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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

Table 3.1.1.1. *Survey of crystallization techniques suitable for the crystallization of low-molecular-weight organic compounds for X-ray crystallography (adapted from van der Sluis, Hezemans & Kroon, 1989)*

Technique	Advantage(s)	Limitation(s)
Evaporation from a single solvent	Simple Inexpensive	Limitation to solvents with adequate vapour pressure Crust formation on tube walls Crystals that are dried are less suitable as seeds, may lose included solvent and become tightly adhered to the crystallization vessel Difficult to reproduce Limited number of solvents give concentration 5–200 mg ml^{-1} for a particular compound
Evaporation from a binary mixture of solvents (volatile solvent and non-volatile precipitant)	No crust formation on the tube walls Crystals are not dried	Stringent demands on solubility, miscibility and volatility of the two solvents Difficult to reproduce
Batch crystallization	No demands on the volatility of the solvent or precipitant Repeated seeding by thermal treatment is easy	Metastable zone with regard to supersaturation must be large High and almost uncontrollable crystallization rate Solvents must be miscible
Liquid–liquid diffusion	Favourable change in supersaturation at the interface during crystallization Repeated seeding by thermal treatment is easy	Density differences required for the two liquids (less stringent if capillaries are used) Viscosity of the liquids greater than water Solvents must be miscible High and almost uncontrollable crystallization rate
Sitting-drop vapour-phase diffusion	Crystallization rate can easily be controlled by changing the diffusion path, solvent, precipitant, or pH Repeated seeding easily implemented Highest number of independent variables to obtain wide variety of conditions	Solvents must be miscible Solvent preferably less volatile than precipitant
Hanging-drop vapour-phase diffusion	Crystallization rate can easily be controlled by changing solvent, precipitant, or pH Easy examination of crystallization outcome in array-like set-up	Only applicable in case of water-based solvents Diffusion rate is fast and difficult to control See previous method
Temperature change	Easily controllable parameter Repeated seeding extremely easily and accurately carried out With Dewar flask inexpensive and simple	Limited to thermally stable compounds and (pseudo)polymorphs
Gel crystallization	Suited for sparingly soluble or easily nucleating compounds	Limited variety of solvents possible Sampling of crystals difficult Laborious
Sublimation	No inclusion of solvent of crystallization	Limited to small hydrophobic molecules Laborious
Solidification	For liquids and gases the only applicable method	Limited to thermostable compounds High change of amorphicity Laborious

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

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 mm^3 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

Table 3.1.1.4. *Reservoir solutions for sparse-matrix sampling (Jancarik & Kim 1991)*

No.	Salt	Buffer	Precipitant (% by mass)
1	0.02 *M* Ca chloride	0.1 *M* Acetate	30% MPD
2			0.4 *M* Na, K tartrate
3			0.4 *M* NH_4 phosphate
4		0.1 *M* Tris	2.0 *M* NH_4 sulfate
5	0.2 *M* Na citrate	0.1 *M* Hepes	40% MPD
6	0.2 *M* Mg chloride	0.1 *M* Tris	30% PEG 4000
7		0.1 *M* Cacodylate	1.4 *M* Na acetate
8	0.2 *M* Na citrate	0.1 *M* Cacodylate	30% 2-Propanol
9	0.2 *M* NH_4 acetate	0.1 *M* Citrate	30% PEG 4000
10	0.2 *M* NH_4 acetate	0.1 *M* Acetate	30% PEG 4000
11		0.1 *M* Citrate	1.0 *M* NH_4 phosphate
12	0.2 *M* Mg chloride	0.1 *M* Hepes	30% 2-Propanol
13	0.2 *M* Na citrate	0.1 *M* Tris	30% PEG 400
14	0.2 *M* Ca chloride	0.1 *M* Hepes	28% PEG 400
15	0.2 *M* NH_4 sulfate	0.1 *M* Cacodylate	30% PEG 8000
16		0.1 *M* Hepes	1.5 *M* Li sulfate
17	0.2 *M* Li sulfate	0.1 *M* Tris	30% PEG 4000
18	0.2 *M* Mg acetate	0.1 *M* Cacodylate	20% PEG 8000
19	0.2 *M* NH_4 acetate	0.1 *M* Tris	30% 2-Propanol
20	0.2 *M* NH_4 sulfate	0.1 *M* Acetate	25% PEG 4000
21	0.2 *M* Mg acetate	0.1 *M* Cacodylate	30% MPD
22	0.2 *M* Na acetate	0.1 *M* Tris	30% PEG 4000
23	0.2 *M* Mg chloride	0.1 *M* Hepes	30% PEG 400
24	0.2 *M* Ca chloride	0.1 *M* Acetate	20% 2-Propanol
25		0.1 *M* Imidazole	1.0 *M* Na acetate
26	0.2 *M* NH_4 acetate	0.1 *M* Citrate	30% MPD
27	0.2 *M* Na citrate	0.1 *M* Hepes	20% 2-Propanol
28	0.2 *M* Na acetate	0.1 *M* Cacodylate	30% PEG 8000
29		0.1 *M* Hepes	0.8 *M* Na, K tartrate
30	0.2 *M* NH_4 sulfate		30% PEG 8000
31	0.2 *M* NH_4 sulfate		30% PEG 4000
32			2.0 *M* NH_4 sulfate
33			4.0 *M* Na formate
34		0.1 *M* Acetate	2.0 *M* Na formate
35		0.1 *M* Hepes	1.6 *M* Na, K phosphate
36		0.1 *M* Tris	8% PEG 8000
37		0.1 *M* Acetate	8% PEG 4000
38		0.1 *M* Hepes	1.4 *M* Na citrate
39		0.1 *M* Hepes	2% PEG 400, 2.0 *M* Na sulfate
40		0.1 *M* Citrate	20% 2-Propanol + 20% PEG 4000
41		0.1 *M* Hepes	10% 2-Propanol + 20% PEG 4000
42	0.05 *M* K phosphate		20% PEG 8000
43			30% PEG 1500
44			0.2 *M* Mg formate
45	0.2 *M* Zn acetate	0.1 *M* Cacodylate	18% PEG 8000
46	0.2 *M* Ca acetate	0.1 *M* Cacodylate	18% PEG 8000
47		0.1 *M* Acetate	2.0 *M* NH_4 sulfate
48		0.1 *M* Tris	2.0 *M* NH_4 sulfate
49	1.0 *M* Li sulfate		2% PEG 8000
50	1.0 *M* Li sulfate		15% PEG 8000

Abbreviations: tris: 2-amino-2-(hydroxymethyl)-1,3-propanediol; hepes: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. Buffers: Na acetate buffer, pH = 4.6; Na citrate buffer, pH = 5.6; Na cacodylate buffer, pH = 6.5; Na hepes buffer, pH = 7.5; tris/HCl buffer, pH = 8.5.

conditions. For diffractometry purposes, it is customary to bathe the crystal in the X-ray beam, so that elongated crystals may require cutting with a razor blade in order to trim them to an appropriate size. Large crystals of hard materials can be ground into spheres or cylinders (Jeffery, 1977), so that corrections can be readily made to the observed intensities for systematic errors in absorption (see Chapter 6.3). Crystals that have elongated prismatic or needle shapes are often useful if data are collected using oscillation geometry, since the crystal can be translated in the X-ray beam at intervals during data collection to minimize radiation damage (Subsection 3.4.1.5). In general, all shapes can be accommodated, but those that are grossly asymmetric (*e.g.* very thin plates) may give elongated or distorted

Table 3.1.2.1. *Use of crystal properties for selection and preliminary study of crystals, adapted from MacGillavry & Henry (1962); morphological, optical, and mechanical properties*

Crystal property	Uses and comments	Relation with structure
Morphological properties		
Crystal habit	Setting crystal parallel to edge, or to symmetry axis, derived from goniometric measurement Habit can be influenced by solvent, crystallization conditions, trace impurities Well formed crystals can be accurately measured for analytical corrections for absorption	Morphological determination of crystal class may narrow down choice of space group Best-developed faces correspond to net planes with large density of lattice or pseudo-lattice nodes (Bravais' law, extended by Donnay & Harker) Prominent faces tend to be parallel to important bond systems Face development correlates inversely with surface free energy
Twinning	Twins may be hard to detect by morphological or diffraction methods. Investigate under the polarizing microscope: optical anomalies strongly indicate mimetic twinning, stacking faults, *etc*. Mechanical twinning may occur when a single crystal is cut or ground. In such cases, the crystal should be shaped by use of a solvent	May indicate hemimorphy or pseudo-hemimorphy of the cell or supercell; see Chapter 1.3 Pseudo-symmetrical stacking
Etch figures; epitaxy	See *IT* A (2002), Section 10.2.3 (pp. 805–806), and chemical properties below	
Optical properties		
Refractive index; birefringence (see IT *A*, Section 10.5.4, p. 790)	Checking quality of crystal: homogeneous extinction, interference figures Extinction direction is used for setting badly formed or ground crystals Magnitude of refractive index may be used for identification of crystal orientation	High refractive index may indicate close packing Shape and orientation of indicatrix may be useful for finding orientation of large atomic or ionic groups with strongly anisotropic polarizability (*e.g.* flat or rod-shaped groups)
Optical activity	Distinguishes between optical antipodes in studies of absolute configuration	Difficult to measure, or even detect, in optically biaxial crystals. No obvious relation with structure
Pleochroism	Identification of crystal orientation through dependence of colour on direction of light vibration	Extended conjugated-bond systems have strong absorption of light vibrating parallel to system; weak absorption perpendicular to system String-like arrangement of some atoms [*e.g.* iodine in poly(vinyl alcohol)] produces strong absorption parallel to string In inorganic compounds, absorption is greatest for light vibrating along directions in which ions are distorted
Reflection of light		Opaque substances contain loosely bound electrons
Raman effect		May give information on the orientation and symmetry of scattering groups
Mechanical properties		
Cleavage	Useful for obtaining good surfaces for crystal setting Useful for improving crystal shape	Correlates with bond-strength anisotropy
Hardness	Anisotropy of hardness may produce ellipsoids instead of spheres when an abrasion chamber is used	Hardness gives an indication of bond strength and bond density Hardness may be very sensitive to impurities, changes in texture through ageing or heat treatment, *etc*.
Plasticity	Single crystals: avoid cutting or grinding Polycrystalline material: plastic deformation is often strongly anisotropic, and may then be used to produce single or double orientation	Non-directive bonding between large strongly bonded units (long-chain paraffins, layer structures) Plastic flow may also be associated with mechanical twinning or lattice imperfections

Table 3.1.2.1. *Use of crystal properties for selection and preliminary study of crystals (cont.)*

Crystal property	Relation with structure
Magnetic properties	
Paramagnetism; diamagnetism	In an isomorphous series of paramagnetic salts, the values of the average susceptibility and of magnetic anisotropy are dependent on the nature of the paramagnetic ion. The shape of the coordination polyhedron may be found from the crystal anisotropies In aliphatic non-conjugated organic crystals, the numerically largest diamagnetic susceptibility is along the direction in which lie the largest molecular directions In crystals containing aromatic compounds or molecules with coplanar conjugated bonds, the numerically largest molecular diamagnetic susceptibility is normal to the plane of the molecular orbitals, and may thus indicate the molecular orientations
Ferromagnetism; antiferromagnetism; ferrimagnetism	Neutron diffraction by magnetic compounds may give information about the directions of the resultant spin and orbital moments. X-ray diffraction effects are usually unimportant In magnetic materials, the interatomic distances, and, in antiferromagnetic oxides, the valency angles at the oxygen ions are related to the diameter of the electron shell
Nuclear magnetic resonance	The line width in NMR spectra is related to the distances between the nuclei with magnetic moments
Electrical properties	
Ferroelectricity; pyroelectricity	See *IT* A (2002), Section 10.2.5, p. 807. Ferroelectricity indicates (i) a structure of polar symmetry, and (ii) the probability of another high-symmetry structure of nearly equal energy, derivable from the ferroelectric by a displacive transition. Often there are several related structures, some ferroelectric and some antiferroelectric Pyroelectricity indicates noncentrosymmetry. Second-harmonic generation is ordinarily a more sensitive test
Piezoelectricity	Piezoelectricity gives information on symmetry; it occurs only in ten crystal classes. See *IT* A, Section 10.2.6
Thermodynamic properties	
Heat capacity ('specific heat')	Anomalies indicate polymorphic transitions, disorder, approach to melting point, and temperature variation gives Einstein and/or Debye characteristic temperatures
Melting point	Atoms in crystals with a low melting point often have large thermal movements; diffraction experiments should preferably be carried out at low temperatures Anomalies in the variation of melting point in a series of homologues indicate a change in packing or bond type
Density	For measurement, see Chapter 3.2. Necessary for determination of number of formula weights per cell. May indicate liquid of crystallization, isomorphous replacement, degree of approach to close packing, first-order transitions with change of temperature or pressure
Thermal expansion	Thermal expansion is usually greatest in directions normal to layers or chains. Abrupt variation with change of temperature or pressure indicates a second-order transition
Chemical properties	
Chemical analysis	Gives kinds of atoms in the structure and (in conjunction with the density) the number of each kind in the unit cell
Attack of surface	May be used to shape crystals Etch figures are sensitive indicators of point-group symmetry (see *IT* A, Section 10.2.3). Change of orientation of etch figures on a face may reveal twinning. Rows of etch pits may reveal grain or sub-grain boundaries
Oriented growth on parent crystal	Epitaxy often reveals similarity of lattice parameters and even of atomic arrangement in the interface Grain boundaries and twinning orientations may be marked by epitaxic growth, or by oriented growth of crystals or reaction products on the mother crystal ('topotaxy')

reflections, leading to poor data quality in certain regions of the diffraction pattern.

The ultimate test of the quality of a crystal and its suitability for a structure analysis is the quality of the diffraction pattern. Ideally, the reflections should appear in the case of monochromatic radiation as single spots without satellites, tails, or streaks between the spots. The diffraction pattern should be indexable in terms of a single lattice.

3.1.2.3. *Optical examination [see IT A (2002), Section 10.2.4]*

Optical examination of a crystal under a polarizing microscope should be a prerequisite before mounting the specimen for a diffraction experiment. The presence of satellite crystals, inclusions, and other crystal imperfections will degrade the data quality, indicating the selection of a better specimen. The external morphology can often give a strong indication regarding

the nature of the crystal system. A preliminary examination under crossed polars will often show whether the crystal is isotropic, uniaxial or biaxial (see, for example, Hartshorne & Stuart, 1960; Bunn, 1961; Ladd & Palmer, 1985). Crystals that comprise two or more fragments will often be revealed by displaying both dark and light regions simultaneously. For uniaxial crystals, a birefringent orientation is always presented to the incident light beam if the unique axis is perpendicular to the microscope axis, and extinction will occur whenever the unique axis is parallel to the crosswires (assuming that the crosswires are parallel to the planes of polarization of the polarizer and analyser). If the unique axis is parallel to the microscope axis, a uniaxial crystal presents an isotropic cross section and will remain extinguished for all rotations of the crystal. Biaxial crystals have three principal refractive indices associated with light vibrating parallel to the three mutually perpendicular directions in the crystal. The two optic axes and their correspondingly isotropic cross sections that derive from this property are not directly related to the crystallographic axes. In the orthorhombic system, the three vibration directions are parallel to the crystallographic axes, often enabling identification of this crystal system. A monoclinic crystal lying with its unique axis parallel to the crosswires will always show straight extinction. If the crystal is oriented so that the unique axis lies along the microscope axis then, in general, the extinction directions will be oblique. In the triclinic case, the three mutually perpendicular vibration directions are arbitrarily related to the crystal axes. Even if it is not possible to discover the nature of the crystal system unequivocally, the extinction directions should at least enable the principal symmetry directions to be identified and therefore suggest how the crystals should be mounted for optimum data collection (see Chapter 3.4).

3.1.2.4. *Twinning (see Chapter 1.3)*

If at all possible, twinned crystals should *not* be used for structure analysis studies, but the recognition of twinning is critical, since unnoticed or misinterpreted twinning can prevent structure determination or lead to errors in the final structure solution. A distinction should be made between multiple crystal growth, whereby single crystals grow on or from the faces of a given single crystal, or from a common nucleation point, in non-specific orientations, and crystallographic twinning (see, for example, Phillips, 1971; Bishop, 1972). In the latter case, the relationship between the lattices of twinned crystals is normally that of rotation of 180° about a central lattice line, or reflection across a lattice plane. If the lattice is not geometrically symmetrical about the line or plane, two lattices with differing orientations will be produced, and the corresponding reciprocal lattices will be visible in the diffraction patterns. In ideal circumstances, the two patterns can be deconvoluted. If the lattice is geometrically symmetrical about the twin axis or plane, then the two reciprocal lattices will coincide and there may be no obvious signs of twinning in the diffraction pattern (merohedry). If the twins are present in almost equal amounts, the result will be an apparent mirror plane and perpendicular twofold axis in the Laue symmetry. It is therefore very important to examine carefully the Laue symmetry, preferably from a number of different crystals, if twinning is suspected. In some of these crystals, one twin component may be predominant, causing a breakdown in the pseudosymmetry.

Morphological evidence (a concave shape indicating an intersection between the two twin components) and optical examination under a polarizing microscope should also be employed to test for twinning. For lattices that are twinned in a geometrically nonsymmetrical manner, the different twin components will show extinction at different orientations. However, perfect optical extinction is not positive evidence of lack of twinning, since the geometrical symmetry plane (or axis) on which twinning takes place may be parallel to a symmetry plane (or axis) in the optical properties of the crystal.

Intensity statistics can also be used to detect twinning, particularly in the case of crystals twinned by merohedry (*e.g.* Britton, 1972; Fisher & Sweet, 1980). If crystallization conditions cannot be found that eliminate twinning, it is still possible, although difficult, to undertake structure analysis. Recent examples include Sr_3CuPtO_6 (Hodeau *et al.*, 1992), $RbLiCrO_4$ (Makarova, Verin & Aleksandrov, 1993), a serine protease from rat mast cells (Reynolds *et al.*, 1985) and plastocyanin from the green alga *Chlamydomonas reinhardtii* (Redinbo & Yeates, 1993).

3.6. Specimens for neutron diffraction

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Specimen preparation for neutron diffraction presents few of the problems encountered in electron diffraction and electron microscopy (Chapter 3.5). This is because – with the exception of a few isotopes – the atomic absorption cross section for slow neutrons is several orders of magnitude less than that for electrons. Whereas thin sections must be prepared from bulk samples for examination by electron microscopy, the bulk samples themselves are used in neutron diffraction.

For structural studies with single crystals, the size of crystal required depends on the magnitude of the incident neutron flux. The fluxes available worldwide from different sources are tabulated by Bacon (1987). If a flux of 10^{14} neutrons cm^{-2} s^{-1} is assumed, a crystal of linear dimension about 1 mm is necessary. Corrections for extinction, absorption, and multiple scattering are easier to apply if the crystal is in the form of a flat plate or sphere. Crystals containing hydrogen give rise to a uniform background from incoherent scattering. This background can be removed by deuteration, but for measuring Bragg intensities this is rarely essential. The sample can be examined *in vacuo* or in an inert atmosphere by sealing it inside a silica tube, which causes very little attenuation of the neutron beam for a wall thickness of 0.5 mm.

Structural studies on polycrystalline samples are often undertaken using a cylindrical volume of material, enclosed in a holder of aluminium or vanadium. Apart from its cost, vanadium is an ideal container because its atomic coherent scattering cross section (3.3×10^{-30} m^2) is negligible compared with its incoherent cross section (530×10^{-30} m^2); consequently, the container contributes significantly to the background, but gives no diffraction lines. Aluminium has negligible absorption and incoherent scattering, and weak coherent scattering. It therefore produces no background scattering, but it does give rise to diffraction peaks, which may be superimposed on those of the sample. Unlike in single-crystal work, it is usually necessary to replace hydrogen atoms by deuterium. By using a cylindrical sample, effects due to preferred orientation can be reduced by rotating the cylinder about its vertical axis.

Coherent inelastic scattering studies, used to investigate the lattice dynamics of crystalline solids [see Section 4.1.1 in *IT* B (1992)], require single crystals of high purity and crystalline perfection (mosaic spread less than 0.3°). Counting rates are, perhaps, one thousand times less than for structural studies, so that the crystal size is measured in centimetres rather than millimetres. Crystals up to 50 cm^3 in volume may be used. For such large crystals, there is an upper limit of about 10×10^{-30} m^2 to the atomic absorption cross section. This is considerably less than the effective absorption cross section of hydrogen (arising from incoherent scattering), so that hydrogenous compounds must be deuterated for neutron work in which coherent inelastic scattering is measured.

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