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).

references