Mother liquors are defined as the solutions that contain all compounds (buffer, crystallizing agent, *etc.*) at the final concentration for crystallization except the macromolecule. Samples of macromolecules often contain quantities of salt of unknown composition, and it is therefore wise to dialyse new batches against well characterized buffers. Whatever the crystallization method used, it almost always requires a high concentration of macromolecule. This may imply concentration steps using devices operating under nitrogen pressure, by centrifugation, or by lyophilization (notice that lyophylization may denature proteins and that non-volatile salts also lyophilize and will accumulate). Dialysis against high-molecular-weight PEG may also be used. During concentration, pH and ionic strength may vary and, if not kept at the appropriate values, denaturation of samples may occur.

4.1.4.4. *Strategic concerns: a summary*

Homogeneity: Perhaps the most important property of a system to be crystallized is its purity. Crystallization presupposes that identical units are available for incorporation into a periodic lattice. If crystallization fails, reconsidering purification protocols often helps achieve success.

Stability: No homogeneous molecular population can remain so if its members alter their form, folding, or association state. Hence, it is crucial that macromolecules in solution are not allowed to denature, aggregate, or undergo conformational changes.

Solubility: Before a molecule can be crystallized, it must be solubilized. This means creation of monodisperse solutions free from aggregates and molecular clusters. Solubility and crystallizability strongly depend on substances (organic solvents and PEGs) that reduce the ionic strength of the solution (Papanikolau & Kokkinidis, 1997).

Supersaturation: Crystals grow from systems displaced from equilibrium so that restoration requires formation of the solid state. Thus, the first task is to find ways to alter the properties of the crystallizing solutions, such as by pH or temperature change, to create supersaturated states.

Association: In forming crystals, molecules organize themselves through self-association to produce periodically repeating three-dimensional arrays. Thus, it is necessary to facilitate positive molecular interactions while avoiding the formation of precipitate or unspecific aggregates, or phase separation.

Nucleation: The number, size and quality of crystals depend on the mechanisms and rates of nuclei formation. In crystallization for diffraction work, one must seek to induce limited nucleation by adjustment of the physical and chemical properties of the system.

Variety: Macromolecules may crystallize under a wide spectrum of conditions and form many polymorphs. Thus, one should explore as many opportunities for crystallization as possible and explore the widest spectrum of biochemical, chemical and physical parameters.

Control: The ultimate value of any crystal is dependent on its perfection. Perturbations of the mother liquor are, in general, deleterious. Thus, crystallizing systems have to be maintained at an optimal state, without fluctuations or shock, until the crystals have matured.

Impurities: Impurities can contribute to a failure to nucleate or grow quality crystals. Thus, one must discourage their presence in the mother liquor and their incorporation into the lattice.

Perfection: Crystallization conditions should be such as to favour crystal perfection, to minimize defects and high mosaicity of the growing crystals, and to minimize internal stress and the incorporation of impurities. Predictions from crystal-growth theories may help to define such conditions (Chernov, 1997*b*, 1999).

Preservation: Macromolecular crystals may degrade and lose diffraction quality upon ageing. Thus, once grown, crystals may be stabilized by temperature change, addition of more crystallizing agent, or by some other suitable alteration in the mother liquor.

4.1.5. Techniques for physical characterization of crystallization

Crystallization comprises four stages. These are prenucleation, nucleation, growth and cessation of growth. It proceeds from macromolecules in a solution phase that then 'aggregate' upon entering a supersaturated state and which eventually undergo a phase transition. This leads to nuclei formation and ultimately to crystals that grow by different mechanisms. Each of these stages can be monitored by specific physical techniques. Although systematic characterization of crystallization is usually not carried out in practice, characterization of individual steps and measurement of the physical properties of crystals obtained under various conditions may help in the design of appropriate experimental conditions to obtain crystals of a desired quality (*e.g.* of larger size, improved morphology, increased resolution or greater perfection) reproducibly.

4.1.5.1. *Techniques for studying prenucleation and nucleation*

Dynamic light scattering (DLS) relies on the scattering of monochromatic light by aggregates or particles moving in solution. Because the diffusivity of the particles is a function of their size, measurement of diffusion coefficients can be translated into hydrodynamic radii using the Stokes–Einstein equation. By making measurements as a function of scattering angle, information regarding aggregate shape can also be obtained. For single-component systems, the method is straightforward for determining the size of macromolecules, viruses and larger particles up to a few μm. For polydisperse and concentrated systems, the problem is more complex, but with the use of autocorrelation functions and advances in signal detection (Peters *et al.*, 1998), DLS provides good estimates of aggregate-size distribution.

In bio-crystallogenesis, investigations based on light scattering have been informative in delineating events prior to the appearance of crystals subsequently observable under the light microscope, that is, the understanding of prenucleation and nucleation processes. Many studies have been carried out with lysozyme as the model (Kam *et al.*, 1978; Durbin & Feher, 1996), though not exclusively, and they have developed with two objectives. One is to analyse the kinetics and the distribution of molecular-aggregate sizes as a function of supersaturation. The aim is to understand the nature of the prenuclear clusters that form in solution and how they transform into crystal nuclei (Kam *et al.*, 1978; Georgalis *et al.*, 1993; Malkin & McPherson, 1993, 1994; Malkin *et al.*, 1993). Such a quantitative approach has sought to define the underlying kinetic and thermodynamic parameters that govern the nucleation process. The second objective is to use light-scattering methods to predict which combinations of precipitants, additives and physical parameters are most likely to lead to the nucleation and growth of crystals (Baldwin *et al.*, 1986; Mikol, Hirsch & Giegé, 1990; Thibault *et al.*, 1992; Ferré D'Amaré & Burley, 1997). A major goal here is to reduce the number of empirical trials. The analyses depend on the likelihood that precipitates are usually linear, branched and extended in shape, since they represent a kind of random polymerization process (Kam *et al.*, 1978). Aggregates leading to nuclei, on the other hand, tend to be more globular and three-dimensional in form. Thus, a mother liquor that indicates a nascent precipitate can be identified as a failure, while those that have the character of globular aggregates hold promise for further exploration and refinement. Other analyses have been based on discrimination between polydisperse and monodisperse protein

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solutions, which suggests that polydispersity hampers crystallization, while monodispersity favours it (Mikol, Hirsch & Giegé, 1990).

A more quantitative approach is based on measurement of the second virial coefficient $B2$, which serves as a predictor of the interaction between macromolecules in solution. Using static light scattering, it was found that mother liquors that yield crystals invariably have second viral coefficients that fall within a narrow range of small negative values. Recently, a correlation between the associative properties of proteins in solution, their solubility and $B2$ coefficient was highlighted (George *et al.*, 1997). If this proves to be a general property, then it could serve as a powerful diagnostic for crystallization conditions.

Related methods, such as fluorescence spectroscopy (Crossio & Jullien, 1992), osmotic pressure (Bonneté *et al.*, 1997; Neal *et al.*, 1999), small-angle X-ray scattering (Ducruix *et al.*, 1996; Finet *et al.*, 1998) and small-angle neutron scattering (Minezaki *et al.*, 1996; Gripon *et al.*, 1997; Ebel *et al.*, 1999) have been used to investigate specific aspects of protein interactions under precrystallization conditions and have produced, in several instances, complementary answers to those from light-scattering studies. Of particular interest are the neutron-scattering studies that provided evidence for two opposite effects of agarose and silica gels on lysozyme nucleation, the agarose gel being a promoter and the silica gel an inhibitor of nucleation (Vidal *et al.*, 1998*a*,*b*).

4.1.5.2. *Techniques for studying growth mechanisms*

A number of microscopies and other optical methods can be used for studying the crystal growth of macromolecules. These are time-lapse video microscopy with polarized light, schlieren and phase-contrast microscopy, Mach–Zehnder and phase-shift Mach–Zehnder interferometry, Michelson interferometry, electron microscopy (EM), and atomic force microscopy (AFM). Each of these methods provides a unique kind of data that are complementary and, in combination, have yielded answers to many relevant questions.

Time-lapse video microscopy has been used to measure growth rates (*e.g.* Koszelak & McPherson, 1988; Lorber & Giegé, 1992; Pusey, 1993). It was valuable in revealing unexpected phenomena, such as capture and incorporation of microcrystals by larger crystals, contact effects, consequences of sedimentation, flexibility of thin crystals, fluctuations in growth rates and initiation of twinning (Koszelak *et al.*, 1991).

Several optical-microscopy and interferometric methods are suited to monitoring crystallization (Shlichta, 1986) and have been employed in bio-crystallogenesis (Pusey *et al.*, 1988; Robert & Lefaucheux, 1988). Information concerning concentration gradients that appear as a consequence of incorporation of molecules into the solid state can be obtained by schlieren microscopy, Zierneke phase-contrast microscopy, or Mach–Zehnder interferometry. These methods, however, suffer from a rather shallow response dependence with respect to macromolecule concentration (Cole *et al.*, 1995). This can be overcome by introduction of phase-shift methods and has been successfully achieved in the case of Mach–Zehnder interferometry. With this technique, gradients of macromolecular concentration, to precisions of a fraction of a mg per ml, have been mapped in the mother liquor and around growing crystals. Classical Mach–Zehnder interferometry has been used to monitor diffusion kinetics and supersaturation levels during crystallization, as was done in dialysis setups (Snell *et al.*, 1996) or in counter-diffusion crystal-growth cells (García-Ruiz *et al.*, 1999).

Michelson interferometry can be used for direct growth measurements on crystal surfaces (Komatsu *et al.*, 1993). It depends on the interference of light waves from the bottom surface of a crystal growing on a reflective substrate and from the top surface, which is developing and, therefore, changes as a function of time with regard to its topological features. Because growth of a crystal surface is generally dominated by unique growth centres produced by dislocations or two-dimensional nuclei, the surfaces and the resultant interferograms change in a regular and periodic manner. Changes in the interferometric fringes with time provide accurate measures of the tangential and normal growth rates of a crystal (Vekilov *et al.*, 1992; Kuznetsov *et al.*, 1995; Kurihara *et al.*, 1996). From these, physical parameters such as the surface free energy and the kinetic coefficients which underlie the crystallization process can be determined.

EM (Durbin & Feher, 1990) and especially AFM are powerful techniques for the investigation of crystallization mechanisms and their associated kinetics. The power of AFM lies in its ability to investigate crystal surfaces *in situ*, while they are still developing, thus permitting one to visualize directly, over time, the growth and change of a crystal face at near nanometre resolution. The method is particularly useful for delineating the growth mechanisms involved, identifying dislocations, quantifying the kinetics of the changes and directly revealing the effects of impurities on the growth of protein crystals (Durbin & Carlson, 1992; Konnert *et al.*, 1994; Malkin *et al.*, 1996; Nakada *et al.*, 1999). AFM has also been applied to the visualization of growth characteristics of crystals made of RNA (Ng, Kuznetsov *et al.*, 1997) and viruses (Malkin *et al.*, 1995). A typical example, Fig. 4.1.5.1, shows two images of the surface of a RNA crystal with spiral growth at low supersaturation and growth

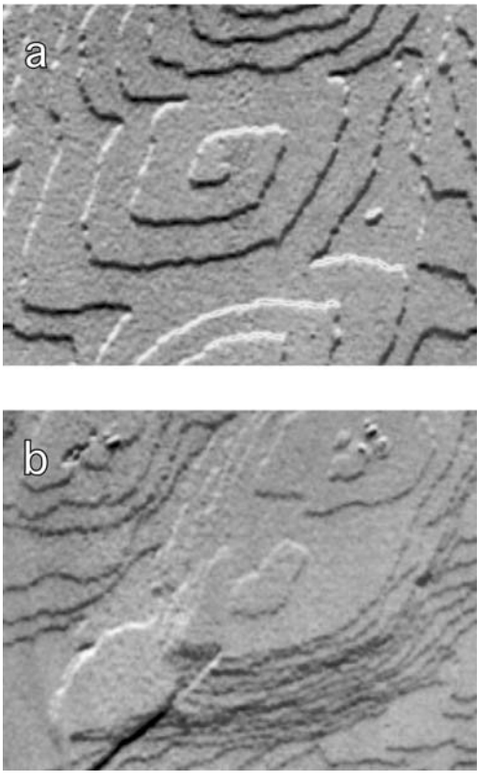

Fig. 4.1.5.1. Visualization of the surface of yeast $tRNA^{Phe}$ crystals by AFM. (*a*) Spiral growth with screw dislocations occurring at lower supersaturation and (*b*) growth by two-dimensional nucleation occurring at higher supersaturation, showing growth and coalescence of islands and expansions of stacks. Notice that supersaturation and type of growth mechanisms are very temperature-sensitive and are modulated by temperature variation, since in (*a*), crystals grew at 15 °C and in (*b*), at 13 °C. Reproduced with permission from Ng, Kuznetsov *et al.* (1997). Copyright (1997) Oxford University Press.

by two-dimensional nucleation at higher supersaturation. A noteworthy outcome of the study was the sensitivity of growth to minor temperature changes. A variation of 2–3 °C was observed to be sufficient to transform the growth mechanism from one regime (spiral growth) to another (by dislocation).

4.1.5.3. *Techniques for evaluating crystal perfection*

The ultimate objective of structural biologists is to analyse crystals of high perfection, in other words, with a minimum of disorder and internal stress. The average disorder of the molecules in the lattice is expressed in the resolution limit of diffraction. Wilson plots provide good illustrations of the diffraction quality for protein crystals. Other sources of disorders, such as dislocations and related defects, as well as the mosaic structure of the crystal, may strongly influence the quality of the diffraction data. They are responsible for increases in the diffuse background scatter and a broadening of diffraction intensities. These defects are difficult to monitor with precision, and dedicated techniques and instruments are required for accurate analysis (reviewed by Chayen *et al.*, 1996).

Mosaicity can be defined experimentally by X-ray rocking-width measurements. An overall diagnostic of crystal quality can be obtained by X-ray diffraction topography. Both techniques have been refined with lysozyme as a test case and are being used for comparative analysis of crystals grown under different conditions, both on earth and in microgravity. For lysozyme and thaumatin, improvement of the mosaicity, as revealed by decreased rocking widths measured with synchrotron radiation, was observed for microgravity-grown crystals (Snell *et al.*, 1995; Ng, Lorber *et al.*, 1997).

Illustration of mosaic-block character in a lysozyme crystal was provided by X-ray topography (Fourme *et al.*, 1995). Comparison of earth and microgravity-grown lysozyme crystals showed a high density of defects in the earth-grown control crystals, while in the microgravity-grown crystals several discrete regions were visible (Stojanoff *et al.*, 1996). X-ray topographs have also been used to compare the orthorhombic and tetragonal forms of lysozyme crystals (Izumi *et al.*, 1996), to monitor temperature-controlled growth of tetragonal lysozyme crystals (Stojanoff *et al.*, 1997), to study the effects of solution variations during growth on the perfection of lysozyme crystals (Dobrianov *et al.*, 1998), and to quantify local misalignments in lysozyme crystal lattices (Otalora *et al.*, 1999).

4.1.6. Use of microgravity

4.1.6.1. *Why microgravity?*

In microgravity, two interrelated parameters, convection and sedimentation, can be controlled. In weightlessness, the elimination of flows that occur in the medium in which the crystal grows theoretically has consequences that may account either for improvements in crystal quality, or crystal deterioration (Chernov, 1997*a*; Carter *et al.*, 1999). The absence of sedimentation permits growth in suspension unperturbed by contact with containing-vessel walls and other crystals. However, protein-crystal movements, some consistent with Marangoni convection (Boggon *et al.*, 1998) and others of diverse origins (García-Ruiz & Otalora, 1997), have been recorded during microgravity growth. On the other hand, it has been proposed that a reduced flow around the crystals minimizes hydrodynamic forces acting on and between the growing crystals and, as a consequence, may favour incorporation of misoriented molecules that act as impurities (Carter *et al.*, 1999). This divergent view of microgravity effects could account for the diversity of results observed in crystallization experiments conducted in this environment, some showing enhanced diffraction qualities of the space-grown crystals (see below), others showing no effect (*e.g.* Vaney *et al.*, 1996) or even decreased crystal quality (*e.g.* Hilgenfeld *et al.*, 1992).

Experiments dealing with the crystallization of proteins and other macromolecules in microgravity have been carried out now for 15 years (DeLucas *et al.*, 1994; Giegé *et al.*, 1995; McPherson, 1996; Boggon *et al.*, 1998). The design of experiments has been based on different strategies. One consists of screening the crystallization of the largest number of proteins, with the intention of obtaining crystals of enhanced quality. In these experiments, monitoring of parameters during growth is restricted, and earth-grown direct control crystals are often not feasible. A second strategic objective is the more thorough study of a few model cases to unravel the basic processes underlying crystal growth. Here, the idea is to monitor as many parameters as possible during flight and, if possible, to conduct ground controls in the same types of crystallization devices, using identical protein samples. In both cases, assessment of the diffraction qualities of the crystals is essential, but precise measurements have only been carried out for the past few years. Altogether, a variety of observations and measurements recorded by many groups of investigators appears to demonstrate, some would say prove, that crystals of biological macromolecules grown in space are superior, in a number of important respects, to equivalent crystals grown in conventional laboratories on earth. This is of some importance, not only from the standpoint of physical phenomena and their understanding, but in a more practical sense as well.

4.1.6.2. *Instrumentation*

Crystallization in microgravity requires specific instrumentation (reviewed by DeLucas *et al.*, 1994; Giegé *et al.*, 1995; McPherson, 1996). A number of reactors have been focused on this goal, some based on current methods used on the ground (batch, dialysis, vapour diffusion), others on more microgravity-relevant approaches, such as free interface diffusion with crystallization vessels of rather large size. The instruments based on this latter method, however, generally cannot be used on earth for control experiments, since with gravity, mixing of the macromolecule and crystallizing-agent solutions occurs by convection. An interesting variation of the classical free interface diffusion system is the hardware using step-gradient diffusion (Sygusch *et al.*, 1996). One of its advantages over more conventional systems is that it provides the possibility of uncoupling nucleation from growth by reducing supersaturation at a constant temperature once nuclei have appeared. A versatile instrument designed by the European Space Agency (ESA) and built by Dornier GmbH is the Advanced Protein Crystallization Facility or APCF (Bosch *et al.*, 1992). The APCF was manifested on a number of US space-shuttle missions and yielded significant comparative 'earth/space' results. It allows monitoring of growth kinetics and can accommodate free interface diffusion (see Fig. 4.1.2.1*d*), dialysis or vapour diffusion. Straightforward ground controls can be conducted with the dialysis cells. A new generation of instruments, the Protein Diagnostic Facility or PCDF, exclusively dedicated to diagnostic measurements of protein-crystal growth, is being developed by ESA and will be installed in the International Space Station (Plester *et al.*, 1999).

4.1.6.3. *Present results: a summary*

Significant and reproducible microgravity experiments have been carried out with a substantial number of model proteins (including lysozyme, thaumatin, canavalin and several plant viruses). The observations in support of microgravity-enhanced crystal growth are primarily of the following nature:

Visual quality and size: The largest dimensions achieved for crystals grown in space were higher than for corresponding crystals

references