3. PREPARATION AND EXAMINATION OF SPECIMENS

Table 3.1.1.2. *Commonly used ionic and organic precipitants, adapted from McPherson (1985a)*

(*a*) Ionic compounds	(*b*) Organic solvents*
Ammonium† or sodium sulfate	Ethanol
Sodium or ammonium citrate	Isopropanol
Sodium, potassium or ammonium chloride	2-Methyl-2,4-pentanediol (MPD)
Sodium or ammonium acetate	Dioxane
Magnesium sulfate	Acetone
Cetyltrimethylammonium salts	Butanol
Calcium chloride	Dimethyl sulfoxide
Ammonium nitrate	2,5-Hexanediol
Sodium formate	Methanol
Lithium chloride	1,3-Propanediol
	1,3-Butyrolactone
	Poly(ethylene glycol) 600–20000 (PEG)

* The volatility of solvents such as ethanol and acetone may cause handling problems. † Ammonium sulfate can cause problems when used as a precipitant, since pH changes occur owing to ammonium transfer following ammonium/ammonia equilibrium; this effect has been studied in detail by Mikol, Rodeau & Giegé (1989). Monaco (1994) has suggested that ammonium succinate is a useful substitute for ammonium sulfate.

increased, a phenomenon termed 'salting-in'. As the ionic strength is increased, the ions added compete with one another and the macromolecules for the surrounding water. The resulting removal of water molecules from the solute leads to a decrease in the solubility, a phenomenon termed 'salting-out'. Different ions will affect the solubility of the protein in different ways. Small highly charged ions will be more effective in the salting-out process than large low-charged ions. Commonly used ionic precipitants are listed in Table 3.1.1.2, column (*a*) (McPherson, 1985*a*).

pH and counterions. The net charge on a macromolecule in solution can be modified either by changing the pH (adding or removing protons) or by binding ions (counterions). In general terms, the protein solubility will increase with the overall net charge and will be least soluble when the net charge is zero (isoelectric point). In the latter case, the molecules can pack in the crystalline form without an overall, destabilizing accumulation of charge.

Temperature. Temperature has a marked affect on many of the factors that govern the solubility of a macromolecule. The dielectric constant decreases with increase in temperature, and the entropy terms in the free energy tend to dominate the enthalpy terms (Blundell & Johnson, 1976). The temperature coefficient of solubility varies with ionic strength and the presence of organic solvents. McPherson (1985*b*) gives a useful account of protein crystallization by variation of pH and temperature.

Organic solvents. Addition of organic solvents can produce a marked change in the solubility of a macromolecule in aqueous solution (care should be taken to avoid denaturation). This is partly due to a lowering of the dielectric constant, but may also involve specific solvation and displacement of water at the surface of the macromolecule. Generally, the solubility decreases with decrease of temperature when substantial amounts of organic solvent are present. Commonly used organic precipitants are listed in Table 3.1.1.2, column (*b*) (McPherson, 1985*a*).

3.1.1.5. *Screening procedures for the crystallization of biological macromolecules*

Optimal conditions for crystal growth are often very difficult to predict *a priori*, although many proteins crystallize close to their pI. In order to surmount the problem of testing a very large range of conditions, Carter & Carter (1979) devised the *incomplete factorial method*, in which a very coarse matrix of crystallization conditions is explored initially. Finer grids are then investigated around the most promising sets of coarse conditions. This technique has been further refined to yield the sparse-matrix sampling technique described by Jancarik & Kim (1991). Table 3.1.1.3 lists the crystallization parameters used by these authors. The 50 conditions constituting the sparse matrix are given in Table 3.1.1.4. A recent update of this matrix and a set of stock solutions in the form of a crystal screen kit can be obtained commercially from Hampton Research (1994). Further developments in screening methods are described in Volume D50 (Part 4) of *Acta Crystallographica* (1994).

3.1.1.6. *Automated protein crystallization*

Several liquid-handling systems have been described that can automatically set up, reproducibly, a range of crystallization conditions (different protein concentrations, ionic strengths, amounts of organic precipitant, *etc*.) for the hanging-drop, sitting-drop, and microbatch methods. A useful introduction describing a system for mixing both buffered protein solutions and the corresponding reservoirs is given by Cox & Weber (1987). Chayen, Shaw Stewart, Maeder & Blow (1990) describe an automatic dispenser involving a bank of Hamilton syringes driven by stepper motors under computer control that can be used to set up small samples (2 µl or less) for microbatch crystallization (or hanging drops). Further systems have been described by Oldfield, Ceska & Brady (1991), Eiselé (1993), Soriano & Fontecilla-Camps (1993), Sadaoui, Janin & Lewit-Bentley (1994), and Chayen, Shaw Stewart & Baldock (1994).

3.1.1.7. *Membrane proteins*

Integral membrane proteins can be considered as those whose polypeptide chains span the lipid bilayer at least once. The external membrane segments exposed to an aqueous environment are hydrophilic, but it is the tight interaction of the hydrophobic segments of the chain with the quasisolid lipid bilayer that constitutes the major problem in their crystallization. Crystallization trials require disruption of the membrane, isolation of the protein, and solubilization of the resultant hydrophobic region (McDermott, 1993). Organic solvents, chaotropic agents, and amphipathic detergents can be used to disrupt the membrane, but detergents such as β-octyl glucoside are most commonly

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Table 3.1.1.3. *Crystallization matrix parameters for sparse-matrix sampling, adapted from Jancarik & Kim (1991)*

Precipitating agents			
Non-volatile	Salts	Volatile	Mixture
2-Methyl-2,4-pentanediol (MPD) Poly(ethylene glycol)(PEG) 400 PEG 4000 PEG 8000	Na, K tartrate NH_4 phosphate NH_4 sulfate Na acetate Li sulfate Na formate Na, K phosphate Na citrate Mg formate	2-Propanol	NH_4 sulfate + PEG 2-Propanol + PEG
Range of pH: 4.6, 5.6, 7.5, 8.5			
Salts, additives:	Ca chloride, Na citrate, Mg chloride, NH_4 acetate, NH_4 sulfate, Mg acetate, Zn acetate, Ca acetate		

used, since they minimize the loss of protein integrity. The several classes of detergent employed tend to be non-ionic or zwitterionic at the pH used, have a maximum hydrocarbon chain length of 12 carbon atoms, and possess a critical micelle concentration. The key to crystallizing membrane protein–detergent complexes appears to be the attainment of conditions in which the protein surfaces are moderately supersaturated and, in addition, the detergent micellar collar is at, or near, its solubility limit (Scarborough, 1994). Most successful integral membrane protein crystallizations are near the micellar aggregation point of the detergent (Garavito & Picot, 1990).

3.1.2. Selection of single crystals

3.1.2.1. *Introduction*

The final results of a structure analysis cannot be better than the imperfections of the crystal allow, and effort invested in producing crystals giving a clearly defined, high-resolution diffraction pattern is rarely wasted. The selection of twinned crystals, aggregates, or those with highly irregular shapes can lead to poor diffraction data and may prohibit a structure solution. There are many properties of crystals that can be examined prior, or in addition, to an X-ray or neutron diffraction study. These are summarized in Table 3.1.2.1. Many of these properties can yield useful information about the crystal packing and the overall molecular shape. For example, the shape and orientation of the optical indicatrix may be used to find the orientation of large atomic groups that possess shapes such as flat discs or rods and therefore also have strong anisotropic polarizability. A morphological examination can reveal information not only about the crystal quality but also in many cases about the crystal system, whilst identification of extinction directions can assist in crystal mounting. It is regrettable that many modern practitioners of the science of crystallography give little more than a cursory optical examination to their specimens before commencing data collection and a structure analysis.

3.1.2.2. *Size, shape, and quality*

A frequently occurring question involves the size and shape of single crystals required for successful diffraction studies. Among other factors, the intensity of diffraction is dependent on the volume of the crystal specimen bathed by the X-ray or neutron beam and is inversely proportional to the square of the unit-cell volume (see Chapter 6.4). Hence, small crystals with large unit cells will tend to give rise to weak diffraction patterns. This can be compensated for by increasing the incident intensity, *e.g.* using a synchrotron-radiation source in the case of X-rays. How large should a crystal be, and what is the smallest crystal size that can be accommodated? X-ray collimators, or slit systems, with diameters in the range 0.1 to 0.8 mm are commonly employed for single-crystal diffraction studies. For many diffractometers, the primary beam is arranged to have a plateau of uniform intensity with dimensions 0.5×0.5 mm. For most small inorganic and organic compounds, crystals with dimensions slightly smaller than this will suffice, depending on the strength of diffraction, although successful structure determinations have been reported on very small crystals (0.1 mm and less) with both conventional and synchrotron X-ray sources (Helliwell *et al.*, 1993). Microfocus beam lines at the third generation of synchrotron sources such as ESRF are designed to examine crystals routinely in the 10 μm range (Riekel, 1993). In the case of a biological macromolecule of molecular weight 50 kDa and using a conventional X-ray source (a rotating-anode generator), a crystal of 0.1 mm in all dimensions will probably give diffraction patterns from which the basic crystal system and unit-cell parameters can be deduced, but a crystal of 0.3 mm in each dimension, *i.e.* roughly 30 times the volume, would be required for the collection of high-resolution data (Blundell & Johnson, 1976). The higher intensity and smaller beam divergence inherent in a synchrotron X-ray source mean that high-resolution data of good quality could be obtained with the smaller crystal. Indeed, useful intensity data have been obtained with crystals with a maximum dimension of 50 μm (Subsection 3.4.1.5). At cryogenic temperatures, radiation damage is greatly reduced, and increased exposure times can be utilized (at the expense of increased background) to compensate for a small crystal volume. In the case of neutrons, the sample size is generally larger than for X-rays, owing to lower neutron flux and higher beam divergence. For a steady-state high-flux reactor such as that at the Institut Laue–Langevin (France), a crystal volume of 6 mm³ or larger is recommended for biological samples. Unfortunately, crystals of this size are not readily obtainable in most cases.

The shape or habit of a single crystal is normally determined by the internal crystal structure and the growth