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

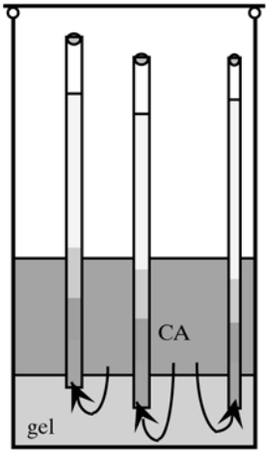

Fig. 4.1.2.3. Principle of the gel acupuncture method for the crystallization of proteins by counter-diffusion. Capillaries containing the macromolecule solution are inserted into a gel, which is covered by a layer of crystallizing agent (CA); the setup is closed by a glass plate. The crystallizing-agent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

references