International Tables for Crystallography (2006). Vol. F, Section 4.1.2, pp. 81–86.

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

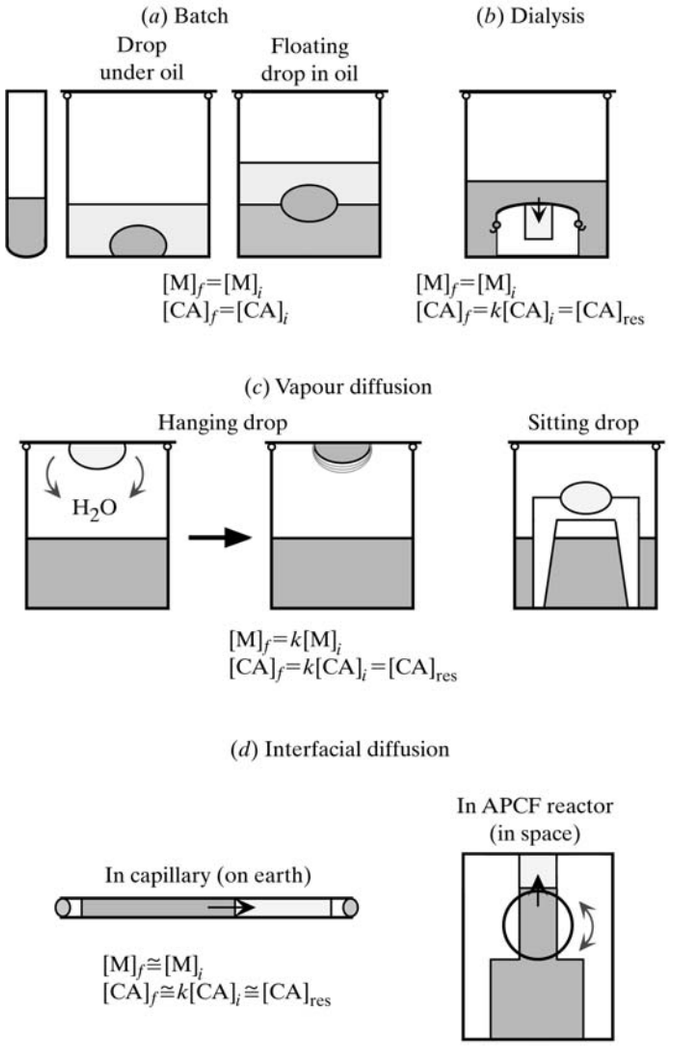

Fig. 4.1.2.1. Principles of the major methods currently used to crystallize biological macromolecules. (*a*) Batch crystallization in three versions. (*b*) Dialysis method with Cambridge button. (*c*) Vapour diffusion crystallization with hanging and sitting drops. (*d*) Interface crystallization in 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

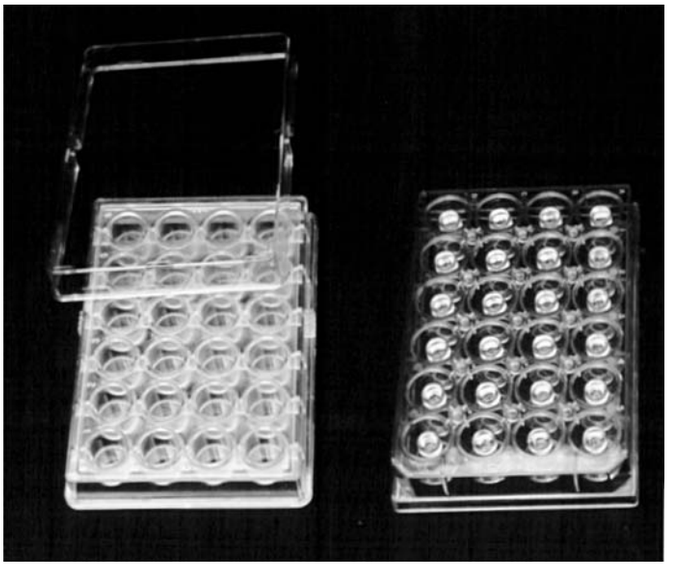

Fig. 4.1.2.2. Two versions of boxes for vapour diffusion crystallization. On the left, a Linbro tissue-culture plate with 24 wells widely used for hanging-drop assays (it may also be used for sitting drops, dialysis and batch crystallization). On the right, a Cryschem multichamber plate, with a post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

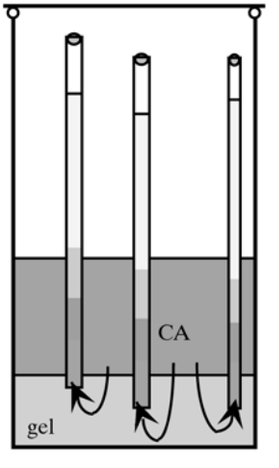

Fig. 4.1.2.3. Principle of the gel acupuncture method for the crystallization of proteins by counter-diffusion. Capillaries containing the macromolecule solution are inserted into a gel, which is covered by a layer of crystallizing agent (CA); the setup is closed by a glass plate. The crystallizing-agent 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

references