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# 4. CRYSTALLIZATION

## 4.1. General methods

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### 4.1.1. Introduction

Crystallization of biological macromolecules has often been considered unpredictable, but presently we know that it follows the same principles as the crystallization of small molecules (Giegé *et al.*, 1995; McPherson *et al.*, 1995; Rosenberger, 1996; Chernov, 1997*a*). It is, similarly, a multiparametric process. The differences from conventional crystal growth arise from the biochemical properties of proteins or nucleic acids compared to quantitative aspects of the growth process and to the unique features of macromolecular crystals. Crystallization methods must reconcile these considerations. The methods described below apply for most proteins, large RNAs, multimacromolecular complexes and viruses. For small DNA or RNA oligonucleotides, crystallization by dialysis is not appropriate, and for hydrophobic membrane proteins special techniques are required.

Macromolecular crystals are, indeed, unique. They are composed of ∼50% solvent on average, though this may vary from 25 to 90%, depending on the particular macromolecule (Matthews, 1985). The protein or nucleic acid occupies the remaining volume so that the entire crystal is, in many ways, an ordered gel with extensive interstitial spaces through which solvent and other small molecules may freely diffuse. In proportion to molecular mass, the number of bonds that a conventional molecule forms with its neighbours in a crystal far exceeds the few exhibited by crystalline macromolecules. Since these contacts provide lattice interactions responsible for the integrity of the crystals, this largely explains the difference in properties between crystals of small molecules and macromolecules. Because macromolecules are labile and readily lose their native structures, the only conditions that can support crystal growth are those that cause little or no perturbation of their molecular properties. Thus, crystals must be grown from solutions to which they are tolerant, within a narrow range of pH, temperature and ionic strength. Because complete hydration is essential for the maintenance of the structure, crystals of macromolecules are always, even during data collection, bathed in the mother liquor (except in cryocrystallography).

Although morphologically indistinguishable, there are important differences between crystals of low-molecular-mass compounds and crystals of macromolecules. Crystals of small molecules exhibit firm lattice forces, are highly ordered, are generally physically hard and brittle, are easy to manipulate, can usually be exposed to air, have strong optical properties and diffract X-rays intensely. Crystals of macromolecules are, by comparison, generally smaller in size, are soft and crush easily, disintegrate if allowed to dehydrate, exhibit weak optical properties and diffract X-rays poorly. They are temperature-sensitive and undergo extensive damage after prolonged exposure to radiation. The liquid channels and solvent cavities that characterize these crystals are primarily responsible for their often poor diffraction behaviour. Because of the relatively large spaces between adjacent molecules and the consequently weak lattice forces, every molecule in the crystal may not occupy exactly equivalent orientations and positions. Furthermore, because of their structural complexity and their potential for conformational dynamics, macromolecules in a particular crystal may exhibit slight variations in their folding patterns or in the dispositions of side groups.

Although the dominant role of the solvent is a major contributor to the poor quality of many protein crystals, it is also responsible for their value to biochemists. Because of the high solvent content, the individual macromolecules in crystals are surrounded by hydration layers that maintain their structure virtually unchanged from that found in bulk solvent. As a consequence, ligand binding, enzymatic and spectroscopic characteristics, and other biochemical features are essentially the same as for the native molecule in solution. In addition, the sizes of the solvent channels are such that conventional chemical compounds, such as ions, substrates or other ligands, may be freely diffused into and out of the crystals. Thus, many crystalline enzymes, though immobilized, are completely accessible for experimentation through alteration of the surrounding mother liquor (Rossi, 1992).

Unlike most conventional crystals (McPherson, 1982), protein crystals are, in general, not initiated from seeds, but are nucleated *ab initio* at high levels of supersaturation, usually reaching 200 to 1000%. It is this high degree of supersaturation that, to a large part, distinguishes protein-crystal formation from that of conventional crystals. That is, once a stable nucleus has formed, it subsequently grows under very unfavourable conditions of excessive supersaturation. Distant from the metastable zone, where ordered growth could occur, crystals rapidly accumulate nutrient molecules, as well as impurities; they also concomitantly accumulate statistical disorder and a high frequency of defects that exceeds those observed for most conventional crystals.

### 4.1.2. Crystallization arrangements and methodologies

#### 4.1.2.1. *General considerations*

Many methods can be used to crystallize macromolecules (McPherson, 1982, 1998; Ducruix & Giegé, 1999), the objectives of which bring the macromolecules to an appropriate state of supersaturation. Although vapour-phase equilibrium and dialysis techniques are favoured, batch and free interface diffusion methods are often used (Fig. 4.1.2.1). Besides the current physical and chemical parameters that affect crystallization (Table 4.1.2.1), macromolecular crystal growth is affected by the crystallization method itself and the geometry of the arrangements used. Generally, in current methods, growth is promoted by the non-equilibrium nature of the crystallization process, which seldom occurs at constant protein concentration. This introduces changes in supersaturation and hence may lead to changes in growth mechanism. Crystallization at constant protein concentration can, however, be achieved in special arrangements based on liquid circulation cells (Vekilov & Rosenberger, 1998).

#### 4.1.2.2. *Batch crystallizations*

Batch methods are the simplest techniques used to produce crystals of macromolecules. They require no more than just mixing the macromolecular solution with crystallizing agents (usually called precipitants) until supersaturation is reached (Fig. 4.1.2.1*a*). Batch crystallization has been used to grow crystals from samples of a millilitre and more (McPherson, 1982), to microdroplets of a few µl (Bott *et al.*, 1982), to even smaller samples in the µl range in

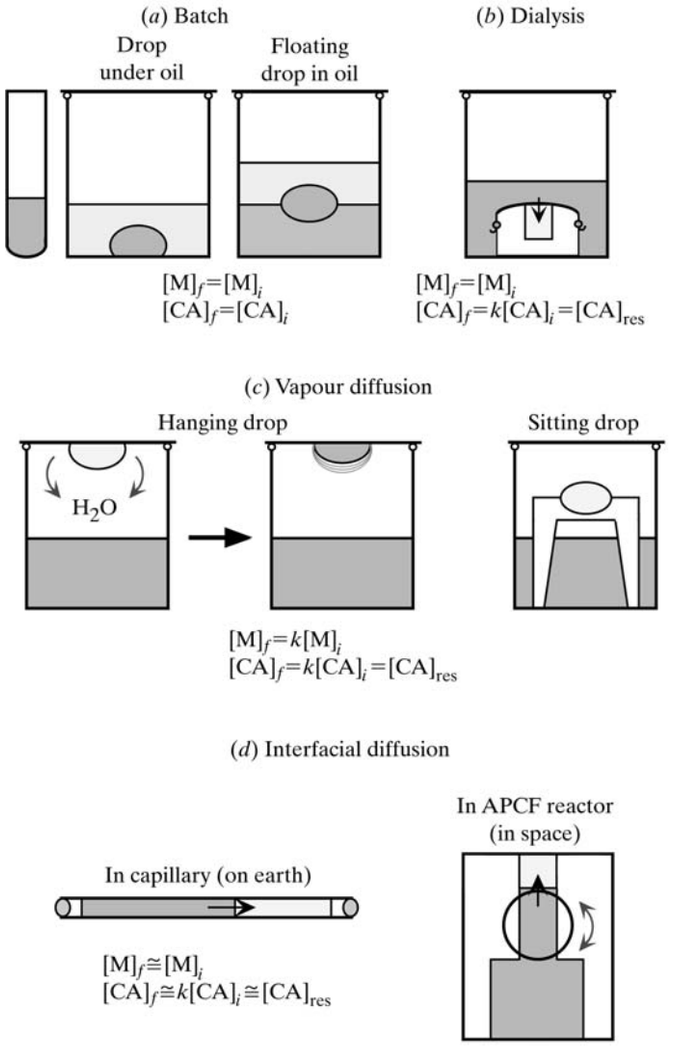


Fig. 4.1.2.1. Principles of the major methods currently used to crystallize biological macromolecules. (*a*) Batch crystallization in three versions. (*b*) Dialysis method with Cambridge button. (*c*) Vapour diffusion crystallization with hanging and sitting drops. (*d*) Interface crystallization in a capillary and in an arrangement for assays in microgravity. The evolution of the concentration of macromolecule, [M], and crystallizing agent (precipitant), [CA], in the different methods is indicated (initial and final concentrations in the crystallization solutions are $[M]_i$, $[M]_f$, $[CA]_i$ and $[CA]_f$, respectively; $[CA]_{res}$ is the concentration of the crystallizing agent in the reservoir solution, and $k$ is a dilution factor specified by the ratio of the initial concentrations of crystallizing agent in the drop and the reservoir). In practice, glass vessels in contact with macromolecules should be silicone treated to obtain hydrophobic surfaces.

capillaries (Luft *et al.*, 1999*a*). Because one begins at high supersaturation, nucleation is often excessive. Large crystals, however, can be obtained when the degree of supersaturation is near the metastable region of the crystal–solution phase diagram.

An automated system for microbatch crystallization and screening permits one to investigate samples of less than 2 µl (Chayen *et al.*, 1990). Reproducibility is guaranteed because samples are dispensed and incubated under oil, thus preventing evaporation and uncontrolled concentration changes of the components in the microdroplets. The method was subsequently adapted for crystallizing proteins in drops suspended between two oil layers (Chayen, 1996; Lorber & Giegé, 1996). Large drops (up to 100 µl) can be deployed, and direct observation of the crystallization process is possible (Lorber & Giegé, 1996). The absence of contacts between the mother liquor and any solid surfaces yields a reduced number of nucleation sites and larger crystals. Batch crystallization can also be conducted under high pressure (Lorber *et al.*, 1996) and has also been adapted for crystallizations on thermal gradients with samples of ~7 µl accommodated in micropipettes (Luft *et al.*, 1999*b*). This latter method allows rapid screening to delineate optimal temperatures for crystallization and also frequently yields crystals of sufficient quality for diffraction analysis.

Batch methods are well suited for crystallizations based on thermonucleation. This can be done readily by transferring crystallization vessels from one thermostated cabinet to another maintained at a higher or lower temperature, depending on whether the protein has normal or retrograde solubility. In more elaborate methods, the temperature of individual crystallization cells is regulated by Peltier devices (Lorber & Giegé, 1992). Local temperature changes can also be created by thermonucleators (DeMattei & Feigelson, 1992) or in temperature-gradient cells (DeMattei & Feigelson, 1993). A variation of classical batch crystallization is the sequential extraction procedure (Jakoby, 1971), based on the property that the solubility of many proteins in highly concentrated salt solutions exhibits significant (but shallow) temperature dependence.

### 4.1.2.3. *Dialysis methods*

Dialysis also permits ready variation of many parameters that influence the crystallization of macromolecules. Different types of systems can be used, but all follow the same general principle. The macromolecule is separated from a large volume of solvent by a semipermeable membrane that allows the passage of small molecules but prevents that of the macromolecules (Fig. 4.1.2.1*b*). Equilibration kinetics depend on the membrane molecular-weight exclusion size, the ratio of the concentrations of precipitant inside and outside the macromolecule chamber, the temperature and the geometry of the dialysis cell. The simplest technique uses a dialysis bag (*e.g.* of inner diameter ~2 mm), but this usually requires at least 100 µl of macromolecule solution per trial.

Crystallization by dialysis has been adapted to small volumes (10 µl or less per assay) in microdialysis cells made from capillary tubes closed by dialysis membranes or polyacrylamide gel plugs (Zeppenzauer, 1971). Microdialysis devices exist in a variety of forms, some derived from the original Zeppenzauer system (Weber & Goodkin, 1970); another is known as the Cambridge button. With this device, protein solutions are deposited in 10–50 µl depressions in Plexiglas microdialysis buttons, which are then sealed by dialysis membranes fixed by rubber O-rings and subsequently immersed in an exterior solution contained in the wells of Linbro plates (or other vessels). The wells are sealed with glass cover slips and vacuum grease. Another dialysis system using microcapillaries was useful, for example, in the crystallization of an enterotoxin from *Escherichia coli* (Pronk *et al.*, 1985). In the double dialysis procedure, the equilibration rate is stringently reduced, thereby improving the method as a means of optimizing crystallization conditions (Thomas *et al.*, 1989). Equilibration rates can be manipulated by choosing appropriate membrane molecular-weight exclusion limits, distances between dialysis membranes, or relative volumes.

### 4.1.2.4. *Vapour diffusion methods*

Crystallization by vapour diffusion was introduced to structural biology for the preparation of tRNA crystals (Hampel *et al.*, 1968). It is well suited for small volumes (as little as 2 µl or less) and has became the favoured method of most experimenters. It is practiced in a variety of forms and is the method of choice for robotics applications. In all of its versions, a drop containing the

Table 4.1.2.1. *Factors affecting crystallization*

| Physical | Chemical | Biochemical |
|---|---|---|
| Temperature variation | pH | Purity of the macromolecule or impurities |
| Surface | Precipitant type | Ligands, inhibitors, effectors |
| Methodology or approach to equilibrium | Precipitant concentration | Aggregation state of the macromolecule |
| Gravity | Ionic strength | Post-translational modifications |
| Pressure | Specific ions | Source of macromolecule |
| Time | Degree of supersaturation | Proteolysis or hydrolysis |
| Vibrations, sound or mechanical perturbations | Reductive or oxidative environment | Chemical modifications |
| Electrostatic or magnetic fields | Concentration of the macromolecules | Genetic modifications |
| Dielectric properties of the medium | Metal ions | Inherent symmetry of the macromolecule |
| Viscosity of the medium | Crosslinkers or polyions | Stability of the macromolecule |
| Rate of equilibration | Detergents, surfactants or amphophiles | Isoelectric point |
| Homogeneous or heterogeneous nucleants | Non-macromolecular impurities | History of the sample |

macromolecule to be crystallized together with buffer, precipitant and additives is equilibrated against a reservoir containing a solution of precipitant at a higher concentration than that in the drop (Fig. 4.1.2.1*c*). Equilibration proceeds by diffusion of the volatile species until the vapour pressure of the drop equals that of the reservoir. If equilibration occurs by water (or organic solvent) exchange from the drop to the reservoir (*e.g.* if the initial salt concentration in the reservoir is higher than in the drop), it leads to a volume decrease of the drop, so that the concentration of all constituents in the drop increase. The situation is the inverse if the initial concentration of the crystallizing agent in the reservoir is lower than that in the drop. In this case, water exchange occurs from the reservoir to the drop. Crystallization of several macromolecules has been achieved using this 'reversed' procedure (Giegé *et al.*, 1977; Richard *et al.*, 1995; Jerusalmi & Steitz, 1997).

Hanging drops are frequently deployed in Linbro tissue-culture plates. These plates contain 24 wells with volumes of ∼2 ml and inner diameters of 16 mm. Each well is covered by a glass cover slip of 22 mm diameter. Drops are formed by mixing 2–10 µl aliquots of the macromolecule with aliquots of the precipitant and additional components as needed. A ratio of two between the concentration of the crystallizing agent in the reservoir and in the drop is most frequently used. This is achieved by mixing a droplet of protein at twice the desired final concentration with an equal volume of the reservoir at the proper concentration (to prevent drops from falling into the reservoir, their final volume should not exceed 25 µl). When no crystals or precipitate are observed in the drops, either sufficient supersaturation has not been reached, or, possibly, only the metastable region has been attained. In the latter case, changing the temperature by a few degrees may be sufficient to initiate nucleation. In the former case, the concentration of precipitant in the reservoir must be increased. A variant of the hanging-drop procedure is the HANGMAN method. It utilizes a clear, non-wetting adhesive tape that both supports the protein drops and seals the reservoirs (Luft *et al.*, 1992).

Sitting drops can be installed in a variety of different devices. Arrangements consisting of Pyrex plates with a variable number of depressions (up to nine) installed in sealed boxes were used for tRNA crystallization (Dock *et al.*, 1984). Drops of mother liquor are dispensed into the depressions and reservoir solutions with precipitant are poured into the bottom sections of the boxes. These systems are efficient for large drop arrays and can be used for both screening and optimizing crystallization conditions. Multi-chamber arrangements are suitable for the control of individual assays (Fig. 4.1.2.2). They often consist of polystyrene plates with 24 wells which can be individually sealed. Sitting drops can also be placed on microbridges (Harlos, 1992) or supported by plastic posts in the centres of the wells. Reservoir solutions are contained in the wells in which the microbridges or support posts are placed. Plates with 96 wells, sealed with clear sealing tape, are convenient for large-matrix screening. Most of these plates are commercially available and can often be used for a majority of different vapour diffusion crystallization methodologies (hanging, sitting or sandwich drops, the latter being maintained between two glass plates). A crystallization setup in which drops are deployed in glass tubes which are maintained vertically and epoxy-sealed on glass cover slips is known as the plug-drop design (Strickland *et al.*, 1995). Plug-drop units are placed in the wells of Linbro plates surrounded by reservoir solution and the wells are then sealed as usual. With this geometry, crystals do not adhere to glass cover slips, as they may with sandwich drops.

Vapour phase equilibration can be achieved in capillaries (Luft & Cody, 1989) or even directly in X-ray capillaries, as described for ribosome crystallization (Yonath *et al.*, 1982). This last method may even be essential for fragile crystals, where transferring from

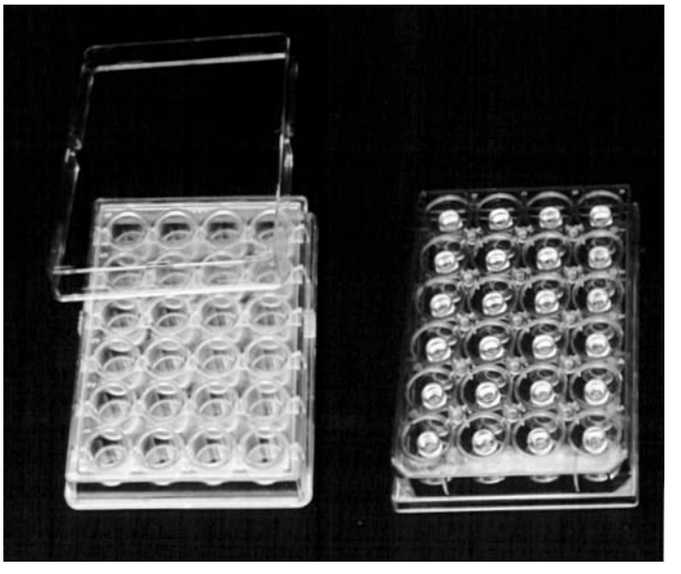

Fig. 4.1.2.2. Two versions of boxes for vapour diffusion crystallization. On the left, a Linbro tissue-culture plate with 24 wells widely used for hanging-drop assays (it may also be used for sitting drops, dialysis and batch crystallization). On the right, a Cryschem multichamber plate, with a post in the centre of each well, for sitting drops.

crystallization cells to X-ray capillaries can lead to mechanical damage. Vapour diffusion methods permit easy variations of physical parameters during crystallization, and many successes have been obtained by affecting supersaturation by temperature or pH changes. With ammonium sulfate as the precipitant, it has been shown that the ultimate pH in the drops of mother liquor is imposed by that of the reservoir (Mikol *et al.*, 1989). Thus, varying the pH of the reservoir permits adjustment of that in the drops. Sitting drops are also well suited for carrying out epitaxic growth of macromolecule crystals on mineral matrices or other surfaces (McPherson & Schlichta, 1988; Kimble *et al.*, 1998).

The kinetics of water evaporation (or of any other volatile species) determine the kinetics of supersaturation and, consequently, those of nucleation. Kinetics measured from hanging drops containing ammonium sulfate, polyethylene glycol (PEG) or 2-methyl-2,4-pentanediol (MPD) are influenced significantly by experimental conditions (Mikol, Rodeau & Giegé, 1990; Luft *et al.*, 1996). The parameters that chiefly determine equilibration rates are temperature, initial drop volume (and initial surface-to-volume ratio of the drop and its dilution with respect to the reservoir), water pressure, the chemical nature of the crystallizing agent and the distance separating the hanging drop from the reservoir solution. Based on the distance dependence, a simple device allows one to vary the rate of water equilibration and thereby optimize crystal-growth conditions (Luft *et al.*, 1996). Evaporation rates can also be monitored and controlled in a weight-sensitive device (Shu *et al.*, 1998). Another method uses oil layered over the reservoir and functions because oil permits only very slow evaporation of the underlying aqueous solution (Chayen, 1997). The thickness of the oil layer, therefore, dictates evaporation rates and, consequently, crystallization rates. Likewise, evaporation kinetics are dependent on the type of oil (paraffin or silicone oils) that covers the reservoir solutions or crystallization drops in the microbatch arrangement (D'Arcy *et al.*, 1996; Chayen, 1997).

The period for water equilibration to reach 90% completion can vary from ~25 h to more than 25 d. Most rapid equilibration occurs with ammonium sulfate, it is slower with MPD and it is by far the slowest with PEG. An empirical model has been proposed which estimates the minimum duration of equilibration under standard experimental conditions (Mikol, Rodeau & Giegé, 1990). Equilibration that brings the macromolecules very slowly to a supersaturated state may explain the crystallization successes with PEG as the crystallizing agent (Table 4.1.2.2). This explanation is corroborated by experiments showing an increase in the terminal crystal size when equilibration rates are reduced (Chayen, 1997).

### 4.1.2.5. *Interface diffusion and the gel acupuncture method*

In this method, equilibration occurs by direct diffusion of the precipitant into the macromolecule solution (Salemme, 1972). To minimize convection, experiments are conducted in capillaries, except under microgravity conditions, where larger diameter devices may be employed (Fig. 4.1.2.1*d*). To avoid too rapid mixing, the less dense solution is poured gently onto the most dense solution. One can also freeze the solution with the precipitant and layer the protein solution above.

Convection in capillaries can be reduced by closing them with polyacrylamide gel plugs instead of dialysis membranes (Zeppenzauer, 1971). A more versatile version of this technique is the gel acupuncture method, which is a counter-diffusion technique (García-Ruiz & Moreno, 1994). In a typical experiment, a gel base is formed from agarose or silica in a small container and an excess of a crystallizing agent is poured over its surface. This agent permeates the gel by diffusion, forming a gradient. A microcapillary filled with the macromolecule and open at one end is inserted at its open end into the gel (Fig. 4.1.2.3). The crystallizing agent then enters the capillary from the gel and forms an upward gradient in the microcapillary, promoting crystallization along its length as it rises by pure diffusion. The effect of the gel is to control this gradient and the rate of diffusion. The method operates with a variety of gels and crystallizing agents, with different heights of these agents over the gel and with open or sealed capillaries. It has been useful for crystallizing several proteins, some of very large size (García-Ruiz *et al.*, 1998).

### 4.1.2.6. *Crystallization in gelled media*

Because convection depends on viscosity, crystallization in gels represents an essentially convection-free environment (Henisch, 1988). Thus, the quality of crystals may be improved in gels. Whatever the mechanism of crystallization in gels, the procedure will produce changes in the nucleation and crystal-growth processes, as has been verified with several proteins (Robert & Lefaucheux, 1988; Miller *et al.*, 1992; Cudney *et al.*, 1994; Robert *et al.*, 1994; Thiessen, 1994; Vidal *et al.*, 1998*a*,*b*). Two types of gels have been used, namely, agarose and silica gels. The latter seem to be the most adaptable, versatile and useful for proteins (Cudney *et al.*, 1994). With silica gels, it is possible to use a variety of different crystallizing agents, including salts, organic solvents and polymers such as PEG. The method also allows the investigator to control pH and temperature. The most successful efforts have involved direct diffusion arrangements, where the precipitant is diffused into a protein-containing gel, or vice versa. As one might expect, nucleation and growth of crystals occur at slower rates, and their number seems to be reduced and their size increased. This finding is supported by small-angle neutron-scattering data showing that silica gels act as nucleation inhibitors for lysozyme (Vidal *et al.*, 1998*a*). Unexpectedly, in agarose gels, the effect is reversed. Here, the gel acts as a nucleation promoter and crystallization has been correlated with cluster formation of the lysozyme molecules (Vidal *et al.*, 1998*b*).

Crystals grown in gels require special methods for mounting in X-ray capillaries, but this can, nonetheless, be done quite easily since the gels are soft (Robert *et al.*, 1999). Gel growth, because it suppresses convection, has also proven to be a useful technique for

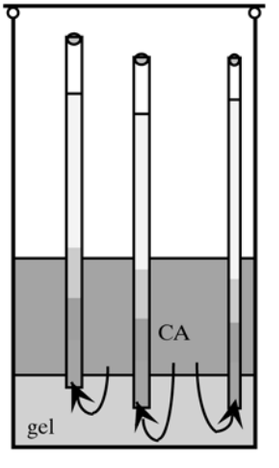


Fig. 4.1.2.3. Principle of the gel acupuncture method for the crystallization of proteins by counter-diffusion. Capillaries containing the macromolecule solution are inserted into a gel, which is covered by a layer of crystallizing agent (CA); the setup is closed by a glass plate. The crystallizing-agent solution diffuses through the gel to the capillaries. The kinetics of crystal growth can be controlled by varying the CA concentration, the capillary volume (diameter and height) and its height in the gel.

Table 4.1.2.2. *Crystallizing agents for protein crystallization*

(*a*) Salts.

| Chemical | | No. of macromolecules | No. of crystals |
|---|---|---|---|
| Ammonium salts: | sulfate | 802 | 979 |
| | phosphate | 20 | 21 |
| | acetate | 13 | 13 |
| | chloride, nitrate, citrate, sulfite, formate, diammonium phosphate | 1–3 | 1–3 |
| Calcium salts: | chloride | 12 | 12 |
| | acetate | 6 | 8 |
| Lithium salts: | sulfate | 33 | 34 |
| | chloride | 17 | 19 |
| | nitrate | 2 | 2 |
| Magnesium salts: | chloride | 32 | 32 |
| | sulfate | 13 | 14 |
| | acetate | 6 | 7 |
| Potassium salts: | phosphate | 42 | 79 |
| | chloride | 15 | 17 |
| | tartrate, citrate, fluoride, nitrate, thiocyanate | 1–3 | 1–3 |
| Sodium salts: | chloride | 148 | 186 |
| | acetate | 43 | 46 |
| | citrate | 34 | 36 |
| | phosphate | 28 | 36 |
| | sulfate, formate, nitrate, tartrate | 3–10 | 3–10 |
| | acetate buffer, azide, citrate–phosphate, dihydrogenphosphate, sulfite, borate, carbonate, succinate, thiocyanate, thiosulfate | 1 or 2 | 1 or 2 |
| Other salts: | sodium–potassium phosphate | 60 | 65 |
| | phosphate (counter-ion not specified) | 33 | 39 |
| | caesium chloride | 18 | 24 |
| | phosphate buffer | 10 | 11 |
| | trisodium citrate, barium chloride, sodium–potassium tartrate, zinc(II) acetate, cacodylate (arsenic salt), cadmium chloride | 1 or 2 | 1–3 |

(*b*) Organic solvents.

| Chemical | No. of macromolecules | No. of crystals |
|---|---|---|
| Ethanol | 63 | 93 |
| Methanol, isopropanol | 27 or 25 | 31 or 28 |
| Acetone | 13 | 13 |
| Dioxane, 2-propanol, acetonitrile, DMSO, ethylene glycol, *n*-propanol, tertiary butanol, ethyl acetate, hexane-1,6-diol | 2–11 | 3–11 |
| 1,3-Propanediol, 1,4-butanediol, 1-propanol, 2,2,2-trifluoroethanol, chloroform, DMF, ethylenediol, hexane-2,5-diol, hexylene-glycol, *N*,*N*-bis(2-hydroxymethyl)-2-aminomethane, *N*-lauryl-*N*,*N*-dimethylamine-*N*-oxide, *n*-octyl-2-hydroxyethylsulfoxide, pyridine, saturated octanetriol, *sec*-butanol, triethanolamine–HCl | 1 | 1 |

(*c*) Long-chain polymers.

| Chemical | No. of macromolecules | No. of crystals |
|---|---|---|
| PEG 4000 | 238 | 275 |
| PEG 6000 | 189 | 251 |
| PEG 8000 | 185 | 230 |
| PEG 3350 | 48 | 54 |
| PEG 1000, 1500, 2000, 3000, 3400, 10 000, 12 000 or 20 000; PEG monomethyl ether 750, 2000 or 5000 | 2–18 | 2–20 |
| PEG 3500, 3600 or 4500; polygalacturonic acid; polyvinylpyrrolidone | 1 | 1 |

Table 4.1.2.2. *Crystallizing agents for protein crystallization* (*cont.*)

(*d*) Low-molecular-mass polymers and non-volatile organic compounds.

| Chemical | No. of macromolecules | No. of crystals |
|---|---|---|
| MPD | 283 | 338 |
| PEG 400 | 40 | 45 |
| Glycerol | 33 | 34 |
| Citrate, Tris–HCl, MES, PEG 600, imidazole–malate, acetate | 2–11 | 4–12 |
| PEG monomethyl ether 550, Tris–maleate, PEG 200, acetate, EDTA, HEPES | 2 | 2 |
| Sucrose, acetic acid, BES, CAPS, citric acid, glucose, glycine–NaOH, imidazole–citrate, Jeffamine ED 4000, maleate, MES–NaOH, methyl-1,2,2-pentanediol, *N*,*N*-bis-(2-hydroxymethyl)-2-aminomethane, *N*-lauryl-*N*,*N*-dimethylamine-*N*-oxide, *n*-octyl-2-hydroxyethylsulfoxide, rufianic acid, spermine–HCl, triethanolamine–HCl, triethylammonium acetate, Tris–acetate, urea | 1 | 1 or 2 |

Abbreviations: BES: *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; CAPS: 3-(cyclohexylamino)-1-propanesulfonic acid; DMF: dimethylformamide; DMSO: dimethyl sulfoxide; EDTA: (ethylenedinitrilo)tetraacetic acid; HEPES: *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); MES: 2-(*N*-morpholino)ethanesulfonic acid; MPD: 2-methyl-2,4-pentanediol; PEG: polyethylene glycol; Tris: tris(hydroxymethyl)aminomethane.

analysing concentration gradients around growing crystals by interferometric techniques (Robert & Lefaucheux, 1988; Robert *et al.*, 1994). In one respect, gel growth mimics crystallization under microgravity conditions (Miller *et al.*, 1992). Finally, it is a useful approach to preserving crystals better once they are grown.

#### 4.1.2.7. *Miscellaneous crystallization methods*

Besides the commonly used methods, less conventional techniques using tailor-made crystallization arrangements exist. Among them are methods where the macromolecules are crystallized in unique physical environments, such as at high pressure (Suzuki *et al.*, 1994; Lorber *et al.*, 1996), under levitation (Rhim & Chung, 1990), in centrifuges (Karpukhina *et al.*, 1975; Lenhoff *et al.*, 1997), in magnetic fields (Ataka *et al.*, 1997; Sazaki *et al.*, 1997; Astier *et al.*, 1998), in electric fields (Taleb *et al.*, 1999) and in microgravity (see Section 4.1.6). The effects of the various physical parameters manipulated in these methods are manifold. Among others, they may alter the conformation of the macromolecule (pressure), orient crystals (magnetic field), influence nucleation (electric field), or suppress convection (microgravity). Thus, formation of new crystal forms may be initiated, and, in favourable cases, crystal quality improved.

In conclusion, it must be recalled that temperature also represents a parameter that can trigger nucleation, regardless of the crystallization method. Temperature-induced crystallization can be carried out in a controlled manner, but it often occurs unexpectedly as a consequence of uncontrolled temperature variations in the laboratory.

#### 4.1.2.8. *Seeding*

It is often desirable to reproduce crystals grown previously, where either the formation of nuclei is limiting, or spontaneous nucleation occurs at such a profound level of supersaturation that poor growth results. In such cases, it is desirable to induce growth in a directed fashion at low levels of supersaturation. This can be accomplished by seeding a metastable, supersaturated protein solution with crystals from earlier trials. Seeding also permits one to uncouple nucleation and growth. Seeding techniques fall into two categories employing either microcrystals as seeds (Fitzgerald & Madson, 1986; Stura & Wilson, 1990) or macroseeds (Thaller *et al.*, 1985). In both cases, the fresh solution to be seeded should be only slightly supersaturated so that controlled, slow growth can occur.

When seeding with microcrystals, the danger is that too many nuclei will be introduced into the fresh supersaturated solution and masses of crystals will result. To overcome this, a stock solution of microcrystals is serially diluted over a very broad range. Some dilution sample in the series will, on average, have no more than one microseed per µl; others will have several times more, or none at all. An aliquot (∼1 µl) of each sample in the series is then added to fresh crystallization trials. This empirical test, ideally, identifies the correct sample to use for seeding by yielding only one or a small number of single crystals when crystal growth is completed.

The second approach involves crystals large enough to be manipulated and transferred under a microscope. Again, the most important consideration is to eliminate spurious nucleation by transfer of too many seeds. It has been proposed that this drawback may be overcome by laser seeding, a technique that permits non-mechanical, *in situ* manipulation of individual seeds as small as 1 µm (Bancel *et al.*, 1998). Even if a single large crystal is employed, microcrystals adhering to its surface may be carried across to the fresh solution. To avoid this, the macroseed is washed by passing it through a series of intermediate transfer solutions. In doing so, not only are microcrystals removed, but, if the wash solutions are chosen properly, some limited dissolution of the seed surface may take place. This has the effect of freshening the seed-crystal surfaces and promoting new growth once it is introduced into the new protein solution.

### 4.1.3. Parameters that affect crystallization of macromolecules

#### 4.1.3.1. *Crystallizing agents*

Crystallizing agents for macromolecules fall into four categories: salts, organic solvents, long-chain polymers, and low-molecular-mass polymers and non-volatile organic compounds (McPherson, 1990). The first two classes are typified by ammonium sulfate and ethanol; higher polymers, such as PEG 4000, are characteristic of the third. In the fourth are placed compounds such as MPD and low-molecular-mass PEGs. A compilation of crystallizing agents and their rates of success, as taken from the CARB/NIST database (Gilliland *et al.*, 1994), is presented in Table 4.1.2.2.

Salts exert their effects by dehydrating proteins through competition for water molecules (Green & Hughes, 1955). Their ability to do this is roughly proportional to the square of the valences of the ionic species composing the salt. Thus multivalent

ions, particularly anions, are the most efficient. One might think there would be little variation between different salts, so long as their ionic valences were the same, or that there would be little variation between two different sulfates, such as $Li_2SO_4$ and $(NH_4)_2SO_4$. This, however, is often not the case. In addition to salting out (a dehydration effect) or lowering the chemical activity of water, there are specific protein–ion interactions that have other consequences (Riès-Kautt & Ducruix, 1991, 1999). These result from the polyvalent character of individual proteins, their structural complexity, and the dependence of their physical properties on environmental conditions and interacting molecules. It is never sufficient, therefore, when attempting to crystallize a protein to examine only one or two salts and ignore a broader range. Changes in salt sometimes produce crystals of varied quality, morphology and diffraction properties.

It is usually not possible to predict the molarity of a salt required to crystallize a particular protein without some prior knowledge of its behaviour. In general, it is a concentration just a few per cent less than that which yields an amorphous precipitate. To determine the precipitation point with a particular agent, a 10 µl droplet of a 5–15 mg ml$^{-1}$ macromolecule solution is placed in the well of a depression slide and observed under a microscope as increasing amounts of salt solution or organic solvent (in 1–2 µl increments) are added. If the well is sealed between additions with a cover slip, the increases can be made over a period of many hours. Indeed, the droplet should equilibrate for 10–30 min after each addition, or longer in the neighbourhood of the precipitation point.

The most common organic solvents used are ethanol, methanol, acetone and MPD. They have been frequently used for crystallizing nucleic acids, particularly tRNAs and duplex oligonucleotides (Dock *et al.*, 1984; Dock-Bregeon *et al.*, 1999). This, in part, stems from the greater tolerance of polynucleotides to organic solvents and their polyanionic character, which appears to be more sensitive to dielectric effects than proteins. Organic solvents should be used at low temperature (especially when volatile), and should be added slowly and with good mixing.

PEGs are polymers of various length that are useful in crystallogenesis (McPherson, 1976; Table 4.1.2.2). The low-molecular-mass species are oily liquids, while those with $M_r >$ 1000 exist as either waxy solids or powders at room temperature. The sizes specified by manufacturers are mean $M_r$ values and the distributions around these means vary. In addition to volume-exclusion properties, PEGs share characteristics with salts that compete for water and produce dehydration, and with organic solvents that reduce the dielectric properties of the medium. PEGs also have the advantage of being effective at minimal ionic strength and providing low-electron-density media. The first feature is important because it provides better affinities for ligand binding than do high-ionic-strength media. Thus, there is greater ease in obtaining heavy-atom derivatives and in forming protein–ligand complexes. The second characteristic, their low electron density, implies a lower noise level for structures derived by X-ray diffraction.

The most useful PEGs in crystallogenesis are those in the range 2000–6000. Sizes are not generally completely interchangeable for a given protein, and thus this parameter has to be optimized by empirical means. An advantage of PEG over other agents is that most proteins crystallize within a rather narrow range of PEG concentration (~4–18%). In addition, the exact PEG concentration at which crystals form is rather insensitive, and if one is within 2–3% of the optimal value, some success will be achieved. The advantage is that when conducting initial trials, one can use a fairly coarse selection of concentrations. This means fewer trials with a corresponding reduction in the amount of material expended.

Since PEG is not volatile, this agent must be used like salt and equilibrated with the protein by dialysis, slow mixing, or vapour diffusion. This latter approach has proved the most popular. When the reservoir concentration is in the range 5–12%, the protein solution to be equilibrated should be at an initial concentration of about half that. When the target PEG concentration is higher than 12%, it is advisable to initiate the equilibration at no more than 4–5% below the final value. This reduces time lags during which the protein might denature. Crystallization of proteins with PEG has proved most successful when ionic strength is low, and most difficult when high. If crystallization proceeds too rapidly, addition of some neutral salt may be used to slow growth. PEG can be used over the entire pH range and a broad temperature range. It should be noted that solutions with PEG may serve as media for microbes, particularly moulds, and if crystallization is attempted at room temperature or over extended periods of time, then retardants, such as azide (~0.1%), must be included in the protein solutions.

### 4.1.3.2. *Other chemical, physical and biochemical variables*

Many physical, chemical and biological variables influence, to a greater or lesser extent, the crystallization of macromolecules (Table 4.1.2.1). The difficulty in arriving at a just assignment of importance for each factor is substantial for several reasons. Every protein (or nucleic acid) is different in its properties, and this even applies to proteins that differ by no more than one or a few amino acids. In addition, each factor may differ in importance. Because of that, there are few means available to predict, in advance, the specific values of a variable or sets of conditions that might be most profitably explored. Furthermore, the various parameters under control are not independent and their interrelations may be difficult to discern. Thus, it is not easy to give firm guidelines regarding physical or chemical factors that can increase the probability of success in crystallizing a particular macromolecule. Among physical parameters, only temperature and pH have been studied carefully; for pressure or magnetic and electric fields, rather few investigations have been carried out (see above), and virtually nothing is known about the effects of sound, vibrations or viscosity on the growth or final quality of protein crystals.

Temperature may be of great importance or it may have little bearing at all. In general, it is wise to conduct parallel investigations at 4 and 20 °C. Even if no crystals are observed at either temperature, differences in the solubility behaviour of a protein with different crystallizing agents and with various effector molecules may give some indication as to whether temperature is likely to play an important role (Christopher *et al.*, 1998). Generally, the solubility of a protein is more sensitive to temperature at low ionic strength than at high. One must remember, however, that diffusion rates are less, and equilibration occurs more slowly, at lower than higher temperatures, so the time required for crystal formation may be longer at lower temperatures. Although most crystallization trials are done at low (~4 °C) or medium (~20 °C) temperatures, higher temperatures in the range 35–40 °C should not be ignored, particularly for molecules that tend to aggregate and for nucleic acids (Dock-Bregeon *et al.*, 1988).

Another important variable is pH. This is because the charge character of a macromolecule and all of its consequences are intimately dependent on the ionization state of its components. Not only does its net charge change with pH (and the charge distribution), but so do its dipole moment, conformation and often its aggregation state. Thus, an investigation of the behaviour of a specific macromolecule as a function of pH is an essential analysis that should be carried out in performing crystallization assays. As with temperature, the procedure is first to conduct trials at coarse intervals over a broad pH range and then to refine trials in the neighbourhoods of those that showed promise. In refining the pH for optimal growth, it should be recalled that the difference between

amorphous precipitate, microcrystals and large single crystals may be only a ΔpH of no more than 0.5.

#### 4.1.3.3. *Additives*

Intriguing questions with regard to optimizing crystallization conditions concern which additional compounds should comprise the mother liquor in addition to solvent, macromolecule and crystallizing agent (Sauter, Ng *et al.*, 1999). Polyamines and metal ions are useful for nucleic acids. Some useful effectors for proteins are those that maintain their structure in a single, homogeneous and invariant state (Timasheff & Arakawa, 1988; Sousa *et al.*, 1991). Such effectors, sometimes named cosmotropes (Jerusalmi & Steitz, 1997), are polyhydric alcohols, like glycerol, sugars, amino acids or methylamino acids. Sulfobetaines also show remarkable properties (Vuillard *et al.*, 1994). Reducing agents, like glutathione or 2-mercaptoethanol, which prevent oxidation, may be important additives, as may chelating compounds, like EDTA, which protect proteins from heavy- or transition-metal ions. Inclusion of these compounds may be desirable when crystallization requires a long period of time to reach completion. When crystallization is carried out at room temperature in PEG or in low-ionic-strength solutions, the growth of microbes that may secrete enzymes that can alter the integrity of the macromolecule under study must be prevented (see below).

Substrates, coenzymes and inhibitors can fix a macromolecule in a more compact and stable form. Thus, a greater degree of structural homogeneity may be imparted to a population of macromolecules by complexing them with a natural ligand before attempting crystallization. In terms of crystallization, complexes have to be treated as almost entirely separate problems. This may permit a new opportunity for growing crystals if the native molecule is obstinate. Just as natural substrates or inhibitors are often useful, they can also have the opposite effect of obstructing crystal formation. In such cases, care must be taken to eliminate them from the mother liquor and from the purified protein before crystallization is attempted. Finally, it should be noted that the use of inhibitors or other ligands may sometimes be invoked to obtain a crystal form different from that grown from the native protein.

### 4.1.4. How to crystallize a new macromolecule

#### 4.1.4.1. *Rules and general principles*

The first concern is to obtain a macromolecular sample of highest quality; second, to collate all biochemical and biophysical features characterizing the macromolecule in order to design the best crystallization strategy; and finally, to establish precise protocols that ensure the reproducibility of experiments. It is also important to clean and sterilize by filtration (over 0.22 μm porosity membranes) all solutions in contact with pure macromolecules to remove dust and other solid particles, and to avoid contamination by microbes. Inclusion of sodium azide in crystallizing solutions may discourage invasive bacteria and fungi. In vapour-diffusion assays, such contamination can be prevented by simply placing a small grain of thymol in the reservoir. Thymol, however, can occasionally have specific effects on crystal growth (Chayen *et al.*, 1989) and thus may serve as an additive in screenings as well.

Crystallization requires bringing the macromolecule to a supersaturated state that favours nucleation. Use of phase diagrams may be important for this purpose (Haas & Drenth, 1998; Sauter, Lorber *et al.*, 1999). If solubilities or phase diagrams are unavailable, it is nevertheless important to understand the correlation between solubility and the way supersaturation is reached in the different crystallization methods (see Fig. 4.1.2.1). In dialysis, the macromolecule concentration remains constant during equilibration. The initial concentration of the crystallizing agent in the exterior solution leaves the macromolecule in an undersaturated state. With increasing concentration of the agent in the exterior solution, a state of supersaturation can be attained, leading to crystallization or precipitation. In a vapour-diffusion experiment, where the concentration of crystallizing agent in the reservoir exceeds that in the drop, the macromolecule will begin to concentrate from an undersaturated to a supersaturated state, with both macromolecule and crystallizing-agent concentrations increasing. Crystals appear in the metastable region. For crystals that appear first, the trajectory of equilibration is complex and the remaining concentration of macromolecule in solution will converge towards a point located on the solubility curve. In batch crystallization using a closed vessel, three situations can occur: if the concentration of the macromolecule is undersaturated, crystallization never occurs (unless another parameter such as temperature is varied); if it belongs to the supersaturated region between solubility and precipitation curves, crystals can grow until the remaining concentration of the macromolecule in solution equals its solubility; if supersaturation is too high, the macromolecule precipitates immediately, although in some cases, crystals can grow from precipitates by Ostwald ripening (Ng *et al.*, 1996).

#### 4.1.4.2. *Purity and homogeneity*

The concept of purity assumes a particular importance in crystallogenesis (Giegé *et al.*, 1986; Rosenberger *et al.*, 1996), even though some macromolecules may crystallize readily from impure solutions (Judge *et al.*, 1998). In general, macromolecular samples should be cleared of undesired macromolecules and small molecules and, in addition, should be pure in terms of sequence integrity and conformation. Contaminants may compete for sites on growing crystals and generate growth disorders (Vekilov & Rosenberger, 1996), and it has been shown that only p.p.m. amounts of foreign molecules can induce formation of non-specific aggregates, alter macromolecular solubility, or interfere with nucleation and crystal growth (McPherson *et al.*, 1996; Skouri *et al.*, 1995). These effects are reported to be reduced in gel media (Hirschler *et al.*, 1995; Provost & Robert, 1995).

Microheterogeneities in purified samples can be revealed by analytical methods, such as SDS–PAGE, isoelectric focusing, NMR and mass spectroscopy. Although their causes are multiple, the most common ones are uncontrolled fragmentation and post-synthetic modifications. Proteolysis represents a major difficulty that must be overcome during protein isolation. Likewise, nucleases are a common cause of heterogeneity in nucleic acids, especially in RNAs that are also sensitive to hydrolytic cleavage at alkaline pH and metal-induced fragmentation. Fragmentation can be inhibited by addition of protease or nuclease inhibitors during purification (Lorber & Giegé, 1999). Conformational heterogeneity may originate from ligand binding, intrinsic flexibility of the macromolecule backbones, oxidation of cysteine residues, or partial denaturation. Structural homogeneity may be improved by truncation of the flexible parts of the macromolecule under study (Price & Nagai, 1995; Berne *et al.*, 1999).

#### 4.1.4.3. *Sample preparation*

Preparation of solutions for crystallization experiments should follow some common rules. Stocks should be prepared with chemicals of the purest grade dissolved in double-distilled water and filtered through 0.22 μm membranes. The chemical nature of the buffer is an important parameter, and the pH of buffers, which must be strictly controlled, is often temperature-dependent, especially that of Tris buffers. Commercial PEG contains contaminants, ionic (Jurnak, 1986) or derived from peroxidation, and thus repurification is recommended (Ray & Puvathingal, 1985).

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

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

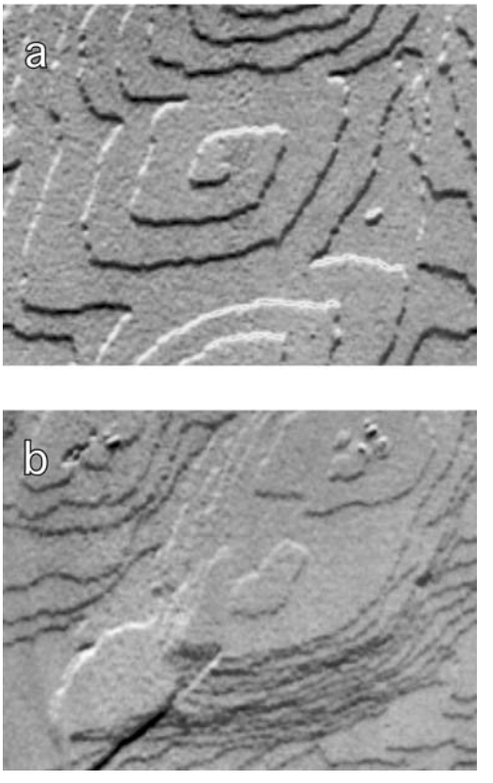


Fig. 4.1.5.1. Visualization of the surface of yeast $tRNA^{Phe}$ crystals by AFM. (*a*) Spiral growth with screw dislocations occurring at lower supersaturation and (*b*) growth by two-dimensional nucleation occurring at higher supersaturation, showing growth and coalescence of islands and expansions 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

grown at 1*g*. Space-grown crystals were observed to be consistently less marred by cracks, striations, secondary nucleation, visible flaws, inclusions, or aggregate growth. When large numbers of crystals were produced in experiments, morphometric analysis (scoring based on size) of the entire population generally showed a statistically significant tendency toward larger average sizes (*e.g.* DeLucas *et al.*, 1994; Koszelak *et al.*, 1995; Ng, Lorber *et al.*, 1997).

*Maximum resolution and Wilson plots*: The first quantitative measurements to support conclusions based on visual inspection were those comparing the maximum resolutions of diffraction patterns from corresponding crystals grown on the ground and in space. A striking improvement of resolution was found for paralbumin, where space-grown crystals diffract to 0.9 Å resolution, but earth-grown crystals are not suitable for diffraction analysis (Declercq *et al.*, 1999).

An analytical procedure for comparing X-ray data is the comparative Wilson plot. Reports have appeared in which the maximum obtainable resolution of X-ray diffraction was greater for crystals grown in space than for equivalent crystals produced on earth. Another product of a Wilson plot is the ratio, over the entire resolution range, of the average intensity to the background scatter, taken in small resolution increments across the entire $\sin(2\theta)$ range. This $I/\sigma$ ratio is, in a way, the peak-to-noise ratio for the measurable X-ray data. Again, as for resolution, the $I/\sigma$ ratio for X-ray diffraction data collected from crystals grown in space was in several cases reported to be greater than for the corresponding earth-grown crystals [*e.g.* for satellite tobacco mosaic virus (McPherson, 1996) and thaumatin (Ng, Lorber *et al.*, 1997)].

*Mosaicity*: An additional criterion used to support the enhanced quality of crystals grown in microgravity is the mosaic spread of X-ray diffraction intensities recorded from space- and earth-grown samples. Several reports indicate that for at least some protein crystals (lysozyme, thaumatin), the width and shape of diffraction intensities are improved for crystals grown in microgravity (*e.g.* Snell *et al.*, 1995; Stojanoff *et al.*, 1996; Ng, Lorber *et al.*, 1997).

*Impurity incorporation*: Impurities can be incorporated in growing crystals and their partitioning between the crystal and the mother liquor shown (Thomas *et al.*, 1998). Based on theoretical considerations, such partitioning should depend on the presence or absence of convection and, therefore, should be gravity-dependent. This is actually the case as demonstrated with lysozyme, for which the microgravity-grown crystals incorporated 4.5 times less impurity (a lysozyme dimer) than the earth controls (Carter *et al.*, 1999).

*Crystallographic structure*: In a case study with tetragonal hen egg-white lysozyme crystals, a significant improvement of resolution from 1.6 to 1.35 Å resolution, an average decrease of *B* factors, and an improved electron density and water structure have been noticed for the space-grown crystals (Carter *et al.*, 1999).

Altogether, the above examples suggest an overall positive effect of microgravity on protein-crystal growth. To date, however, and because of the youth of microgravity science, in particular in its newest developments, it is not possible to make generalizations for all proteins. Even for the same protein, divergent conclusions can be reached; for example, the quality of the X-ray structure of lysozyme was shown to be improved (Carter *et al.*, 1999) or unaffected by microgravity (Vaney *et al.*, 1996). In this case, the contradiction may originate from different levels of impurities present in the protein batches used in the two studies and/or from non-identical growth conditions in different hardware.

### 4.1.6.4. *Interpretation of data*

It is conceivable that the alteration of fluid properties by gravity, the occurrence of density fluctuations, or some other property such as these could affect nucleation. Based on computer simulations, it has been suggested that crystals nucleate under different supersaturation and supersaturation rates on ground and in space (Otalora & García-Ruiz, 1997). There is, however, no definitive evidence at this time for how gravity affects nucleation, although it has been observed with thaumatin that the total number of crystals grown in space was less relative to those grown on earth (Ng, Lorber *et al.*, 1997). Gravity expresses itself in fluids, including crystallization mother liquors, by altering mass and heat transport, and it is acknowledged that transport has a real, and in some cases profound, effect on several aspects of growth. It therefore seems reasonable to expect that the growth of crystals is altered once a critical nucleus has formed and that this is important in understanding the effects of microgravity.

Transport would seem to be of particular importance for macromolecular crystallization, because the size of the entities involved requires them to have extremely low diffusivities, two to three orders of magnitude less than for conventional molecules. Elimination of fluid convection may, however, dramatically affect the movement and distribution of macromolecules in the fluid and their transport and absorption to crystal surfaces (Pusey *et al.*, 1988). In addition, most macromolecules, particularly at high concentration, tend to form large non-specific aggregates and clusters in solution. These may very well be a major source of contaminants that become incorporated into the crystal lattices of macromolecules and are, therefore, a major influence on the growth process. By virtue of their size and low diffusivity, the movement of aggregates and large impurities in solution is even more significantly altered. Finally, some macromolecular crystals may grow by the direct addition of three-dimensional nuclei or volume elements of a liquid protein phase, and all macromolecular crystals are, at the very least, affected by these processes. The transport of three-dimensional nuclei or liquid protein droplets, again, by virtue of their size, should be altered in the absence of gravity.

Protein crystals grow in relatively large volumes of mother liquor, hence the consumption of molecules by growing crystals does not significantly exhaust the solution of protein nutrient for a long period of time. Thus, normal crystal growth may proceed to completion at high supersaturation and never approach the metastable phase of supersaturation where growth might proceed more favourably. In earth's gravity, there is continuous density-driven convective mixing in the solution owing to gradients arising from temperature and from incorporation of molecules by the growing crystal. The effects of diffusive transport in the laboratory are almost negligible in comparison to microgravity because of the very slow rate of diffusion of large macromolecules. Because of convective mixing, protein crystals nucleated on earth are continuously exposed to the full concentration of protein nutrient present in the bulk solvent. Convection thus maintains, at the growing crystal interface, excessive and unfavourable supersaturation as growth proceeds. This provides an explanation as to why microgravity may significantly improve the quality of protein crystals. The mechanism for enhanced order and reduction of defects may not be directly due to convective turbulence at growing crystal surfaces, but to reduction of the concentration of nutrient molecules and impurities in the immediate neighbourhood of the growing crystals.

As a macromolecular crystal forms in microgravity, a concentration gradient or 'depletion zone' is established around the nucleus. Because protein diffusion is slow and that of impurities may even be slower, the depletion zone is quasi-stable. The net effect is that the surfaces of the growing crystal interface with a local solution phase at a lower concentration of protein nutrient and impurities than exists in the bulk solvent. The crystal, as it grows, experiences a reduction in its local degree of supersaturation and essentially

creates for itself an environment equivalent to the metastable region where optimal growth might be expected to occur.

#### 4.1.6.5. *The future of crystallization under microgravity*

Several years ago, investigations of macromolecular crystallization under microgravity diverged along two paths. The objective of the first was to produce high-quality crystals for biotechnology and research applications, *e.g.* X-ray diffraction analysis. The crystals themselves were the product, and scientific results were of lesser importance. The goal of the second line of investigation was a definition and description, in a quantitative sense, of the mechanisms by which the quality of crystals was improved (or altered) in microgravity. Understanding and, in the end, controlling the physics of the process was the real objective. This second interest was ably supported by extensive ground-based research based on a variety of sophisticated techniques.

The confluence of results from these two streams has significantly altered prevailing circumstances and attitudes. Persuasive explanations for the observed improvements in size and quality of macromolecular crystals grown in microgravity have emerged, and a convincing theoretical framework now exists for understanding the phenomena involved. Physical methods, such as interferometry and AFM, have revealed the unsuspected variety, structure and density of dislocations and defects inherent in macromolecular crystals. These arrays of defects, which provide the key to the improvement attained in microgravity, have been shown to be far more complex, extensive and dense than those commonly associated with conventional small-molecule crystals. Thus, macromolecular crystals are more sensitive to the unusually high degrees of supersaturation at which they are usually grown and to the mass-transport mechanisms responsible for bringing nutrient to their growing surfaces. The self-regulating nature of protein crystallization in microgravity, through the establishment of local concentration gradients of reduced supersaturation, explains why the diffusive transport that predominates in space produces a significant difference in ultimate crystal quality.

There are currently a number of powerful systems under development by the USA, Europe and Japan. These will be deployed on the International Space Station, where they will form the core facilities for the investigation of macromolecular crystallizations in space. These studies will extend and refine our understanding of the physical principles governing microgravity crystal growth and will better identify the properties of macromolecules likely to benefit most from crystallization in microgravity.

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# References

## 4.1


Astier, J. P., Veesler, S. & Boistelle, R. (1998). *Protein crystals orientation in a magnetic field. Acta Cryst.* D**54**, 703–706.

Ataka, M., Katoh, E. & Wakayama, N. L. (1997). *Magnetic orientation as a tool to study the initial stage of crystallization of lysozyme. J. Cryst. Growth*, **173**, 592–596.

Baldwin, E. T., Crumly, K. V. & Carter, C. W. (1986). *Practical, rapid screening of protein crystallization conditions by dynamic light scattering. Biophys. J.* **49**, 47–48.

Bancel, P. A., Cajipe, V. B., Rodier, F. & Witz, J. (1998). *Laser seeding for biomolecular crystallization. J. Cryst. Growth*, **191**, 537–544.

Berne, P. F., Doublié, S. & Carter, C. W. Jr (1999). *Molecular biology for structural biology.* In *Crystallization of nucleic acids and proteins*, edited by A. Ducruix & R. Giegé, 2nd ed. Oxford University Press.

Boggon, T. J., Chayen, N. E., Snell, E. H., Dong, J., Lautenschlager, P., Potthast, L., Siddons, D. P., Stojanoff, V., Gordon, E., Thompson, A. W., Zagalsky, P. F., Bi, R.-C. & Helliwell, J. R. (1998). *Protein crystal movements and fluid flows during microgravity growth. Philos. Trans. R. Soc. London Ser. A*, **356**, 1045–1061.

Bonneté, F., Malfois, M., Finet, S., Tardieu, A., Lafont, S. & Veesler, S. (1997). *Different tools to study interaction potentials in $\gamma$-crystallin solutions: relevance to crystal growth. Acta Cryst.* D**53**, 438–447.

Bosch, R., Lautenschlager, P., Potthast, L. & Stapelmann, J. (1992). *Experiment equipment for protein crystallization in $\mu$g facilities. J. Cryst. Growth*, **122**, 310–316.

Bott, R. R., Navia, M. A. & Smith, J. L. (1982). *Improving the quality of protein crystals through purification by isoelectric focusing. J. Biol. Chem.* **257**, 9883–9886.

Carter, D. C., Lim, K., Ho, J. X., Wright, B. S., Twigg, P. D., Miller, T. Y., Chapman, J., Keeling, K., Ruble, J., Vekilov, P. G., Thomas, B. R., Rosenberger, F. & Chernov, A. A. (1999). *Lower dimer impurity incorporation may result in higher perfection of HEWL crystal grown in $\mu$g – a case study. J. Cryst. Growth*, **196**, 623–637.

Chayen, N. E. (1996). *A novel technique for containerless protein crystallization. Protein Eng.* **9**, 927–929.

Chayen, N. E. (1997). *A novel technique to control the rate of vapour diffusion, giving larger protein crystals. J. Appl. Cryst.* **30**, 198–202.

Chayen, N. E., Boggon, T. J., Cassetta, A., Deacon, A., Gleichmann, T., Habash, J., Harrop, S. J., Helliwell, J. R., Nieh, Y. P., Peterson, M. R., Raftery, J., Snell, E. H., Hädener, A., Niemann, A. C., Siddons, D. P., Stojanoff, V., Thompson, A. W., Ursby, T. & Wulff, M. (1996). *Trends and challenges in experimental macromolecular crystallography. Q. Rev. Biophys.* **29**, 227–278.

Chayen, N. E., Lloyd, L. F., Collyer, C. A. & Blow, D. M. (1989). *Trigonal crystals of glucose isomerase require thymol for their growth and stability. J. Cryst. Growth*, **97**, 367–374.

Chayen, N. E., Shaw Stewart, P. D., Maeder, D. L. & Blow, D. M. (1990). *An automated system for micro-batch protein crystallisation and screening. J. Appl. Cryst.* **23**, 297–302.

Chernov, A. A. (1997*a*). *Crystals built of biological macromolecules. Phys. Rep.* **288**, 61–75.

Chernov, A. A. (1997*b*). *Protein versus conventional crystals: creation of defects. J. Cryst. Growth*, **174**, 354–361.

Chernov, A. A. (1999). *Estimates of internal stress and related mosaicity in solution grown crystals: proteins. J. Cryst. Growth*, **196**, 524–534.

Christopher, G. K., Phipps, A. G. & Gray, R. J. (1998). *Temperature-dependent solubility of selected proteins. J. Cryst. Growth*, **191**, 820–826.

Cole, T., Kathman, A., Koszelak, S. & McPherson, A. (1995). *Determination of the local refractive index for protein and virus crystals in solution by Mach–Zehnder interferometry. Anal. Biochem.* **231**, 92–98.

Crossio, M.-P. & Jullien, M. (1992). *Fluorescence study of precrystallization of ribonuclease A: effect of salts. J. Cryst. Growth*, **122**, 66–70.

Cudney, B., Patel, S. & McPherson, A. (1994). *Crystallization of macromolecules in silica gels. Acta Cryst.* D**50**, 479–483.

D'Arcy, A., Elmore, C., Stihle, M. & Johnston, J. E. (1996). *A novel approach to crystallizing proteins under oil. J. Cryst. Growth*, **168**, 175–180.

Declercq, J.-P., Evrard, C., Carter, D. C., Wright, B. S., Etienne, G. & Parello, J. (1999). *A crystal of a typical EF-hand protein grown under microgravity diffracts X-rays beyond 0.9 Å resolution. J. Cryst. Growth*, **196**, 595–601.

DeLucas, L. J., Long, M. M., Moore, K. M., Rosenblum, W. M., Bray, T. L., Smith, C., Carson, M., Narayana, S. V. L., Harrington, M. D., Carter, D., Clark, A. D. Jr, Nanni, R. G., Ding, J., Jacobo-Molina, A., Kamer, G., Hughes, S. H., Arnold, E., Einspahr, H. M., Clancy, L. L., Rao, G. S. J., Cook, P. F., Harris, B. G., Munson, S. H., Finzel, B. C., McPherson, A., Weber, P. C., Lewandowski, F. A., Nagabhushan, T. L., Trotta, P. P., Reichert, P., Navia, M. A., Wilson, K. P., Thomson, J. A., Richards, R. N., Bowersox, K. D., Meade, C. J., Baker, E. S., Bishop, S. P., Dunbar, B. J., Trinh, E., Prahl, J., Sacco, A. Jr & Bugg, C. E. (1994). *Recent results and new developments for protein crystal growth in microgravity. J. Cryst. Growth*, **135**, 183–195.

DeMattei, R. C. & Feigelson, R. S. (1992). *Controlling nucleation in protein solutions. J. Cryst. Growth*, **122**, 21–30.

DeMattei, R. C. & Feigelson, R. S. (1993). *Thermal methods for crystallizing biological macromolecules. J. Cryst. Growth*, **128**, 1225–1231.

Dobrianov, I., Finkelstein, K. D., Lemay, S. G. & Thorne, R. E. (1998). *X-ray topographic studies of protein crystal perfection and growth. Acta Cryst.* D**54**, 922–937.

Dock, A.-C., Lorber, B., Moras, D., Pixa, G., Thierry, J.-C. & Giegé, R. (1984). *Crystallization of transfer ribonucleic acids. Biochimie*, **66**, 179–201.

Dock-Bregeon, A.-C., Chevrier, B., Podjarny, A., Moras, D., deBear, J. S., Gough, G. R., Gilham, P. T. & Johnson, J. E. (1988). *High resolution structure of the RNA duplex [U(U − A)6A]$_2$. Nature (London)*, **209**, 375–378.

Dock-Bregeon, A.-C., Moras, D. & Giegé, R. (1999). *Nucleic acids and their complexes.* In *Crystallization of nucleic acids and proteins*, 2nd ed. A. Ducruix & R. Giegé, edited by Oxford University Press.

Ducruix, A. & Giegé, R. (1999). Editors. *Crystallization of proteins and nucleic acids: a practical approach*, 2nd ed. Oxford: IRL Press.

Ducruix, A., Guilloteau, J.-P., Riès-Kautt, M. & Tardieu, A. (1996). *Protein interactions as seen by solution X-ray scattering prior to crystallogenesis. J. Cryst. Growth*, **168**, 28–39.

Durbin, S. D. & Carlson, W. E. (1992). *Lysozyme crystal growth studied by atomic force microscopy. J. Cryst. Growth*, **122**, 71–79.

Durbin, S. D. & Feher, G. (1990). *Studies of crystal growth mechanisms by electron microscopy. J. Mol. Biol.* **212**, 763–774.

Durbin, S. D. & Feher, G. (1996). *Protein crystallization. Annu. Rev. Phys. Chem.* **47**, 171–204.

Ebel, C., Faou, P. & Zaccaï, G. (1999). *Protein–solvent and weak protein–protein interactions in halophilic malate dehydrogenase. J. Cryst. Growth*, **196**, 395–402.

Ferré-D'Amaré, A. & Burley, S. K. (1997). *Dynamic light scattering in evaluating crystallizability of macromolecules. Methods Enzymol.* **276**, 157–166.

Finet, S., Bonneté, F., Frouin, J., Provost, K. & Tardieu, A. (1998). *Lysozyme crystal growth, as observed by small angle X-ray scattering, proceeds without crystallization intermediates. Eur. Biophys. J.* **76**, 554–561.

Fitzgerald, P. M. D. & Madson, N. B. J. (1986). *Improvement of limit of diffraction and useful X-ray lifetime of crystals of glycogen debranching enzyme. J. Cryst. Growth*, **76**, 600–606.

**4.1 (*cont.*)**


Fourme, R., Ducruix, A., Ries-Kautt, M. & Capelle, B. (1995). *The perfection of protein crystals probed by direct recording of Bragg reflection profiles with a quasi-planar X-ray wave. J. Synchrotron Rad.* **2**, 136–142.

García-Ruiz, J. M. & Moreno, A. (1994). *Investigations on protein crystal growth by the gel acupuncture method. Acta Cryst.* D**50**, 484–490.

García-Ruiz, J. M., Moreno, A., Otalora, F., Rondon, D., Viedma, C. & Zauscher, F. (1998). *Teaching protein crystallization by the gel acupuncture method. J. Chem. Educ.* **75**, 442–446.

García-Ruiz, J. M., Novella, M. L. & Otalora, F. (1999). *Supersaturation patterns in counter-diffusion crystallization methods followed by Mach–Zehnder interferometry. J. Cryst. Growth*, **196**, 703–710.

García-Ruiz, J. M. & Otalora, F. (1997). *Crystal growth studies in microgravity with the APCF. II. Image analysis studies. J. Cryst. Growth*, **182**, 155–167.

Georgalis, Y., Zouni, A., Eberstein, W. & Saenger, W. (1993). *Formation dynamics of protein precrystallization fractal clusters. J. Cryst. Growth*, **126**, 245–260.

George, A., Chiang, Y., Guo, B., Abrabshahi, A., Cai, Z. & Wilson, W. W. (1997). *Second virial coefficient as predictor in protein crystal growth. Methods Enzymol.* **276**, 100–110.

Giegé, R., Dock, A.-C., Kern, D., Lorber, B., Thierry, J.-C. & Moras, D. (1986). *The role of purification in the crystallization of proteins and nucleic acids. J. Cryst. Growth*, **76**, 554–561.

Giegé, R., Drenth, J., Ducruix, A., McPherson, A. & Saenger, W. (1995). *Crystallogenesis of biological macromolecules. Biological, microgravity, and other physico-chemical aspects. Prog. Cryst. Growth Charact.* **30**, 237–281.

Giegé, R., Moras, D. & Thierry, J.-C. (1977). *Yeast transfer RNA$^{Asp}$: a new high resolution X-ray diffracting crystal form of a transfer RNA. J. Mol. Biol.* **115**, 91–96.

Gilliland, G., Tung, M., Blakeslee, D. M. & Ladner, J. E. (1994). *Biological macromolecule crystallization database, version 3.0: new features, data and the NASA archive for protein crystal growth data. Acta Cryst.* D**50**, 408–413.

Green, A. A. & Hughes, W. L. (1995). *Protein fractionation on the basis of solubility in aqueous solutions of salts and organic solvents. Methods Enzymol.* **1**, 67–90.

Gripon, C., Legrand, L., Rosenman, I., Vidal, O., Robert, M.-C. & Boué, F. (1997). *Lysozyme–lysozyme interactions in under- and super-saturated solutions: a simple relation between the second virial coefficient in $H_2O$ and $D_2O$. J. Cryst. Growth*, **178**, 575–584.

Haas, C. & Drenth, J. (1998). *The protein–water phase diagram and the growth of protein crystals from aqueous solution. J. Phys. Chem.* **102**, 4226–4232.

Hampel, A., Labananskas, M., Conners, P. G., Kirkegard, L., Raj Bhandary, U. L., Sigler, P. B. & Bock, R. M. (1968). *Single crystals of transfer RNA from formyl-methionine and phenylalanine transfer RNA's. Science*, **162**, 1384–1386.

Harlos, K. (1992). *Micro-bridges for sitting-drop crystallizations. J. Appl. Cryst.* **25**, 536–538.

Henisch, H. K. (1988). *Crystals in gels and Liesegang rings.* Cambridge, MA: Cambridge University Press.

Hilgenfeld, R., Liesum, A., Storm, R. & Plaas-Link, A. (1992). *Crystallization of two bacterial enzymes on an unmanned space mission. J. Cryst. Growth*, **122**, 330–336.

Hirschler, J., Charon, M.-H. & Fontecilla-Camps, J. C. (1995). *The effects of filtration on protein nucleation in different growth media. Protein Sci.* **4**, 2573–2577.

Izumi, K., Sawamura, S. & Ataka, M. (1996). *X-ray topography of lysozyme crystals. J. Cryst. Growth*, **168**, 106–111.

Jakoby, W. B. (1971). *Crystallization as a purification technique. Methods Enzymol.* **22**, 248–252.

Jerusalmi, D. & Steitz, T. A. (1997). *Use of organic cosmotropic solutes to crystallize flexible proteins: application to T7 RNA polymerase and its complex with the inhibitor T7 lysozyme. J. Mol. Biol.* **274**, 748–756.

Judge, R. A., Forsythe, E. L. & Pusey, M. L. (1998). *The effect of protein impurities on lysozyme crystal growth. Biotech. Bioeng.* **59**, 776–785.

Jurnak, F. (1986). *Effect of chemical impurities in polyethylene glycol on macromolecular crystallization. J. Cryst. Growth*, **76**, 577–582.

Kam, Z., Shore, H. B. & Feher, G. (1978). *On the crystallization of proteins. J. Mol. Biol.* **123**, 539–555.

Karpukhina, S. Ya., Barynin, V. V. & Lobanova, G. M. (1975). *Crystallization of catalase in the ultracentrifuge. Sov. Phys. Crystallogr.* **20**, 417–418.

Kimble, W. L., Paxton, T. E., Rousseau, R. W. & Sambanis, A. (1998). *The effect of mineral substrates on the crystallization of lysozyme. J. Cryst. Growth*, **187**, 268–276.

Komatsu, H., Miyashita, S. & Suzuki, Y. (1993). *Interferometric observation of the interfacial concentration gradient layers around a lysozyme crystal. Jpn. J. Appl. Phys.* **32**(2), 1855–1857.

Konnert, J. H., D'Antonio, P. & Ward, K. B. (1994). *Observation of growth steps, spiral dislocations and molecular packing on the surface of lysozyme crystals with the atomic force microscope. Acta Cryst.* D**50**, 603–613.

Koszelak, S., Day, J., Leja, C., Cudney, R. & McPherson, A. (1995). *Protein and virus crystal growth on International Microgravity Laboratory-2. Biophys. J.* **69**, 13–19.

Koszelak, S. & McPherson, A. (1988). *Time lapse microphotography of protein crystal growth using a color VRC. J. Cryst. Growth*, **90**, 340–343.

Koszelak, S., Martin, D., Ng, J. & McPherson, A. (1991). *Protein crystal growth rates determined by time lapse microphotography. J. Cryst. Growth*, **110**, 177–181.

Kurihara, K., Miyashita, S., Sazaki, G., Nakada, T., Suzuki, Y. & Komatsu, H. (1996). *Interferometric study on the crystal growth of tetragonal lysozyme crystal. J. Cryst. Growth*, **166**, 904–908.

Kuznetsov, Y. G., Malkin, A. J., Greenwood, A. & McPherson, A. (1995). *Interferometric studies of growth kinetics and surface morphology in macromolecular crystal growth: canavalin, thaumatin, and turnip yellow mosaic virus. J. Struct. Biol.* **114**, 184–196.

Lenhoff, A. M., Pjura, P. E., Dilmore, J. G. & Godlewski, T. S. Jr (1997). *Ultracentrifugal crystallization of proteins: transport-kinetic modelling, and experimental behavior of catalase. J. Cryst. Growth*, **180**, 113–126.

Lorber, B. & Giegé, R. (1992). *A versatile reactor for temperature controlled crystallization of biological macromolecules. J. Cryst. Growth*, **122**, 168–175.

Lorber, B. & Giegé, R. (1996). *Containerless protein crystallization in floating drops: application to crystal growth monitoring under reduced nucleation conditions. J. Cryst. Growth*, **168**, 204–215.

Lorber, B. & Giegé, R. (1999). *Biochemical aspects of macromolecular solutions and crystals.* In *Crystallization of nucleic acids and proteins*, edited by A. Ducruix & R. Giegé, 2nd ed. Oxford University Press.

Lorber, B., Jenner, G. & Giegé, R. (1996). *Effect of high hydrostatic pressure on nucleation and growth of protein crystals. J. Cryst. Growth*, **158**, 103–117.

Luft, J. R., Albright, D. T., Baird, J. K. & DeTitta, G. T. (1996). *The rate of water equilibration in vapor-diffusion crystallizations: dependance on the distance from the droplet to the reservoir. Acta Cryst.* D**52**, 1098–1106.

Luft, J. & Cody, V. (1989). *A simple capillary vapor diffusion apparatus for surveying macromolecular crystallization conditions. J. Appl. Cryst.* **22**, 396.

Luft, J. R., Cody, V. & DeTitta, G. T. (1992). *Experiences with HANGMAN: a macromolecular hanging drop vapor diffusion technique. J. Cryst. Growth*, **122**, 181–185.

Luft, J. R., Rak, D. M. & DeTitta, G. T. (1999*a*). *Microbatch macromolecular crystallization in micropipettes. J. Cryst. Growth*, **196**, 450–455.

Luft, J. R., Rak, D. M. & DeTitta, G. T. (1999*b*). *Microbatch macromolecular crystallization on a thermal gradient. J. Cryst. Growth*, **196**, 447–449.

**4.1 (*cont.*)**

McPherson, A. (1976). *Crystallization of proteins from polyethylene glycol. J. Biol. Chem.* **251**, 6300–6303.
McPherson, A. (1982). *The preparation and analysis of protein crystals.* New York: John Wiley and Sons.
McPherson, A. (1990). *Current approaches to macromolecular crystallization. Eur. J. Biochem.* **189**, 1–23.
McPherson, A. (1996). *Macromolecular crystal growth in microgravity. Crystallogr. Rev.* **6**, 157–305.
McPherson, A. (1998). *Crystallization of biological macromolecules.* Cold Spring Harbor and New York: Cold Spring Harbor Laboratory Press.
McPherson, A., Malkin, A. J. & Kuznetsov, Y. G. (1995). *The science of macromolecular crystallization. Structure*, **3**, 759–768.
McPherson, A., Malkin, A. J., Kuznetsov, Y. G. & Koszelak, S. (1996). *Incorporation of impurities into macromolecular crystals. J. Cryst. Growth*, **168**, 74–92.
McPherson, A. & Shlichta, P. (1988). *Heterogeneous and epitaxial nucleation of protein crystals on mineral surfaces. Science*, **239**, 385–387.
Malkin, A. J., Cheung, J. & McPherson, A. (1993). *Crystallization of satellite tobacco mosaic virus. I. Nucleation phenomena. J. Cryst. Growth*, **126**, 544–554.
Malkin, A. J., Kuznetsov, Yu. G., Land, T. A., DeYoreo, J. J. & McPherson, A. (1995). *Mechanisms of growth for protein and virus crystals. Nature Struct. Biol.* **2**, 956–959.
Malkin, A. J., Kuznetsov, Yu. G. & McPherson, A. (1996). *Defect structure of macromolecular crystals. J. Struct. Biol.* **117**, 124–137.
Malkin, A. J. & McPherson, A. (1993). *Light scattering investigations of protein and virus crystal growth: ferritin, apoferritin and satellite tobacco mosaic virus. J. Cryst. Growth*, **128**, 1232–1235.
Malkin, A. J. & McPherson, A. (1994). *Light-scattering investigations of nucleation processes and kinetics of crystallization in macromolecular systems. Acta Cryst.* D**50**, 385–395.
Matthews, B. W. (1985). *Determination of protein molecular weight, hydration, and packing from crystal density. Methods Enzymol.* **114**, 176–187.
Mikol, V., Hirsch, E. & Giegé, R. (1990). *Diagnostic of precipitant for biomacromolecule crystallization by quasi-elastic light scattering. J. Mol. Biol.* **213**, 187–195.
Mikol, V., Rodeau, J.-L. & Giegé, R. (1989). *Changes of pH during biomacromolecule crystallization by vapor diffusion using ammonium sulfate as the precipitant. J. Appl. Cryst.* **22**, 155–161.
Mikol, V., Rodeau, J.-L. & Giegé, R. (1990). *Experimental determination of water equilibrium rates in the hanging drop method of protein crystallization. Anal. Biochem.* **186**, 332–339.
Miller, T. V., He, X. M. & Carter, D. C. (1992). *A comparison between protein crystals grown with vapor diffusion methods in microgravity and protein crystals using a gel liquid–liquid diffusion ground based method. J. Cryst. Growth*, **122**, 306–309.
Minezaki, Y., Nimura, N., Ataka, M. & Katsura, T. (1996). *Small angle neutron scattering from lysozyme solutions in unsaturated and supersaturated states (SANS from lysozyme solutions). Biophys. Chem.* **58**, 355–363.
Nakada, T., Sazaki, G., Miyashita, S., Durbin, S. D. & Komatsu, H. (1999). *Impurity effects on lysozyme crystallization as directly observed by atomic force microscopy. J. Cryst. Growth*, **196**, 503–510.
Neal, B. L., Asthagiri, D., Velev, O. D., Lenhoff, A. M. & Kaler, E. W. (1999). *Why is the osmotic second virial coefficient related to protein crystallization? J. Cryst. Growth*, **196**, 377–387.
Ng, J., Kuznetsov, Y. G., Malkin, A. J., Keith, G., Giegé, R. & McPherson, A. (1997). *Vizualization of RNA crystals growth by atomic force microscopy. Nucleic Acids Res.* **25**, 2582–2588.
Ng, J., Lorber, B., Witz, J., Théobald-Dietrich, A., Kern, D. & Giegé, R. (1996). *The crystallization of biological macromolecules from precipitates. Evidence for Ostwald ripening. J. Cryst. Growth*, **168**, 50–62.
Ng, J. D., Lorber, B., Giegé, R., Koszelak, S., Day, J., Greenwood, A. & McPherson, A. (1997). *Comparative analysis of thaumatin crystals grown on earth and in microgravity. Acta Cryst.* D**53**, 724–733.
Otalora, F. & García-Ruiz, J. M. (1997). *Crystal growth studies in microgravity with APCF. I. Computer simulation of transport dynamics. J. Cryst. Growth*, **182**, 141–154.
Otalora, F., García-Ruiz, J. M., Gavira, J. A. & Capelle, B. (1999). *Topography and high resolution diffraction studies in tetragonal lysozyme. J. Cryst. Growth*, **196**, 546–558.
Papanikolau, Y. & Kokkinidis, M. (1997). *Solubility, crystallization and chromatographic properties of macromolecules strongly depend on substances that reduce the ionic strength of the solution. Protein Eng.* **10**, 847–850.
Peters, R., Georgalis, Y. & Saenger, W. (1998). *Accessing lysozyme nucleation with a novel dynamic light scattering detector. Acta Cryst.* D**54**, 873–877.
Plester, V., Stapelmann, J., Potthast, L. & Bosch, R. (1999). *The protein crystallization facility, a new European instrument to investigate biological macromolecular crystal growth on board the International Space Station. J. Cryst. Growth*, **196**, 638–648.
Price, S. R. & Nagai, K. (1995). *Protein engineering as a tool for crystallography. Curr. Opin. Biotechnol.* **6**, 425–430.
Pronk, S. E., Hofstra, H., Groendijk, H., Kingma, J., Swarte, M. B. A., Dorner, F., Drenth, J., Hol, W. G. J. & Witholt, B. (1985). *Heat-labile enterotoxin of Escherichia coli. Characterization of different crystal forms. J. Biol. Chem.* **260**, 13580–13584.
Provost, K. & Robert, M.-C. (1995). *Crystal growth of lysozymes in media contaminated by parent molecules: influence of gelled media. J. Cryst. Growth*, **156**, 112–120.
Pusey, M., Witherow, W. K. & Nauman, R. (1988). *Preliminary investigations into solutal flow about growing tetragonal lysozyme crystals. J. Cryst. Growth*, **90**, 105–111.
Pusey, M. L. (1993). *A computer-controlled microscopy system for following protein crystal growth rates. Rev. Sci. Instrum.* **64**, 3121–3125.
Ray, W. J. Jr & Puvathingal, J. M. (1985). *A simple procedure for removing contaminating aldehydes and peroxides from aqueous solutions of polyethylene glycols and of nonionic detergents that are based on the polyoxyethylene linkage. Anal. Biochem.* **146**, 307–312.
Rhim, W.-K. & Chung, S. K. (1990). *Isolation of crystallizing droplets by electrostatic levitation. Methods Companion Methods Enzymol.* **1**, 118–127.
Richard, B., Bonneté, F., Dym, O. & Zaccaï, G. (1995). *Archaea, a laboratory manual*, pp. 149–154. Cold Spring Harbor Laboratory Press.
Riès-Kautt, M. & Ducruix, A. (1991). *Crystallization of basic proteins by ion pairing. J. Cryst. Growth*, **110**, 20–25.
Riès-Kautt, M. & Ducruix, A. (1999). *Phase diagrams.* In *Crystallization of nucleic acids and proteins*, edited by A. Ducruix & R. Giegé, 2nd ed. Oxford University Press.
Robert, M. C., Bernard, Y. & Lefaucheux, F. (1994). *Study of nucleation-related phenomena in lysozyme solutions. Application to gel growth. Acta Cryst.* D**50**, 496–503.
Robert, M.-C. & Lefaucheux, F. (1988). *Crystal growth in gels: principles and applications. J. Cryst. Growth*, **90**, 358–367.
Robert, M.-C., Vidal, O., García-Ruiz, J. M. & Otalora, F. (1999). *Crystallization in gels and related methods.* In *Crystallization of nucleic acids and proteins*, edited by A. Ducruix & R. Giegé, 2nd ed. Oxford University Press.
Rosenberger, F. (1996). *Protein crystallization. J. Cryst. Growth*, **166**, 40–54.
Rosenberger, F., Vekilov, P. G., Muschol, M. & Thomas, B. R. (1996). *Nucleation and crystallization of globular proteins – what do we know and what is missing. J. Cryst. Growth*, **168**, 1–27.
Rossi, G. L. (1992). *Biological activity in the crystalline state. Curr. Opin. Struct. Biol.* **2**, 816–820.
Salemme, F. R. (1972). *A free interface diffusion technique for crystallization of proteins for X-ray crystallography. Arch. Biochem. Biophys.* **151**, 533–540.
Sauter, C., Lorber, B., Kern, D., Cavarelli, J., Moras, D. & Giegé, R. (1999). *Crystallogenesis studies on yeast aspartyl-tRNA synthe-*

### 4.1 (*cont.*)

*tase: use of phase diagram to improve crystal quality. Acta Cryst.* D**55**, 149–156.

Sauter, C., Ng, J. D., Lorber, B., Keith, G., Brion, P., Hosseini, M. W., Lehn, J.-M. & Giegé, R. (1999). *Additives for the crystallization of proteins and nucleic acids. J. Cryst. Growth*, **196**, 365–376.

Sazaki, G., Yoshida, E., Komatsu, H., Nakada, T., Miyashita, S. & Watanabe, K. (1997). *Effects of a magnetic field on the nucleation and growth of protein crystals. J. Cryst. Growth*, **173**, 231–234.

Shlichta, P. J. (1986). *Feasibility of mapping solution properties during the growth of protein crystals. J. Cryst. Growth*, **76**, 656–662.

Shu, Z.-Y., Gong, H.-Y. & Bi, R.-C. (1998). *In situ measurements and dynamic control of the evaporation rate in vapor diffusion crystallization of proteins. J. Cryst. Growth*, **192**, 282–289.

Skouri, M., Lorber, B., Giegé, R., Munch, J.-P. & Candau, S. J. (1995). *Effect of macromolecular impurities on lysozyme solubility and crystallizability. Dynamic light scattering, phase diagram, and crystal growth studies. J. Cryst. Growth*, **152**, 209–220.

Snell, E., Helliwell, J. R., Boggon, T. J., Lautenschlager, P. & Potthast, L. (1996). *First ground trials of a Mach–Zehnder interferometer for implementation into a microgravity protein crystallization facility – the APCF. Acta Cryst.* D**52**, 529–533.

Snell, E. H., Weisgerber, S., Helliwell, J. R., Weckert, E., Hölzer, K. & Schroer, K. (1995). *Improvements in lysozyme protein crystal perfection through microgravity growth. Acta Cryst.* D**51**, 1099–1102.

Sousa, R., Lafer, E. M. & Wang, B.-C. (1991). *Preparation of crystals of T7 RNA polymerase suitable for high resolution X-ray structure analysis. J. Cryst. Growth*, **110**, 237–246.

Stojanoff, V., Siddons, D. P., Monaco, L. A., Vekilov, P. & Rosenberger, F. (1997). *X-ray topography of tetragonal lysozyme grown by the temperature-controlled technique. Acta Cryst.* D**53**, 588–595.

Stojanoff, V., Snell, E. F., Siddons, D. P. & Helliwell, J. R. (1996). *An old technique with a new application: X-ray topography of protein crystals. Synchrotron Radiat. News*, **9**, 25–26.

Strickland, C. L., Puchalski, R., Savvides, S. N. & Karplus, P. A. (1995). *Overexpression of Crithidia fasciculata trypanothione reductase and crystallization using a novel geometry. Acta Cryst.* D**51**, 337–341.

Stura, E. A. & Wilson, I. A. (1990). *Analytical and production seeding techniques. Methods Companion Methods Enzymol.* **1**, 38–49.

Suzuki, Y., Miyashita, S., Komatsu, H., Sato, K. & Yagi, T. (1994). *Crystal growth of hen egg white lysozyme under high pressure. Jpn. J. Appl. Phys.* **33**, 1568–1570.

Sygusch, J., Coulombe, R., Cassanto, J. M., Sportiello, M. G. & Todd, P. (1996). *Protein crystallization in low gravity by step gradient diffusion method. J. Cryst. Growth*, **162**, 167–172.

Taleb, M., Didierjean, C., Jelsch, C., Mangeot, J.-P., Capelle, B. & Aubry, A. (1999). *Crystallization of biological macromolecules under an external electric field. J. Cryst. Growth*, **200**, 575–582.

Thaller, D., Eichele, G., Weaver, L. H., Wilson, E., Karlsson, R. & Jansonius, J. N. (1985). *Seed enlargement and repeated seeding. Methods Enzymol.* **114**, 132–135.

Thibault, F., Langowski, L. & Leberman, R. (1992). *Pre-nucleation crystallization studies on aminoacyl-tRNA synthetases by dynamic light scattering. J. Mol. Biol.* **225**, 185–191.

Thiessen, K. J. (1994). *The use of two novel methods to grow protein crystals by microdialysis and vapor diffusion in an agarose gel. Acta Cryst.* D**50**, 491–495.

Thomas, B. R., Vekilov, P. G. & Rosenberger, F. (1998). *Effects of microheterogeneity in hen egg-white lysozyme crystallization. Acta Cryst.* D**54**, 226–236.

Thomas, D. H., Rob, A. & Rice, D. W. (1989). *A novel dialysis procedure for the crystallization of proteins. Protein Eng.* **2**, 489–491.

Timasheff, S. N. & Arakawa, T. (1988). *Mechanism of protein precipitation and stabilization by co-solvents. J. Cryst. Growth*, **90**, 39–46.

Vaney, M. C., Maignan, S., Riès-Kautt, M. & Ducruix, A. (1996). *High-resolution structure (1.33 Å) of a HEW lysozyme tetragonal crystal grown in the APCF apparatus. Data and structural comparison with a crystal grown under microgravity from SpaceHab-01 mission. Acta Cryst.* D**52**, 505–517.

Vekilov, P. G., Ataka, M. & Katsura, T. (1992). *Laser Michelson interferometry investigation of protein crystal growth. J. Cryst. Growth*, **130**, 317–320.

Vekilov, P. G. & Rosenberger, F. (1996). *Dependence of lysozyme growth kinetics on step sources and impurities. J. Cryst. Growth*, **158**, 540–551.

Vekilov, P. G. & Rosenberger, F. (1998). *Protein crystal growth under forced solution flow: experimental setup and general response of lysozyme. J. Cryst. Growth*, **186**, 251–261.

Vidal, O., Robert, M.-C. & Boué, F. (1998*a*). *Gel growth of lysozyme crystals studied by small angle neutron scattering: case of agarose gel, a nucleation promotor. J. Cryst. Growth*, **192**, 257–270.

Vidal, O., Robert, M.-C. & Boué, F. (1998*b*). *Gel growth of lysozyme crystals studied by small angle neutron scattering: case of silica gel, a nucleation inhibitor. J. Cryst. Growth*, **192**, 271–281.

Vuillard, L., Rabilloud, T., Leberman, R., Berthet-Colominas, C. & Cusack, S. (1994). *A new additive for protein crystallization. FEBS Lett.* **353**, 294–296.

Weber, B. H. & Goodkin, P. E. (1970). *A modified microdiffusion procedure for the growth of single protein crystals by concentration-gradient equilibrium dialysis. Arch. Biochem. Biophys.* **141**, 489–498.

Yonath, A., Müssig, J. & Wittmann, H. G. (1982). *Parameters for crystal growth of ribosomal subunits. J. Cell. Biochem.* **19**, 145–155.

Zeppenzauer, M. (1971). *Formation of large crystals. Methods Enzymol.* **22**, 253–266.

### 4.2

Allen, J. P., Feher, G., Yeates, T. O., Komiya, H. & Rees, D. C. (1987). *Structure of the reaction center from Rhodobacter sphaeroides R-26: the protein subunits. Proc. Natl Acad. Sci. USA*, **84**, 6162–6166.

Bordier, C. (1981). *Phase separation of integral membrane proteins in Triton X-114 solution. J. Biol. Chem.* **256**, 1604–1607.

Buchanan, S. K., Fritzsch, G., Ermler, U. & Michel, H. (1993). *New crystal form of the photosynthetic reaction centre from Rhodobacter sphaeroides of improved diffraction quality. J. Mol. Biol.* **230**, 1311–1314.

Buchanan, S. K., Smith, B. S., Venkatrami, L., Xia, D., Esser, L., Palnitkar, M., Chakraborty, R., van der Helm, D. & Deisenhofer, J. (1999). *Crystal structure of the outer membrane active transporter FepA from Escherichia coli. Nature Struct. Biol.* **6**, 56–63.

Chang, C. H., El-Kabbani, D., Tiede, D., Norris, J. & Schiffer, M. (1991). *Structure of the membrane-bound protein photosynthetic reaction center from Rhodobacter sphaeroides. Biochemistry*, **30**, 5352–5360.

Chang, G., Spencer, R. H., Lee, A. T., Barclay, M. T. & Rees, D. C. (1998). *Structure of the MscL homolog from Mycobacterium tuberculosis: a gated mechanosensitive ion channel. Science*, **282**, 2220–2226.

Cowan, S. W., Garavito, R. M., Jansonius, J. N., Jenkins, J. A., Karlsson, R., König, N., Pai, E. F., Pauptit, R. A., Rizkallah, P. J., Rosenbusch, J. P., Rummel, G. & Schirmer, T. (1995). *The structure of OmpF porin in a tetragonal crystal form. Structure*, **3**, 1041–1050.

Cowan, S. W., Schirmer, T., Rummel, G., Steiert, M., Gosh, R., Pauptit, R. A., Jansonius, J. N. & Rosenbusch, J. P. (1992). *Crystal structures explain functional properties of two E. coli porins. Nature (London)*, **358**, 727–733.

Deisenhofer, J., Epp, O., Miki, K., Huber, R. & Michel, H. (1985). *Structure of the protein subunits in the photosynthetic reaction*