3.1. Preparation, selection, and investigation of specimens

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3.1.1. Crystallization

3.1.1.1. *Introduction*

The preparation of single crystals probably constitutes the most important step in a crystal structure analysis, since without high-quality diffraction data many analyses will prove problematical, if not completely intractable; time and effort invested in crystallization procedures are rarely wasted. There is a wealth of literature available on the subject of growing crystals and this includes the *Journal of Crystal Growth* (Amsterdam: Elsevier). This section does not intend to be a comprehensive review of the subject, but rather to provide some key lines of approach with appropriate references. The field of crystallizing biological macromolecules is itself a growth area and, in consequence, has been given a special emphasis.

Useful general references for growing crystals for structure analysis include Bunn (1961), Stout & Jensen (1968), Blundell & Johnson (1976), McPherson (1976, 1982, 1990), Ducruix & Giegé (1992) and Helliwell (1992). Volume D50 (Part 4) of *Acta Crystallographica* (1994) reports the Proceedings of the Fifth International Conference on Crystallization of Biological Macromolecules (San Diego, California, 1993) and is essential reading for crystallization experiments in this area. A biological macromolecular database for crystallization conditions has also been initiated (Gilliland, Tung, Blakeslee & Ladner, 1994).

3.1.1.2. *Crystal growth*

Crystallization has long been used as a method of purification by chemists and biochemists, although lack of purity can severely hamper the growth of single crystals, particularly if the impurities have some structural resemblance to the molecule being crystallized (Giegé, Theobald-Dietrich & Lorber, 1993; Thatcher, 1993). The process of crystallization involves the ordering of ions, atoms, and molecules in the gas, liquid, or solution phases to take up regular positions in the solid state. The initial stage is nucleation, followed by deposition on the crystallite faces. The latter can be considered as a dynamic equilibrium between the fluid and the crystal, with growth occurring when the forward rate predominates. Factors that affect the equilibrium include the chemical nature of the crystal surface, the concentration of the material being crystallized, and the nature of the medium in and around the crystal. Relatively little research has been done concerning the process of nucleation, but crystal formation appears to be conditional on the appearance of nuclei of a critical size. Too small aggregates will have either a positive or an unfavourable free energy of formation, so that there is a tendency to dissolution, whilst above the critical size the intermolecular interactions will, on average, lead to an overall negative free energy of formation. The rate of nucleation will increase considerably with the degree of supersaturation, and, in order to limit the number of nuclei (and therefore number of crystals growing), the degree of supersaturation must be as low as possible. Supersaturation must be approached slowly, and, when a low degree has been achieved, it must be carefully controlled. Many factors can influence crystallization, but a conceptually simple explanation of crystal growth has been described in detail by Tipson (1956) and elaborated, for example, by Ries-Kautt & Ducruix (1992). These latter authors provide a useful schematic description of the two-dimensional solubility diagram relating the concentration of the molecule being crystallized to the concentration of the crystallizing agent. The presence of foreign bodies, such as dust particles, makes the nucleation process thermodynamically more favourable, and these should be removed by centrifugation and/or filtration. The addition of seed crystals can often be used to control the nucleation process (Thaller, Eichelle, Weaver, Wilson, Karlsson & Jansonius, 1985). In the case of the formation of crystals of macromolecules in solution, Ferré-D'Amaré & Burley (1994) have described the use of dynamic light scattering to screen crystallization conditions for monodispersity. Empirical observations suggest that macromolecules that have the same size under normal solvent conditions tend to form crystals, whereas those systems that are polydisperse, or where random aggregation occurs, rarely give rise to ordered crystals.

3.1.1.3. *Methods of growing crystals*

General strategies for crystallizing low-molecular-weight organic compounds have been reported by van der Sluis, Hezemans & Kroon (1989) and are listed in Table 3.1.1.1. Many of these strategies are also applicable to inorganic compounds. In the case of biological macromolecules, the main methods utilize one or more of the factors described in Subsection 3.1.1.5 and include batch crystallization, the hot-box technique, equilibrium dialysis, and vapour diffusion (see, for example, Blundell & Johnson, 1976; Helliwell, 1992). The growth of macromolecular crystals in silica hydrogels minimizes convection currents, turbidity, and any strain effects due to the presence of the crystallization vessel. Heterogeneous and secondary nucleation are also reduced (Robert, Provost & Lefaucheux, 1992; Cudney, Patel & McPherson, 1994; García-Ruiz & Moreno, 1994; Thiessen, 1994; Robert, Bernard & Lefaucheux, 1994; Bernard, Degoy, Lefaucheux & Robert, 1994; Sica *et al.*, 1994). Various apparata have been described for use with the vapour diffusion technique (see also Subsection 3.1.1.6) and include a simple capillary vapour diffusion device for preliminary screening of crystallization conditions (Luft & Cody, 1989), a double-cell device that decouples the crystal nucleation from the crystal growth, facilitating the control of nucleation and growth (Przybylska, 1989), microbridges for use with sitting drops in the 35–45 µl range (Harlos, 1992), and diffusion cells with varying depths, in order to control the time course of the equilibration between the macromolecule and the reservoir solution (Luft *et al.*, 1994).

3.1.1.4. *Factors affecting the solubility of biological macromolecules*

There are many factors that influence the crystallization of macromolecules (McPherson, 1985*a*; Giegé & Ducruix, 1992; Schick & Jurnak, 1994; Tissen, Fraaije, Drenth & Berendsen, 1994; Carter & Yin, 1994; Spangfort, Surin, Dixon & Svensson, 1994; Axelrod *et al.*, 1994; Konnert, D'Antonio & Ward, 1994; Forsythe, Ewing & Pusey, 1994; Diller, Shaw, Stura, Vacquier & Stout, 1994; Hennig & Schlesier, 1994), but the following are particularly important with respect to solubility (Blundell & Johnson, 1976).

Ionic strength. The solubility of macromolecules in aqueous solution depends on the ionic strength, since the presence of ions modifies the interactions of the macromolecule with the solvent. At low ion concentrations, the solubility of the macromolecule is

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