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

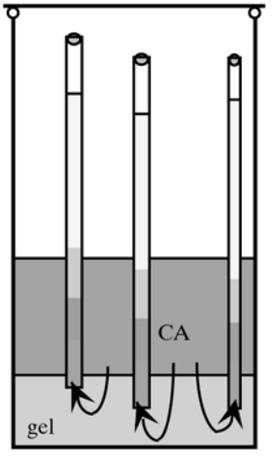

Fig. 4.1.2.3. Principle of the gel acupuncture method for the crystallization of proteins by counter-diffusion. Capillaries containing the macromolecule solution are inserted into a gel, which is covered by a layer of crystallizing agent (CA); the setup is closed by a glass plate. The crystallizing-agent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