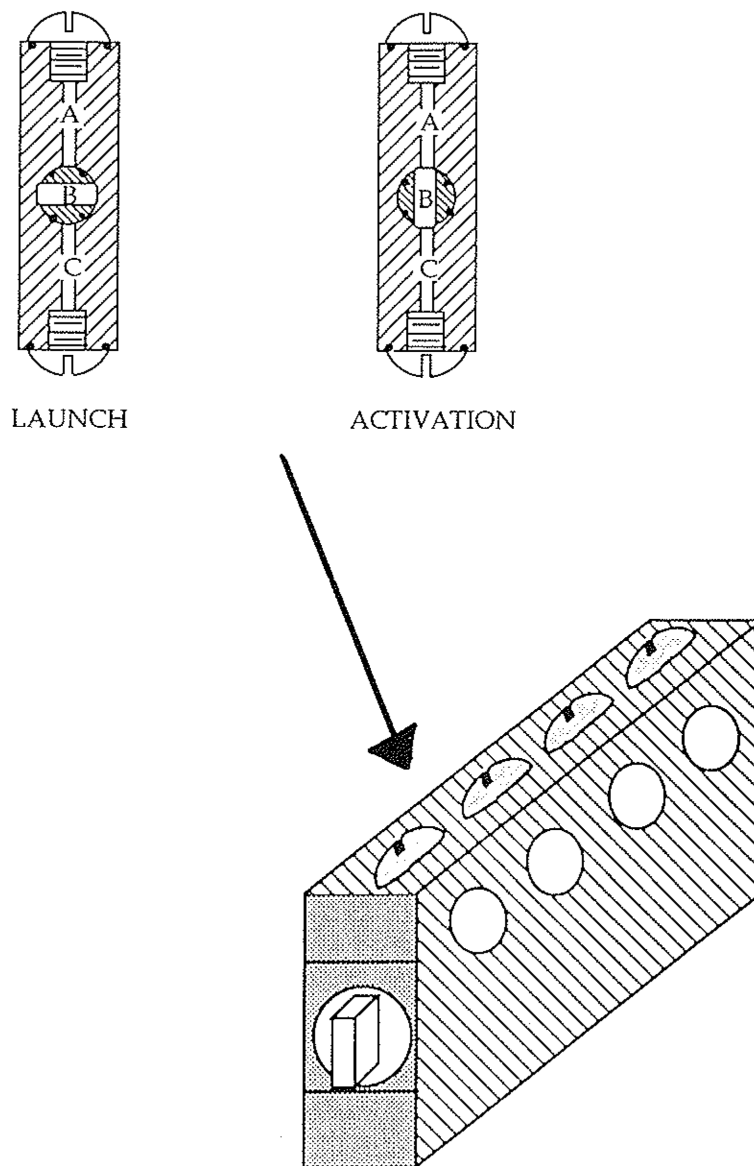

Figure 3. Boundary layer diffusion device. The device contains a chamber for protein (A), a chamber of equal volume containing precipitant (C), and an intermediate chamber containing neutral buffer (B), which is held in a closed, non-equilibrating position during launch, and then rotated to establish a pathway of liquid–liquid diffusion upon activation. Each device contains four crystallization chambers, and 16 devices are used in each flight complement, allowing 64 crystallization experiments to be performed.

500 μl of precipitant and 75 μl of neutral buffer. All photographic path surfaces were polished to the same optical clarity as the vapour-diffusion device.

ASSESSMENT OF PROTEIN CRYSTALLIZATION RESULTS FROM MICROGRAVITY

Protein crystallization experiments cannot be transferred directly from the ground-based laboratory to a microgravity platform. There are numerous requirements that a microgravity experiment must meet that do not apply to a ground-based experiment. Hardware must be designed which is biocompatible and does not interfere with the process of crystallization, but which can also withstand the rigours of launch and recovery. The equipment must be designed to be set up in a field laboratory, protecting the delicate biological samples, preventing the onset of crystal growth until a stable microgravity environment can be reached. The device must be able to be activated once this environment is reached. The hardware must protect the crystals from any damage the recovery process might inflict. The temperature must be maintained at acceptable levels. Lastly, the crews of the manned platforms must be protected from any toxic components present in the experiment. These requirements have been met in several different ways by the groups conducting these experiments. The fact that the microgravity crystallization hardware ends up looking quite dissimilar from the apparatus used by the average crystallographer in the laboratory, means that careful control experiments must be performed to isolate any effects the hardware alone may have on crystal quality. For example, the very first protein microgravity crystalliation experiment (Littke and John, 1984) was performed with the protein lysozyme isolated from hen egg whites (HEWL). The crystals grown in orbit were described as being '1000-fold larger' than those grown in the crystallization hardware on the ground. However, the crystals grown in orbit were below the average size of crystals of HEWL grown in the laboratory, and those grown in the same hardware on Earth were far worse. Clearly, the hardware as designed detrimentally affected crystallization, an effect that microgravity may have overcome to a certain degree.

What criteria *should* be used for judging crystal quality? It is straightforward to visually gauge the size of a crystal, and to even judge its relative 'perfection' in terms of morphology. But the only quality that truly matters is the performance of a given crystal in a diffraction experiment, and its success in providing structural information. The quality that crystallographers are most interested in measuring is the resolution limit of the diffraction data collectable from a crystal. High-resolution data mean that the details of a protein structure are easier to determine; low-resolution data mean that the determination of an unambiguous structure may not be possible. In the case of lysozyme crystals, despite the use of this protein in more microgravity experiments than any other, crystals of HEWL grown in microgravity do not yield higher resolution diffraction data than HEWL crystals grown on the ground. Another example, the protein canavalin (Day and McPherson, 1992), yields beautiful, flawless crystals in microgravity experiments, which nevertheless do not provide diffraction data that are significantly better than crystals grown on the ground.

The results of 10 years of microgravity crystallization experiments from a

number of different groups have consistently shown that 20–25% of the macromolecular crystals grown in orbit yield modestly improved diffraction data, as a function of resolution limits and signal-to-noise ratios throughout all observable resolution shells. For those fractions of the crystals which are improved by growth in the absence of gravity, the resolution limit of the diffraction data is extended by 0.1 Å to 0.5 Å. This level of improvement is not dramatic; it would not usually make a significant difference in determining the structure of a protein from unsuitable crystals. Microgravity crystallization has never yielded crystals that were suitable for diffraction experiments from a crystallization problem where the laboratory-grown crystals were not also usable. However, a small number of space-grown crystals have provided diffraction data, which have at least been utilized during refinement and high-resolution refinement. (1) Microgravity-derived diffraction data from one protein crystallization problem, γ-interferon (Ealick *et al.*, 1991), has been used in a published structure; the determination of that structure did not depend on the improved microgravity data. (2) Crystals of a second protein, satellite tobacco mosaic virus, show a 0.3 Å improvement in diffraction resolution when grown in microgravity (Day and McPherson, 1992); these data will be used in the refinement of the structure of this virus, which had been determined from laboratory-grown crystals (Koszelak, Dodds and McPherson, 1989). While not dramatic, this level of improvement is real and can make the determination of a three-dimensional structure considerably easier or the interpretation of a structure more significant. Other protein crystals which have displayed small improvements in size, internal perfection and scattering power when grown in microgravity include porcine elastase, *Lathyrus ochrus* lectin I protein, canavalin, anti-HPr Fab, anti-Brucella Fab, isocitrate dehydrogenase, and the 'Z' crystal form of ribonuclease S (DeLucas and Bugg, 1990, 1991; McPherson, Greenwood and Day, 1991; DeLucas *et al.*, 1991; Stoddard *et al.*, 1992; Asano *et al.*, 1992).

In addition to the modest improvements in resolution limits summarized above, a small number of the proteins which have been flown and crystallized in microgravity have initially been reported as *exceptional* successes, including lysozyme (Littke and John, 1984), isocitrate lyase (DeLucas and Bugg, 1991), 10-formyl tetrahydrofolate synthetase (10-THFS) and aspartate ammonia lyase (Stoddard *et al.*, 1992). As described above, the conclusions published after the first crystallization of lysozyme in microgravity have been criticized as being misdirected and plagued by a lack of adequate control experiments. After careful ground control studies and further investigations in the laboratory, the other three 'successes' have been reproduced or dramatically exceeded on Earth, as described below. In addition, another protein (albumin) which produced improved or slightly improved crystals in microgravity, subsequently was shown to improve to an equivalent degree through the use of a gel matrix during crystallization, and another protein which was flown *unsuccessfully* on *Mir* (anti-chymotrypsin, a serpin inhibitor) was subsequently successfully crystallized after incorporation of a disulphide cross-link at the C-terminus of the protein (Stoddard *et al.*, 1992; Katz *et al.*, 1993), as described below.

Isocitrate lyase

This protein was originally crystallized as dendritic clusters, subsequently characterized as belonging to space group $P2_12_12_1$. An improved crystal morphology belonging to the same space group was grown in the microgravity experiments conducted on the shuttle missions STS-26 and STS-32. These crystals grew as well-formed prisms and diffracted to considerable higher resolution (DeLucas and Bugg, 1991). However, subsequent careful ground control experiments indicate that this improved crystal morphology was due solely to the altered evaporation and equilibration rate in the crystallization hardware, and could be duplicated on the ground under several different conditions (DeMattei, Feigelson and Weber, 1992).

10-THFS

Initial crystallization trials of tetrahydrofolate synthetase from *Clostridia acidici-urici*, spanning a 2 year period, yielded only long needle-like specimens which did not diffract measurably. Crystallization on *Mir* yielded rhombs which diffracted to approximately 30 Å resolution (Stoddard *et al.*, 1992). However, subsequent intense ground controls in the same hardware on Earth reproduced this result, which appeared to be caused by the temperature profile which existed on *Mir* during crystallization rather than by the microgravity environment. More importantly, two independent investigators have succeeded in growing large, single crystals of this enzyme which diffract to high resolution. The enzyme described above from *C. acidici-urici* produced large orthrhombic crystals, space group $P2_12_12$, which diffract to approximately 2.5 Å resolution after simply reducing the temperature from 20°C to 18°C (D'Ari *et al.*, submitted). Simultaneously, a second group has also produced large, well-diffracting crystals of the same enzyme by using an alternate biological source, *Clostridium thermoaceticum* (Lewinski *et al.*, 1993). Structure determination is proceeding in both laboratories.

Aspartate ammonia lyase

The same mission flown on the *Mir* Space Station also produced crystals of aspartate ammonia lyase (aspartase) from the vapour diffusion set-ups which diffracted to significantly higher resolution (3.2 Å) than their ground-based counterparts, which diffracted to 4.0 Å resolution (Stoddard *et al.*, 1992). However, recent work in the laboratory has yielded crystals of much greater size and volume, simply by utilizing the dialysis equilibration method rather than vapour diffusion and by adding significant amounts of the reducing agent dithiothreitol to the mother liquor. These crystals diffract to better than 2.8 Å resolution and have been characterized as belonging to space group $P2_12_12$. Structure determination is proceeding.

Human serum albumin

Crystals of human serum albumin were grown on the STS-31 shuttle mission and subsequently shown to be larger than Earth-grown specimens and to diffract slightly more strongly (DeLucas and Bugg, 1991). However, crystals grown in gels on the ground were comparable to the microgravity-grown crystals (Miller, He and Carter, 1992).

It is worth noting that the above examples of protein crystallization experiments, in which the results from microgravity were subsequently matched or vastly exceeded by further ground-based crystallization attempts, probably represents a very small number of the total number of crystallizations flown in orbit for which this result has occurred. This is due primarily to the fact that almost all the published experiments from microgravity platforms present detailed analyses only of successful crystallizations, so that accurate book-keeping and comparison of all space-based crystallization attempts with the further utilization of standard laboratory crystallization methods is extremely difficult. Only in the case of the recent crystal growth experiments on *Mir* (Strong *et al.*, 1992; Stoddard *et al.*, 1992) has the detailed publication of all attempted crystallization experiments led to the identification of at least one species (anti-chymotrypsin) which completely failed to crystallize in microgravity and which later produced excellent specimens through the use of targetted disulphide cross-linking, as discussed above.

Earth-bound crystallization: More crystals for the money, more structures for the effort

Microgravity-based crystallization has often, perhaps always, been justified to a large extent because crystallization is a 'bottle neck' in the elucidation of macromolecular three-dimensional structures. Historically, this is true, and continues to be true today. However, currently, this is due not only to the inherent difficulties of the crystallization process, but also because of the incredible advances in data collection hardware, computer power, memory and speed, and molecular modelling and refinement algorithms which have appeared in the past 5–10 years. At the time that the first several dozen X-ray structures of proteins were deduced, there were no area detectors, synchrotron radiation sources, low-temperature cryostats, powerful unix-based workstations or CRAY supercomputers, or simulated annealing algorithms for molecular dynamics and refinement. Therefore, even though today crystallization still represents the most time-consuming portion of structure determination, the rate of new structures being solved continues to accelerate, and the amount of time necessary for any crystallization project to succeed drops dramatically each year: the number of abstracted new structures in *Macromolecular Structures* (Hendrickson and Wuttrich, 1991, 1992) jumped from 134 in 1991 to 165 in 1992, a rate of increase which Hendrickson and Wuttrich

take to indicate that the rate of new structure determinations will climb into the thousands each year by the end of the decade.

In addition to enormous improvements in data collection efficiency and computational power, the success rate of attempted macromolecular crystallizations has itself increased dramatically over the past 10 years. The rapid pace of novel crystallizations is due to a few technologies being exploited by structural laboratories which are increasingly adept in their use: (1) intense, thorough purification of proteins and nucleic acids; (2) genetic manipulation and over-expression of the targets of crystallization; (3) determination of aggregation state, binding stoichoimetry and solution behaviour by electrophoretic, chromatographic and dynamic light scattering techniques; and (4) accurate environmental control and variation during crystallization. In addition, improvements in scattering power (diffraction resolution limits) of data quality crystals are currently possible through four unrelated technologies: (1) optimization of molecular constructs through genetic engineering or chemical modification to eliminate disorder effects; (2) use of low temperature to reduce or eliminate radiation-dependent crystal decay and therefore improve counting statistics, data redundancy and accuracy; (3) the use of extremely sensitive area detectors, particularly phosphor-plate systems; and (4) the availability of synchrotron radiation. In this section of our review, we summarize the importance of various methodologies on macromolecular crystallization and provide an abbreviated sampling of structures solved in the past 2 years through their use. The question we then ask is simple: Do we really need microgravity-based protein crystallization, or would the field of structural biology be far better served by continued effort and investment in these 'standard' methodologies?

In contrast to microgravity-based crystalliation, which in over 10 years has yet to produce a single macromolecular crystal that has been shown to be necessary to produce a structure determination, it is clear from *Table 2* that a number of techniques which have become widely used in the past 10 years have been instrumental in producing an impressive number of important new structures. Of these, two main areas stand out: complete purification of the target macromolecular population to macro- and micro-homogeneity, and the use of DNA recombinant techniques to vary the primary structure of the protein to the advantage of the crystallographer.

CRYSTALLIZABILITY IS DIRECTLY CORRELATED TO PURITY

Studies of virtually every protein under crystallographic study shows that impurities, often undetected by simple denaturing electrophoretic analysis, are almost always deleterious to crystallization. Differences throughout the molecular population with respect to variable isoforms, conformers and post-translational modifications of the same protein are often the culprit in poor crystallization results, and are often inadequately characterized. A prime example is the enzyme alanine racemase, which has produced twinned crystals for the past 10 years (Neidhart *et al.*, 1987). Recently, a single additional purification step, which eliminated a minor subpopulation of the

enzyme, has produced improved crystals, and a structure determination is now forthcoming (D. Ringe and G. Petsko, pers. comm.). Similarly, the periplasmic domain of the Aspartate receptor and the MAX oncogene product could not be crystallized until adequate purification (Milburn *et al.*, 1991; Ferre-D'Amare *et al.*, 1993). Any experienced crystallographer can produce similar cases from their own experiences. As McPherson (1982) accurately states: 'There are numerous sources of microheterogeneity in an otherwise pure preparation, and an awareness of some of the more common of these is essential in diagnosing the source of a particular [crystallization] problem . . . though there are undoubtedly other causes as well, this list can serve as a starting point for scrutiny at the molecular level.' It is not unreasonable, therefore, to state that for those molecular species which do not crystallize readily, the best course to pursue is analysis of purity and solution behaviour, followed by further purification and possibly genetic manipulation as described below.

SUCCESSFUL CRYSTALLIZATION STRATEGIES

Variation in the overall expressed protein construct, *N*- and *C*-termini locations, subunit and lattice contacts by genetic manipulation, combined with accurate and thorough characterization and purification, is the most likely strategy for crystallization. As can be seen from *Table 2*, the most successful technique by which X-ray crystallographers have improved the success rate of protein crystallization is by altering the protein at the genetic level. It was noted many years ago that occasionally a site-directed mutant of an interesting protein was more easily crystallizable than wild-type protein; this effect was shown to be due to the presence of the relevant site in a lattice contact region. Currently, a number of methods have been shown to produce high-quality crystals, the most common of which is simply to crystallize a construct which represents a subset of the entire wild-type sequence, and which constitutes a domain containing the important functionalities of the intact protein. Approximately 10–20% of all new structures being reported in the literature are of this variety.

The clearest example of this strategy is the crystallization of the components of the pol I transcription complex, particularly the MAX transcriptional activator in complex with its cognate DNA (Ferre-D'Amare *et al.*, 1993). This protein was only crystallized after extensive analysis of solution aggregaton and dispersion of over a dozen constructs of various length using dynamic light-scattering assays. The most promising candidate was crystallized and the structure solved in a period of less than 6 months after its identification. A large number of other structures were elucidated in 1990–91 through a similar fashion, particularly functional domains of members of two protein classes which are traditionally difficult to crystallize: membrane-bound receptors proteins (bacterial aspartate chemotaxis receptor, Milburn *et al.*, 1991; human growth hormone receptor, De Vos, Ultsch and Kossiakoff, 1992; LDL receptor, Wilson *et al.*, 1991; CD4, Ryu *et al.*, 1990 and Wang *et al.*, 1990; glucocorticoid receptor, Luisi *et al.*, 1991) and multidomain transcriptional

Table 2. Crystallization methodologies and representative structures

	Technique	Some recent structures
I.	Purification	All species
II.	Genetic manipulation	
	A. Expression of independent domains	MAX b-zip domain/DNA complex
		DNA gyrase B
		Glucocorticoid receptor
		Heat shock protein ATP domain
		CD4
		Aspartate receptor ligand domain
		Variable surface glycoprotein
		Klenow fragment
		HGH receptor
	B. Site mutants to improve lattice contacts	H-ferritin
	C. Artificial disulphide cross-links	Aspartate receptor ligand domain
		Anti-chymotrypsin (serpin inhibitor)
	D. 'Internal' site-directed mutations	Aspartate amino transferase
		Azurin
		Fibroblast growth factor
III.	Determination of aggregation state and solution behaviour	MAX
		USF
		TATA-binding protein
		αl helical protein
		Gene 5 protein
		Ferritin
IV.	Temperature control and variation	10-THFS
		Myosin regulatory domain
		Xylose isomerase
V.	Chemical modification	Mysosin head protein

regulators (MAX, Ferre-D'Amare *et al.*, 1993; GCN4, Ellenberger *et al.*, 1992).

In addition to the wholesale reduction of intact wild-type protein molecules to functional domains, which is a 'divide and conquer' approach to crystalization, more subtle changes in primary sequence can often improve results dramatically. As shown in *Table 2*, changes in internal or inter-subunit cross-linking, single point mutations at the site of a lattice contact, and the alteration of a single site of a post-translational modification (particularly phosphorylation, methylation and glycosylation) can improve crystallization results (Milburn *et al.*, 1991; Lawson *et al.*, 1991; Ago *et al.*, 1991).

CRYSTALLIZABILITY VERSUS SOLUTION AGGREGATION BEHAVIOUR

A number of investigators are now discovering that along with the degree of purity and nature of the genetic construct used to produce the target protein, there is an extremely strong correlation between the degree of non-specific aggregation in solution (polydispersion) and crystallizability, with those molecules which display clean oligomerization and aggregation behaviour (forming a mondisperse solution) often being much more easily crystallized (Burley and Roeder, 1992; Ferre-D'Amare *et al.*, 1993). The most commonly reported technique for assessing aggregation is photon correlation spectros-

copy, also known as dynamic or quasi-elastic light scattering. This method is currently being used by a handful of crystallography laboratories around the world, and is currently primarily responsible for the elucidation of approximately a dozen novel structures in the past couple of years. It is clear that routine application of this technology, in conjunction with genetic and purification methodologies mentioned above, will have an enormous impact on protein crystallization in the future.

TECHNIQUES FOR RESOLUTION IMPROVEMENT

Organizations and investigators have recently backed away from initial predictions that space-based macromolecular crystallization will allow the initial elucidation of structures that cannot otherwise be determined, primarily because microgravity-based crystallization experiments have not produced any specimens in a unique morphology, space group, or dramatically improved scattering power as compared to standard crystallization techniques. As a result, it is now clear that it is far more efficient and cost-effective to discover a new route to a desired novel crystalline species through the application of standard laboratory methodologies, than it is to attempt to use the microgravity environment to achieve the same goal.

Therefore, most claims on the side of microgravity-based protein crystallization are now found at the doorstep of 'improved resolution' or scattering power from many space-grown crystals (DeLucas and Bugg, 1990, 1991; McPherson, Greenwood and Day, 1991; DeLucas *et al.*, 1991; Stoddard *et al.*, 1992; Asano *et al.*, 1992). The average improvement in resolution for these species averages between 0.1 and 0.4 Å, and is presumably caused by improved internal lattice formation. As mentioned above, there are four commonly used ground-based methods for producing similar resolution improvements. The first is improved genetic constructs which eliminate disordered regions (particularly *N*- and *C*-termini in the target proteins). Sensitive area detector systems and synchrotron radiation, taken together, may produce substantial improvements in diffraction limits for a given crystal. Finally, ultra-low temperature data collection, which can often allow collection of much higher resolution data simply by extending the length of scan time across reflection profiles sufficiently to dramatically improve counting statistics for weak data (Hope, 1990) as described below.

The cryo-preservation of crystals at temperatures approaching −190°C can improve the observed diffraction data by as much as that seen with microgravity crystallization. Crystals of biological macromolecules are sensitive to damage caused by X-ray irradiation. The result is an often severe degradation in the quality of the data as they are collected. A technique pioneered by Hope (reviewed 1990) immortalizes crystals by rapidly reducing their temperature to that of liquid nitrogen. When successful, the method results in a sample that is preserved as a vitreous freeze; as long as the crystal is maintained at low temperature, it experiences no damage due to irradiation. The advantage is two-fold: data no longer suffer the inaccuracies introduced by radiation decay, and higher resolution data may be collected by exposing

cryo-preserved crystals to much higher X-ray fluxes, allowing weak, high-resolution terms to be measured accurately. The technique has been instrumental in the recent elucidation of several important structures (Teng, 1990; Joshua-Tor *et al.*, 1988; Brown *et al.*, 1993). The resolution enhancement observed can be as high as 1.0 Å (Pamela J. Bjorkman, pers. comm.).

Conclusions

A theoretical treatment of the conditions which affect macromolecular crystallization indicates that the microgravity environment should be conducive to lattice formation and growth, as discussed in the first section of this review. However, 10 years of microgravity crystallization experiments, covering over 100 protein species and using several different microgravity platforms, indicates that there has yet to be a single crystallization which has yielded any major improvement, leading to significant structural information, which could not be attained through ground-based methodologies at lower cost. Indeed, those cases which have been reported as the most 'successful' microgravity crystallizations (isocitrate lyase, aspartate ammonia lyase, 10-THFS) have all been shown to either have been caused by experimental artifacts, or to have subsequently improved dramatically by simple modifications of standard crystallization trials on Earth. It is therefore worthwhile to perform a cost–benefit analysis of microgravity-based crystallization.

The cost of a single launch of a crystallization payload which runs on either *Mir* or the US Shuttle varies between US$100 000 and well over US$1 million by the time all factors have been accounted for. In some cases, these expenses are not actually shouldered by the individual academic laboratory, but rather by the taxpayer through the support of the relevant space agency, but the final price is the same nonetheless. In comparison, for US$100 000 an individual laboratory could purchase dynamic light-scattering and automated chromatography systems (roughly US$25 000 and US$35 000, respectively) and pay a full-time senior technician or two postdoctoral fellows a year's salary to conduct somewhere between 2000 and 8000 h of purification, cloning and crystallization trials. For the upper price tag, a pair of crystallography laboratories could be fully outfitted with the best data collection hardware available.

Microgravity crystallization is, without question, one method to improve the quality of protein crystals. However, it is by no means the most efficacious method developed in the past decade, and still remains a technique available only to those researchers who are able to work on the US Space Shuttle, or who are rich enough to afford to pay the commercial launch costs which INTOSPACE or Payload Systems charge. Currently, realistic, unsubsidized launch costs begin in the hundreds of thousands of dollars, and go up from there. The cost of a shuttle mission, in its entirety, has been estimated at hundreds of millions of dollars, the proposed space station is estimated to cost in the tens of *billions* of dollars. The methods that we have briefly outlined have been developed in average laboratories, and are available for use by any researcher so inclined. Barring some breakthrough in current results, we

would consider microgravity crystallization a poor justification for building a space station. A vastly greater impact on our understanding of disease and the fundamental processes of life, the development of new drugs and therapeutic agents, could be made instead by investing just 1% of the cost of the proposed space station in basic biomedical research.

The take-home message of this analysis is clear: space-based research, while important in its own right as a driving force for technological innovation, cannot and should not be justified by relationships with well-established research areas, such as structural biology, which actually have few benefits derived from manned space programmes. Additionally, it is clear that in an age of vanishing monetary resources for basic research, it is possible for critically important research areas which are composed solely of many small academic and industrial research groups (such as structural biology) to be adversely affected by the funding of a massive programme in a separate technology simply because both are broadly classified as 'science' and connected to one another in competition for federal support. Finally, it is clear that the greater the potential cost of a research programme to a nation, the greater the need for impartial peer review, criticism and policy advisement from a broad spectrum of the most well-respected and productive principle investigators in that particular research field, in order to maintain accountability among all involved agencies and investigators.

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