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Extracting trends from two decades of microgravity macromolecular crystallization history

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Since the 1980s hundreds of macromolecular crystal growth experiments have been performed in the reduced acceleration environment of an orbiting spacecraft. Significant enhancements in structural knowledge have resulted from X-ray diffraction of the crystals grown. Similarly, many samples have shown no improvement or degradation in comparison to those grown on the ground. A complex series of interrelated factors affect these experiments and by building a comprehensive archive of the results it was aimed to identify factors that result in success and those that result in failure. Specifically, it was found that dedicated microgravity missions increase the chance of success when compared with those where crystallization took place as a parasitic aspect of the mission. It was also found that the chance of success could not be predicted based on any discernible property of the macromolecule available to us.

1. Introduction

There have been a number of improvements in structural knowledge from X-ray data provided by samples crystallized in the colloquially termed 'microgravity' environment of an orbiting spacecraft. These include the nucleosome core particle (Harp *et al.*, 1998), canavalin (Ko *et al.*, 2000), satellite tobacco mosaic virus (Larson *et al.*, 1998), antithrombin (Skinner *et al.*, 1997), parvalbumin (Declercq *et al.*, 1999), collagenase (Broutin-L'Hermite *et al.*, 2000), and insulin (Smith *et al.*, 1996). The mosaicity of microgravity grown crystals of lysozyme (Snell *et al.*, 1995, 2001), insulin (Borgstahl *et al.*, 2001) and thaumatin (Ng *et al.*, 1997) was also found to be dramatically decreased compared to control experiments in standard gravity, giving an insight into the improved crystalline order and a possible explanation for improved X-ray diffraction data. There exist several postulated mechanisms behind the macromolecule crystal X-ray data quality improvements seen from experiments in microgravity notably the reduction in sedimentation and in buoyancy driven convection (Helliwell *et al.*, 2002; Judge *et al.*, 2002; Kundrot *et al.*, 2001). Sedimentation is thought to affect crystal growth because significant movement of the crystal in reference to its surrounding growth medium disturbs the physical environment in which growth takes place. Buoyancy driven convection disturbs the environment close to the crystal surface, thus affecting mass transport to the surface and ultimately the crystal quality.

Microgravity as an environment for macromolecular crystal growth still attracts controversy (Couzin, 1998; Laver, 1999; McPherson & DeLucas, 1999). Since 1981 hundreds of macromolecule crystallization experiments have been performed in microgravity (Kundrot *et al.*, 2001). The experiments themselves are small, require minimal intervention and potentially have high scientific and commercial payback. The results from various experiments appear to be mixed and are often reported individually. To date there has been no complete analysis of these experiments as a group. In this paper we present this analysis for experiments conducted prior to access to the International Space Station.

2. Compiling the data

Improvements seen from the use of microgravity to grow macromolecular crystals are generally reported in the literature. In contrast

inconclusive results or crystallization failures are not typically reported. If we are to uncover correlations between success and the parameters under which the experiments were done, we also need to know when success does not occur. To that end we have obtained published information from professional journals, and publicly available reports submitted to the National Aeronautics and Space Administration (NASA) and the European Space Agency (ESA) as well as NASA archives for experiments conducted on 63 missions (up to and including Space Shuttle mission STS-95 launched in October 1998). Not included are experiments on unmanned satellites, the Russian Space Station MIR or results from sounding rocket experiments. Where information was unclear or unavailable we have tried to contact the investigator or group concerned. From reports generated after Space Shuttle launches, we know that for the Space Shuttle program 581 macromolecular crystallization experiments were conducted, representing 207 different macromolecules. Each of these experiments may have involved several crystallizations of the same macromolecular sample. Our best efforts have located reports or results for 67% of these cases, which corresponds to data for 177 different macromolecules. These reports cover approximately 85% of the total number of unique macromolecular samples flown.

The information was sorted and tabulated with respect to several metrics including the macromolecule identity, the microgravity mission on which it was flown, the crystallization hardware and technique used, and the results. We used the results as reported by each experimenter and categorized them in one of three groups: improved, if the experimenter reported that the crystals were of better quality than obtained on the ground, the same or worse quality, or unknown. The criteria for crystal quality improvement were those defined by the experimenter and included improved X-ray diffraction resolution, higher signal-to-noise ratio, lower mosaicity or combinations of those improvements. The data obtained is summarized in Fig. 1. For each macromolecule the molecular weight and space group were obtained from published reports or from data deposited in the Protein Data Bank (PDB; Berman *et al.*, 2000; Sussman *et al.*, 1998).

3. Analyzing the experimental variables

To analyze the data we posed several questions based on sample, environment and method, *i.e.* if the improvements were related to the macromolecule, if they were a function of the crystal space group, the microgravity environment and duration of microgravity conditions or the crystallization method. The available data allow us to address these questions. For more complex issues, *e.g.* the sample preparation method and sample aging, data were not available to answer those questions in a statistically reliable way.

3.1. Macromolecular properties

To examine the behavior of different macromolecules in the microgravity environment, samples for which there were eight or more reports were tabulated and their improvement rate of microgravity- over ground-grown calculated as a percentage of the available reported outcomes. Reports noting apparatus failure were not included. The missions on which samples were flown are listed divided into dedicated microgravity and parasitic missions (see below) and the molecular weight of the sample was also noted.

We also analyzed the reported diffraction resolution improvements for crystals grown in microgravity listed in a review by Kundrot *et al.* (2001). A total of 30 data points consisting of molecular weight and space group were obtained. The molecular weight was examined as a function of resolution improvement. The frequency at which each space group occurred within the list of experiments with improved results was recorded and expressed as a percentage of the total. These data were compared to the findings of Wukovitz & Yeates (1995) who analyzed the distribution of macromolecule space groups in the PDB. In their analysis the authors make a case that space group distribution is not a random function, but rather is guided by inter-molecular interactions between protein molecules. If the distribution of space groups recorded in our survey is different from the findings by Wukovitz and Yates, the data would then suggest that particular space groups may be more amenable to crystallization in a convection free environment.

In addition to the 30 samples from the review by Kundrot *et al.* (2001) molecular weight information was obtained for as many other experiments as possible. This was either obtained from the reports, references therein, or other references referring to the same material. Molecular weight was examined as a function of improvement and mission quality.

3.2. Microgravity environment and duration

Microgravity is not an accurate term to describe the environment experienced on an orbiting spacecraft. The reduced acceleration is achieved through free fall as the spacecraft orbits the earth. The spacecraft is a single body in which any vibration is transmitted to the rest of the body, there being insufficient mass to damp it. Oscillatory accelerations also known as *g*-jitter arise from crew exercise and activity, the operation of experimental and life-support equipment and harmonic structural vibrations of the spacecraft itself (Boggon *et al.*, 1998; Matsumoto & Yoda, 1999; Snell *et al.*, 1997). These oscillatory accelerations typically range in magnitude from $10^{-5}g$ to $10^{-3}g$ with frequencies varying from 0.01 to 300 Hz. There are also transient accelerations that are random and short in duration, which exhibit a broad band of frequencies. Common sources of these accelerations are the firing of thrusters, docking of spacecraft, launching and retrieving of satellites, closing and opening of hatches and panels, or

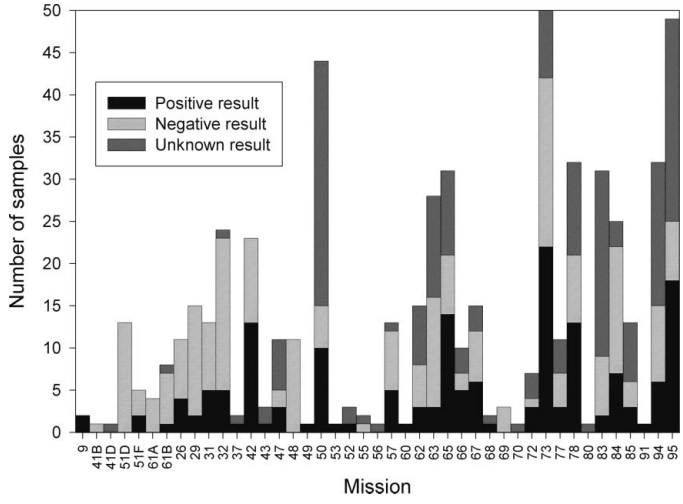

Figure 1
Plot of experimental reports per mission in chronological order. In most cases each sample represents several individual crystallization experiments. Positive results (improvement) appear on the bottom with negative then unknown stacked above them, respectively. For mission STS-73 the bar for unknown results has been truncated. Reports from 19 samples on this mission were not available primarily as the experimental purpose was to test crystallization hardware rather than to grow and analyze the crystals. Mission STS-50 also has a large number of unknown results due to samples being used in a glove box experiment to test sample manipulation.

the start-up of equipment. These transient accelerations typically have peak values as high as $10^{-2}g$.

In examining the quality of the microgravity missions we divided the missions into those that were dedicated to providing a microgravity environment and those that were not. Dedicated microgravity environment missions maintain the attitude of the Orbiter such as to minimize certain secondary parameters, for example the net quasi-steady acceleration, duration and frequency of thruster firings, and attitude changes. There are still, however, disturbances due to minor attitude adjustments, crew activities including exercise and the operation of life support and other equipment. The non-dedicated missions were 'parasitic' missions so called because macromolecule crystallization experiments were present while other experiments or activities such as satellite launches and retrievals took precedence. Principle component spectral analysis (PCSA) for microgravity dedicated and non-microgravity periods during the same mission, STS-62, show significantly more short duration acceleration noise in the non-dedicated microgravity time (DeLombard *et al.*, 1997). Limited acceleration data are available for more recent missions (see Fig. 2) but not for the majority of missions under investigation. For all missions timelines are available for activities that took place. Comparable acceleration data from the Space Acceleration Measurement System (SAMS; DeLombard *et al.*, 1992) were available for missions STS-57, 65, 73, and 94. This includes a parasitic mission, STS-57 and three dedicated microgravity missions. The average root-mean-square acceleration was calculated for each day of the mission and the 95% peak value for the first 5 days and whole mission. A 25 Hz cutoff was imposed on the data due to instrumentation limitations and the data compared with the experimental crystallization results. The calculated values were from the accelerometer locations, on the forward bulkhead for STS-57, under the microgravity glovebox for STS-73 and on racks in the payload bay for STS-65 and 94. No attempt was made to seek or calculate a transfer function for the accelerations seen at the experiment as it proved difficult to determine the experiment location from the available data.

Mission duration is not controlled by the experimenter. On the ground a crystallization experiment can run for as long as is necessary to form the best crystals. Microgravity experimental duration, before the advent of permanent manned space stations, has varied from days up to a couple of weeks. We analyzed reported crystallization improvements as a function of the mission duration only including those missions with eight or more reported experiment results for statistically meaningful comparison between missions. Missions were not included if there was a known failure of the crystallization apparatus and data were also excluded for mission durations shorter than 4 days to avoid results of interrupted or excessively hurried crystallization experiments. Space Shuttle launches are prone to delay due to adverse weather conditions for launch or other logistical factors, with delays ranging from hours to days. The launch of STS-51, as an extreme example, was delayed for 15 days. In case of a delay the investigators have the opportunity to have their experiments reloaded with fresh protein and other solutions, as the crystallization can be sensitive to the age of the macromolecular samples. The information on which cases this refresh was actually performed is not readily available and the analysis is conducted without this knowledge.

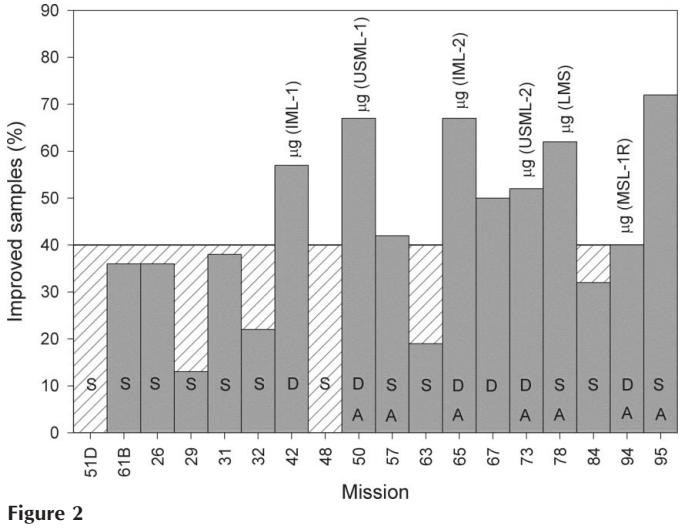

Figure 2
The percentage of improved samples for missions where 10 or more samples were flown. Dedicated microgravity missions are labeled with the mission name above the histogram bars. The missions are also labeled S for single crewed (*i.e.* there were quiet periods when the crew slept), D for double crewed (two shifts resulting in 24 hour activity) and A for missions where accelerometer data is available. The dashed area highlights an improvement rate of 40% or below.

3.3. Crystallization method

The crystallization method itself is a variable. Three main types of crystallization methods have been used in microgravity: dialysis, liquid diffusion and vapor diffusion. Dialysis experiments have been performed using various apparatus including the Advanced Protein Crystallization Facility (APCF; Bosch *et al.*, 1992) and the Diffusion-controlled Crystallization Apparatus for Microgravity (DCAM; Carter, Wright, Miller, Chapman, Twigg, Keeling, Moody, White, Click, Ruble, Ho, Adcock-Downey, Bunick *et al.*, 1999). In these apparatus the macromolecule solution is separated from a precipitant solution by a dialysis membrane or a gel fuse. Liquid diffusion is based on the same principle but there is no dialysis membrane and only a liquid–liquid interface exists between the solutions. Hardware used for these experiments includes the APCF, the Hand Held Diffusion Test Cell (HHDTC), and the Enhanced Nitrogen Dewar (EGN; Koszelak *et al.*, 1996). In the case of the EGN, samples are loaded in Tygon tubes that are flash frozen in liquid nitrogen and sealed. On orbit the tube bundles are allowed to warm to room temperature and the solutions diffuse into each other. Vapor diffusion methods are the most commonly used method in microgravity. Apparatus for vapor diffusion methods includes the APCF, the Vapor Diffusion Apparatus (VDA; DeLucas *et al.*, 1986), and the Protein Crystallization Apparatus for Microgravity (PCAM; Carter, Wright, Miller, Chapman, Twigg, Keeling, Moody, White, Click, Ruble, Ho, Adcock-Downey, Dowling *et al.*, 1999). These apparatus operate analogous to hanging or sitting drop experiments in the laboratory in which a drop containing protein and precipitant approaches equilibrium *via* vapor transport with a larger precipitant reservoir. The Protein Crystallization Facility (PCF; Long *et al.*, 1994), provides an additional crystallization method using temperature as a driving force. The success rate of each of these techniques in microgravity was examined. The data were included in this analysis from all missions, irrespective of mission duration, mission nature, or reported hardware status. We considered the data from the EGN apparatus as a separate case and did not include it with the data for liquid diffusion. Using EGN, the samples are flash frozen and this introduces another factor, that of the ability of macromolecules to respond to a freeze thaw process and bubble formation due to thermal expansion and contraction, which is not present in other liquid diffusion experiments (Barnes *et al.*, 2002; Ciszak *et al.*, 2002). Comparison of

Table 1
Performance of crystallization experiments in microgravity for specific macromolecules.

Listed are the number of reports available for each protein and the percentage of cases where improvement was reported. Also shown is a breakdown of the dedicated and parasitic missions with the number of cases where improvements are seen in parenthesis. Finally molecular weight is listed for each macromolecule.

Macromolecule	No. of times reported	Improved (%)	Dedicated microgravity	Parasitic mission	MW (kDa)
Regularly successful (>70% of the time)					
Insulin	8	88	1 (1)	7 (6)	5.8
Thaumatin	11	90	8 (8)	3 (2)	22
Canavalin	14	71	9 (5)	5 (5)	150
Mixed success (70% > succes s> 30%)					
Serum albumin	13	46	6 (3)	7 (3)	66
STMV	9	56	7 (5)	2 (0)	150
Lysozyme	23	43	5 (2)	18 (8)	14
Difficult (<30%)					
Apoferritin	10	0	6 (0)	4 (0)	480

results from regular liquid diffusion and EGN might therefore skew the results.

Another parameter related to the particular hardware flown is temperature control. Currently PCAM, VDA and DCAM have no independent temperature control but fly inside the Single-Locker Thermal Enclosure System (STES). In this system thermal control is accomplished by heat conduction in which one wall is actively heated or cooled by Peltier thermal electric units. Different apparatus use a slightly different configuration of the crystal growth cells in the enclosure system therefore heating or cooling is not necessarily exactly uniform with the distribution of the growth cells. The APCF on the other hand, uses a different incubator system, which operates on forced air and several Peltier elements, resulting in a very uniform internal temperature, with a variation of ± 0.1 K (Bosch *et al.*, 1992; Snyder *et al.*, 1991). Barnes *et al.* (2002) performed one of the first experiments with the EGN in which the actual temperature was recorded. They noted that the temperature of the EGN, which is not actively controlled, fluctuated as much as 3 K with the ambient cabin temperature after thawing had been completed. This experiment serves as a benchmark to assess temperature changes when no active temperature control is in place.

4. Results

4.1. Macromolecule and crystal space group

The frequent flyers of the microgravity crystallization world are insulin, thaumatin, canavalin, serum albumin, satellite tobacco mosaic virus, lysozyme and apoferritin. For each of these proteins experimental data recorded on eight or more missions have been reported. These results are summarized in Table 1, showing the number of times reported, the percentage of those reports showing improvement and the molecular weight of the macromolecule. Also shown are the numbers of dedicated missions, parasitic missions and the number of times improvements were seen on each. We have defined three groups, regularly successful samples (more than 70% reports of improvement), mixed success (between 30% and 70% improvement) and those that show poor success (less than 30% improvement). The data show that crystal quality improvement in microgravity is macromolecule specific. Some proteins, such as insulin and thaumatin appear to give improved crystallization on a regular basis, while other proteins such as apoferritin do not do so. There is no correlation between molecular weight and crystal quality improvement in these examples. Similarly, an analysis of resolution improvement against molecular weight for the samples listed in the paper by Kundrot *et al.* (2001) showed no correlation.

Table 2
Comparison of the most common macromolecule space groups from experiments performed on the ground (Wukovitz & Yeates, 1995) with the space groups of successful microgravity macromolecule crystallizations.

Space group	Protein Data Bank (%)	Successful microgravity experiments (%)
$P2_12_12_1$	36.1	26.7
$P2_1$	11.1	13.3
$C2$	6.1	10.0
$P4_32_12$	5.7	6.7

To test the hypothesis whether molecules crystallizing in particular space groups show more improvement than others, we list in Table 2 the frequency by which the four most relevant space groups occur in the PDB next to the frequency of space groups observed with improved results for all samples crystallized in microgravity. Since the space group distributions are similar for both cases, we may conclude that no space group is more amenable to improvement in microgravity than any other.

The complete data were divided based on molecular weight into small (less than 25 kDa), medium (less than 200 kDa and greater than 25 kDa), and large macromolecules (greater than 200 kDa). This is the same definition as used by Vergara *et al.* (2003) in a study of results obtained from the APCF apparatus. For the dedicated microgravity missions (Table 3), molecular weight data was available for 78% of the macromolecules flown. From a total of 111 experimental reports, improvement was reported for 43, 60 and 39% of the small, medium and large samples respectively. For parasitic missions macromolecular weight data was available for 62% of the experimental reports. From a total of 72 experimental reports, improvement was reported for 46, 31 and 38% of the small, medium and large samples respectively. Based on this limited data macromolecules of medium molecular weight seem to do better on dedicated flights. However, looking at data from all flights, molecular weight has no significant effect on improvement.

4.2. Microgravity environment and duration

In Table 1 we broke down the results into dedicated microgravity missions and parasitic missions. The sensitivity of the various macromolecules to the reduced acceleration levels is remarkably variable. STMV (satellite tobacco mosaic virus) for example, only shows improvement on dedicated microgravity missions where acceleration levels were relatively low. Insulin, however, always shows improvement in flight experiments even though only one of the eight flight experiments was on a dedicated microgravity mission. Canavalin, serum albumin and lysozyme show improvement on both dedicated microgravity and 'parasitic' missions. For many of these macromolecules however the best result for crystallization of these proteins was obtained on a dedicated mission. On the other hand, crystallization of apoferritin proves difficult to improve even though half of its crystallization trials were on flights, which were dedicated microgravity missions.

Table 3 lists the individual missions broken down into dedicated microgravity and parasitic, the duration, the number of crystallization experiment reports available for each, and the reported success rate. The data is also summarized in Fig. 2. Dedicated microgravity missions show a 55% improvement while parasitic missions show only a 34% improvement. A statistical *t*-test at 95% confidence limits shows the data from dedicated microgravity missions to be statisti-

Table 3
Comparison of experimental results for dedicated microgravity missions with parasitic missions.

The percent improved refers to the number of positive reports as a percentage of the total number of reports received.

Dedicated microgravity mission				Parasitic mission			
Mission	Duration (d)	Experiment reports	Improved (%)	Mission	Duration (d)	Experiment reports	Improved (%)
STS-42	7	23	57	STS-61B	6	7	14
STS-50	13	15	67	STS-29	4	15	13
STS-62	15	8	38	STS-31	4	13	38
STS-65	15	21	67	STS-32	10	23	22
STS-73	16	42	52	STS-57	10	12	42
STS-78	17	21	62	STS-63	8	12	19
STS-94	16	15	40	STS-67†	17	12	50
				STS-84	9	22	32
				STS-95‡	9	25	72
Mean			55	Mean			34

† Although STS-67 was not a dedicated microgravity mission, it was a quiet mission conducting telescope observations without satellite launches. ‡ Similarly STS-95 was also a quiet mission. Parasitic missions STS-51D and STS-48 were not included due to failure of crystallization equipment.

Table 4
Comparison of experimental results based on crystallization technique.

The percent improved refers to the number of positive reports as a percentage of the total number of reports received. The percentage of dedicated missions is the number of dedicated microgravity missions as a percentage of all missions flown using this technique.

Crystallization method	Apparatus	Number of samples	Improvement (%)	Dedicated missions (%)
Temperature	PCF	8	88	14
Dialysis	APCF, DCAM	39	46	72
Liquid diffusion	APCF, HHDTC	74	39	50
Vapor diffusion	PCAM, VDA, APCF	274	40	46

cally significantly better, compared with the data from the parasitic missions. Normalizing to the parasitic mission, STS-57, the average RMS accelerations on STS-65, 73 and 94 (where comparative data were available) were approximately 0.6, 0.1 and 5 times that of STS-57. STS-57 showed a 42% improvement with STS-65, 73 and 94 showing 67, 52 and 40% respectively. The 95% acceleration value for STS-57 was 415 μ*g* over the entire mission and 420 μ*g* for the first 5 d, and STS-65, 73 and 95, having 745, 160 and 515 μ*g* for the whole mission, and 755, 135 and 595 μ*g* for the first 5 d.

The percent of experiments for which improvements are reported as a function of mission duration is illustrated in Fig. 3. Looking at all the data there appears to be a trend where longer duration missions improve the crystal quality, particularly for missions longer than 12 d. Classification of the various missions as parasitic or dedicated microgravity missions reveals that the increased success rate may well be due to a combination of low acceleration values and mission duration. Data listed in Table 3 do not show a correlation between mission duration and crystal quality improvement. The finding that specific macromolecules show sensitivity to mission quality (Table 1) and no correlation to mission duration suggests that mission quality is the predominant factor in crystal quality improvement.

4.3. Crystallization method

The performance of each crystallization technique is listed in Table 4. For the Protein Crystallization Facility (PCF) (first entry in the table), we have found complete reports only for crystallization of insulin, which consistently gives improved crystallization results in microgravity. The limited number of unique samples used in the PCF skews its comparison with other techniques. The success rates for the other methods are similar to each other. The success rate for dialysis is somewhat higher than the rates for liquid and vapor diffusion. The higher rate of success of the dialysis technique may be due to its use on a disproportionably large percentage of missions dedicated to microgravity (72%). Considering the data included in Table 4, with the exclusion of the temperature driven technique of the PCF, the overall reported success rate for microgravity crystallization is 40%.

Based on the data here presented, the quality of the microgravity environment has a positive effect on the outcome. If we analyze the growth method as a function of success rate for missions with 40% or greater improvement (Fig. 2) another picture emerges. In this case there is a clear difference between the success of liquid diffusion and dialysis methods over vapor diffusion on missions showing overall, greater than 40% improvement (Table 5). This includes all the dedicated microgravity missions and also missions STS-57, 67 and 95. By assessing the timelines and typical accelerations associated with activities onboard we can redefine STS-67 and STS-95 as close to an ideal microgravity mission. STS-57 recovered a free-flying satellite so we still classify it as a parasitic mission.

Unfortunately, there are only experimental results available from one parasitic mission on which the dialysis and liquid–liquid diffusion techniques were performed, STS-57. Based on three available reports, we find that dialysis and liquid–liquid diffusion each improved one sample, while liquid–liquid diffusion showed no improvement with another sample. The data in Table 5 suggest that vapor diffusion may not be the most favorable technique to use in microgravity. Unfortunately, there is only limited data to support this claim.

In the laboratory temperature can be controlled with an accuracy better than ±1 K. Convective heat transfer is minimal in microgravity and heating or cooling has to be accomplished by conduction or forced air. As an example, Fig. 4 illustrates temperatures recorded in

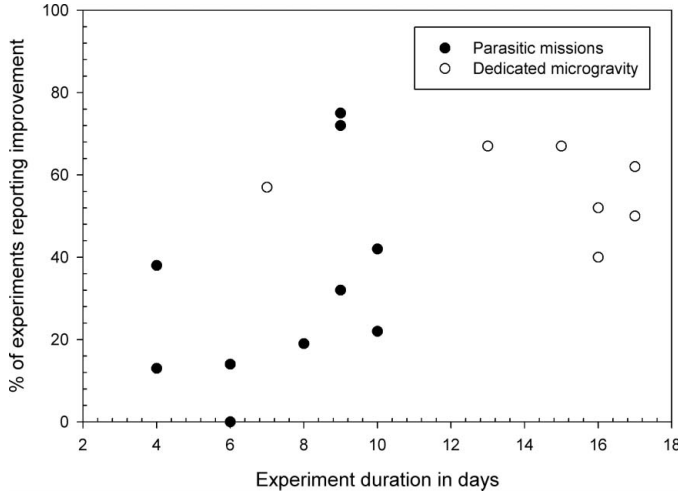

Figure 3
Number of experiments reporting improvement as a function of mission duration. Open circles represent the dedicated microgravity missions, the closed circles are 'parasitic' missions.

Table 5
Improvement listed for different methods of growth on missions that had low acceleration levels compared to those that did not.

Crystallization method	Apparatus	Improvement (%)	
		Missions with greater than or equal to 40% improvement	Missions with less than 40% improvement
Dialysis	APCF, DCAM	71	—
Liquid diffusion	APCF, HHDTC	68	—
Vapor diffusion	PCAM, VDA, APCF	43	32

the STES system in three PCAM cylinders during the STS-95 mission. Temperature control is by conduction and the variation in temperature is a function of the distance of the cylinder from the controlling wall. The data show that the actual temperature close to the controlling wall is within 0.5 K of the set point, however peak deviations from the set point of 1.0 K in the middle cylinder and 1.5 K for the cylinder farthest away from the controlling wall are observed. Therefore the position of the sample within this thermal enclosure affects the temperature control experienced by the experiment. The temperature differences shown in Fig. 4 suggest that this information may be an important parameter especially when comparative analyses are being made.

5. Discussion

We were able to obtain reports on 67% of the macromolecular crystallization experiments conducted in microgravity (Fig. 1). We must therefore take into account that this limited percentage may influence, perhaps even skew the results presented. A first interpretation might lead us to think that the unreported results were all unfavorable *i.e.* the crystallization failed or results were inferior to control experiments. Although certainly some of the unreported experiments failed to produce adequate crystals, it is clear from our data acquisition that there are other reasons for the lack of reports *e.g.* the crystals deteriorated before they could be analyzed or the results of the experiments are proprietary. There are many factors in addition to microgravity that determine the success of a crystallization experiment, all of which have been left out of our analysis. The history of the sample can be important, how it was obtained, purified, its age and preparation history all may contribute to final crystal quality differences. The handling of the crystals after the return to ground, including the experimental analysis, also plays an important role in the results obtained. Unless explicitly reported by the individual investigators, we do not have access to these data and therefore we cannot disregard these influences on the results. However, by analyzing a sufficiently large number of results, we exclude most that are not part of a trend. We have only looked at three distinct areas in as much detail as possible with the data available to us: the characteristics of the sample, the characteristics of the environment and the method of growth.

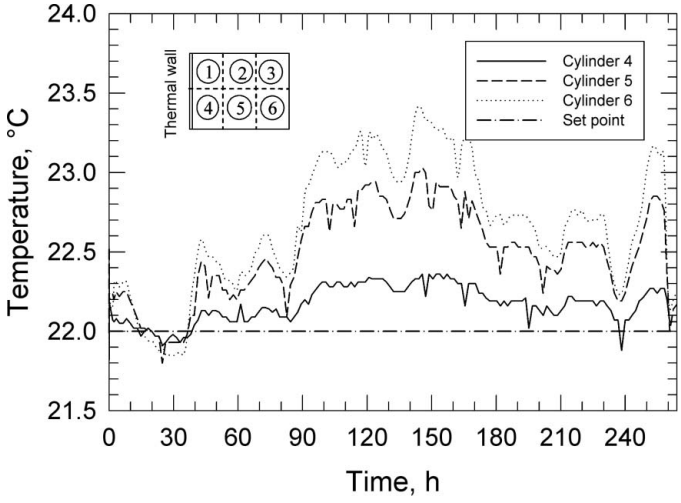

Figure 4
Temperature recorded for a row of PCAM cylinders inside a thermal enclosure controlled to a set point of 22°C. Each cylinder is 381 mm long and 81 mm in diameter and contains 63 sitting-drop vapor-diffusion crystallization experiments. The cylinders are aligned parallel to the thermally controlled wall. Temperature data are reported for the bottom row of three PCAM cylinders, with each cylinder at a different distance with respect to the wall controlling the temperature – cylinder 4, closest, 5 midway and 6 farthest away from the controlling wall.

Table 6
Data analysis from Vergara *et al.* (2003).

'Worse' is used for cases where samples grown in microgravity exhibited poorer diffraction than did ground-grown samples, 'no change' cases where diffraction was the same or crystals grown on the ground and in microgravity were not suitable for diffraction analysis, and 'improved' cases where microgravity grown crystals exhibited better diffraction than ground-grown samples.

Molecular weight		Samples	Worse (%)	No change (%)	Improved (%)
Small	<28 kDa	15	0	26	74
Medium	>28 kDa and <200 kDa	18	6	44	50
Large	>200 kDa	13	8	61	31

Crystallization of some macromolecule samples consistently improves in microgravity while others do not. To date no technique has been experimentally confirmed that will predict this. An elegant macromolecule-dependent rationale based on the kinetic Péclet number has been proposed by Vekilov *et al.* (1996). The kinetic Péclet number is a dimensionless number and is expressed as the ratio of bulk mass transport and the crystal interface kinetics. Based on its definition, the Péclet number changes, dependent on whether transport is the growth limiting step ($Pe_k \geq 1$) or crystal surface kinetics are the limiting step ($Pe_k < 0.1$). The total mass transport significantly depends on whether diffusion is the only mechanism (predicted to be the case for acceleration levels below $10^{-5}g$) or convective transport significantly contributes to total mass transport. Based on kinetic data available in this study it was postulated that canavalin and STMV should show greater crystalline perfection in microgravity while thaumatin and lysozyme were not expected to show improvement. From our analysis of the 'frequent fliers' of the microgravity program it is clear that canavalin does regularly improve in microgravity experiments. While STMV exhibits mixed success the best results both for canavalin and for STMV crystallization are reported on dedicated microgravity missions while a diffusion-dominated mass transfer is more likely to occur. Thaumatin however behaves contrary to the postulations in that it is regularly reported to improve in microgravity (Barnes *et al.*, 2002; Ng *et al.*, 1997). Improvements are also reported for lysozyme although not consistently (Snell *et al.*, 2001). In a recent paper by Vergara *et al.* (2003) the authors studied all the results from a single piece of hardware, the APCF. Their results are listed in Table 6. Their study appears to show that smaller macromolecules are more likely to show improvement in microgravity in contrast to our analysis showing no significant variation based on molecular weight. While we observe trends contrary to Vekilov's theory, these trends do not invalidate the theory. More extensive kinetic measurements of individual macromolecules and the crystallization process are needed to assess or improve its predicting power.

The space group in which the sample molecules crystallize is a readily available descriptor of the packing within the crystal. Wukovitz and Yeates (1995) have developed a model explaining why certain space groups are more prevalent in macromolecular crystals and also why these are different from the space groups most commonly found for small molecule crystals. The authors showed that small organic molecules prefer to crystallize in space groups that efficiently fill space while macromolecules crystallize in space groups in which it is easiest to achieve intermolecular connectivity, while maintaining maximum freedom of movement, which allows for conformational flexibility. This model is in good agreement with the frequency distribution of space groups in the PDB. A reduced convection environment for growth is unlikely to have an effect on intermolecular interactions where Brownian motion is a more persuasive force. However, the space group is an easily obtainable metric for the experiments we studied. As such we examined it as a function of improved crystal samples and saw a distribution similar to that in the PDB. Not surprisingly, the space group is not a useful predictor for crystal quality improvement in microgravity.

The environment on non-dedicated microgravity missions is noisy, experiencing frequent, relatively large magnitude transient accelerations (up to $10^{-2}g$) and high magnitude *g*-jitter (up to $3 \times 10^{-3}g$). These disturbances can induce convective flows capable of disrupting concentration gradients, with the disruption predicted to continue long after the disturbance has ceased to exist (Savino & Monti, 1996). Early in the crystallization process transient accelerations can cause premature nucleation. Long *et al.* (1994) mention this as the probable cause of showers of small crystals during experiments on STS-49 in which the mission required continual manoeuvering of the Shuttle. Later in the crystallization process, transient accelerations can result in bulk sedimentation or movement of the crystals within their environment. As a second example, on the Spacehab-1 mission (STS-47) lysozyme crystallization experiments were conducted with limited direct observation (Helliwell *et al.*, 1996; Snell *et al.*, 1995; Weisgerber & Helliwell, 1993). Well into the mission, and after crystal growth had largely been completed, a sudden and dramatic motion of the crystals occurred attributed to the retrieval of the EURECA satellite. The gravitational acceleration, at that moment onboard the Shuttle was in excess of $3 \times 10^{-3}g$.

Ramachandran *et al.* (1995) developed numerical models for fluid flow and mass transport under different *g*-levels. They proposed that the classical solution to the vertically heated flat plate could be used in an approximation to describe the velocity and mass transport in the vicinity of a crystal. This gave a Sherwood number (a dimensionless number that describes the ratio of total mass transport to molecular diffusivity) for protein crystal growth conditions of 1.0 at $10^{-5}g$, *i.e.* at this gravity level the transport is diffusion limited. For dedicated microgravity missions this regime can be frequently achieved. However, Lee & Chernov (2002) used simulations to predict that the acceleration levels at which diffusion controlled mass transport dominates depend on the specific properties of the macromolecule, its crystal growth kinetics and on the crystal size. Different macromolecules would therefore have different steady state acceleration thresholds at which they experience diffusion controlled mass transport. Based on these theories higher success rates are expected on dedicated microgravity mission environments, as these are better able to accommodate a wide variety of macromolecule experiments that have varying diffusion controlled mass transport acceleration thresholds. Our qualitative analysis of the results as a function of mission type clearly supports this idea. However, the result is not so clear from the quantitative comparable acceleration data from the four missions, STS-57, 65, 73 and 95. In order to explain this we need to consider the effect of *g*-jitter, an acceleration varying in magnitude, direction and frequency. Lower frequency *g*-jitter components cause net particle drift therefore they are more detrimental than higher frequencies, which result in fluctuating movements. For example, on the IML-2 mission (STS-65), Snell *et al.* (1997) report that the vibration caused by a water pump ($4.4 \times 10^{-3}g$ at 79–81 Hz) had no significant effect on the growth of the crystals, while accelerations associated with crew exercise (reaching a maximum of $1 \times 10^{-3}g$ at 2.1–2.5 Hz) correlated directly with variation observed in the crystal growth rates for a crystal attached to a wall. The increased gravity levels during these periods induced acceleration within the crystallization chamber periodically breaking down the diffusion-dominated system. This observed variation in growth rates took place during a dedicated microgravity mission (STS-65), which our results indicate had good success (70% improved). Furthermore, the X-ray mosaicity analysis of the crystals showed an improvement in crystal perfection of threefold over the ground controls (Snell *et al.*, 1995). Limited flow may therefore not be detrimental to crystal quality and may in fact be beneficial resulting in fresh media for crystal growth. Normal operational *g*-jitter (also experienced on dedicated missions) appears not to be as detrimental to macromolecule crystal growth as the larger transient accelerations, the bumps and jolts associated with launching or chasing satellites, extra vehicle activities (EVAs) *etc*. The acceleration data examined in this study, although useful for general mission comparison, is that located at the accelerometer position (away from the experiment site) and has a lower cutoff at 25 Hz, above the low frequency components (2.1–2.5 Hz) seen to cause effects on the crystal growth (Snell *et al.*, 1997). Unfortunately, no comparable data are available in the frequencies of most interest.

The crystallization method was investigated because there may be effects masked on the ground that become more pronounced in microgravity. For example, vapor diffusion is a very popular technique but Marangoni convection becomes a significant factor in microgravity (Boggon *et al.*, 1998; Chayen *et al.*, 1997). Marangoni flow, a convective flow due to surface tension differences, has been perceived as a negative effect on crystal growth and therefore vapor diffusion has been abandoned in the APCF apparatus. From the data in our analysis dialysis and liquid diffusion contribute to the improvements listed in Table 5 to a greater extent than does vapor diffusion. There appears to be a gain by switching from vapor diffusion to dialysis or liquid diffusion for a dedicated microgravity mission.

Temperature and the control of the temperature can be a key factor in the crystallization. Fig. 4 illustrates a considerable variation between sample position and the temperature set point for the standard incubator used for most NASA crystallization experiments *e.g.* PCAM, DCAM, or VDA. Convection is greatly reduced in microgravity so distribution of heat has to occur through conduction pathways or forced convection. The response time for temperature changes while using conductive pathways temperature is long, making a stable and even temperature distribution difficult to establish. Other systems, such as the APCF, use forced air convection as part of the apparatus and achieve a temperature control better than ± 0.1 K (Bosch *et al.*, 1992; Snell *et al.*, 1995; Snyder *et al.*, 1991). We do not have complete data available to conclusively investigate temperature control as a factor in crystal quality improvement. Some experiments have been conducted in incubators, others under (variable, uncontrolled) ambient conditions. Based on the results displayed in Fig. 4, it is obvious that the set point of the incubator in most cases is not an accurate indicator of the temperature of the experiment.

The results from our study lead to two important questions: how can we optimize future experiments and what data need to be recorded for a future more detailed analysis? The quality of the acceleration environment is the key aspect of improving the experimental success rate. By comparing crystallization experiments on parasitic missions with those in a quieter environment, the experimental success rate was improved significantly, from 34 to 55%. However, after the International Space Station became operational, macromolecular crystallization experiments have been predominantly performed on this platform, as opposed to the Space Shuttle, for which acceleration data are available. To date, there exists no detailed study of acceleration levels on the International Space Station, nor has the effect of the use of this platform on crystallization been reported. Considering its increased size (and increased distance between the experiment location and the center of mass), the complexity and the variety of experiments conducted on the ISS, the acceleration environment can be expected to be significantly different from that on the Space Shuttle Orbiter. The measurement, minimization and correlation of acceleration measurements with observations while experiments are conducted will be important in understanding future experimental results and further optimization. Key accelerations that appear to affect crystallization are those associated with crew exercise because of their associated vibration frequencies. If experiments could be automated and samples could be recovered, a free-flyer approach (an unmanned satellite) may well be the best way to optimize the success rate for microgravity crystallization experiments. Marangoni convection effects are important in hanging drop and sitting drop experiments (Chayen *et al.*, 1997; Savino & Monti, 1996) and it is not surprising to see that this crystallization method, according to data collected for this review, reduce the experimental success rate. It is well known that the crystallization temperature and temperature stability requirements vary widely with the nature of the protein sample. Because the temperature regulation depends on the hardware used in the experiment, it is advisable that individual investigators determine these requirements in ground-based experiments before experiments are conducted in microgravity.

Our results provide no other suggestions on how to improve the experiment and specific targeted experiments are needed to provide this information. A history of the experiment is needed, including sample preparation, detail on the loading of the hardware, flight duration and acceleration analysis. Crystal quality analysis details are needed as part of the experiment report. The data analysis on the crystals should occur with a uniform definition of the diffraction criteria used. Ideally samples should be studied on the same X-ray source using the same experimental procedure. Practically this is almost impossible for a large group of individual experiments as most investigators are not interested in how microgravity aids crystallization or how much the quality of their crystal is improved, as long as it does improve: their product is the resulting macromolecular structure. The only way to satisfactorily answer the questions we have posed, *i.e.* if the improvements were related to the sample, the microgravity environment and duration of microgravity conditions or the crystallization method, is to perform dedicated experiments to answer these questions. These experiments have not been performed to date and are a prerequisite for the optimum use of microgravity.

There are three improvements or combinations of improvements that microgravity-grown crystals have shown to improve X-ray diffraction analysis; increased resolution, decreased mosaicity and increased crystal volume. Resolution enhancements reflect an improved short-range order within the crystal, mosaicity an improved long-range order. We may be able to take advantage of the improved long-range order by optimizing the technique to obtain the highest possible signal-to-noise ratio in the data. The crystal volume is also a parameter that may somewhat increase the signal-to-noise ratio in the data. However, these three different improvements may not have the same cause. The reduced buoyancy driven convection may drive the improvements in short-range and long-range order whereas volume improvements may come from the reduction in sedimentation. Marangoni convection and transient accelerations may well drive the volume effects but could be a limiting factor in other improvements. The combination of effects is complex and any analysis of the cause of the effects is equally complex.

6. Conclusion

We have compiled data and results for 67% of all the macromolecular crystallization experiments flown in microgravity up to and including the STS-95 mission launched in October of 1998. This data corresponds to 85% of the total number of unique macromolecules flown. Using this data we have explored crystal improvement as a function of macromolecule characteristics, the experimental environment such as acceleration noise, experiment duration, growth-method and temperature control.

From the data we have available we cannot identify any predictive technique to establish if a crystallization experiment will benefit from microgravity. That does not mean that one does not exist. However, we do find qualitatively that a dedicated mission with a low acceleration environment significantly improves the chance for a successful outcome of a macromolecular crystallization. This conclusion is supported by theoretical studies and limited experimental observations. To conclusively prove the influence of the reduced acceleration environment on crystallization requires a dedicated study. To improve the success of crystallization experiments in general and to study the influence of the acceleration environment in particular, it is clear that dedicated monitoring of the acceleration environment at the experiment location, over time and at appropriate frequencies is required. Implicitly, based on the data summarized here, it is clear that microgravity has a demonstrated positive effect and that carefully maintaining the microgravity environment is a key factor in improving the crystallization success rate. Perhaps improvements in automation, reduction of mass and volume of the experimental hardware and the development of alternate recovery methods will allow for optimization of crystallization on a free-flying, unmanned satellite.

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