Chapter 2
Making an Extract

2.1 The Raw Material

To many people embarking on a protein purification project, much of this section will not be relevant, for they have no choice of raw material. A wide variety of different circumstances could prevail; the laboratory may be working solely on a particular organ or species, and the only considerations about the raw material may be questions of availability in sufficient quantities at times when needed, and the possibility of frozen storage before use.

At the next level, the researcher may have some choice of tissue to use for purifying the protein, or, alternatively, the tissue/organ may be defined but the species source allowed some variation. Species used for protein purification have been chosen principally on the ease of raising or growing. With animals, the principal species used are the rat (especially for liver studies), rabbit (especially for skeletal muscle), and meat animals, mainly cow and pig, for organs which are rather small in size in the laboratory animals (e.g., heart, brain, kidney, thymus). In addition, one must not forget the human animal, on behalf of whom so many laboratory animals are sacrificed, on the assumption that what is true for the rat is likely to be true for the human. Studies on proteins from human tissues have of necessity been limited to those materials relatively easily obtained, such as blood and placenta. But studies on a wider variety of species have become commoner, partly through the realization that many important features of metabolic control, and enzyme localization and characteristics do differ markedly even within the vertebrate phylum, and humans are not always the same as rats!

Invertebrates' proteins have been rather neglected, mainly due to the fact that most invertebrates are very small, and a large number are

required to get a reasonable amount of starting material. If the organ of interest is not dissected from each individual, an arduous task with insects, whole-body homogenates cause great problems because of the large quantity of digestive juices, including proteolytic enzymes, that are released into the extract. Most well-known invertebrate proteins and enzymes have been purified from the larger crustacea such as crayfish and crabs. Many a feast of lobster claws has been enjoyed in laboratories isolating enzymes from the tail muscle!

Plant biochemistry has long been the poor relation to animal work, largely because of the medical implications of the latter, but also because plants pose particular difficulties to the protein chemist. The variation of quality with season can be overcome only by growing the plants in special growth chambers; otherwise, one has to put up with this variation in material grown in the open. Of the vast variety of species, those studied have been, for obvious reasons, mainly those of economic importance, and these are not necessarily the best choices biochemically. Nevertheless, one or two plants have tended to predominate because of the ease of extraction of their leaves, especially when it is easy to isolate chloroplasts from these extracts. Most commonly used is spinach, *Spinacia oleracea*, or alternatively the (not closely) related silver beet *Beta vulgaris*, which is easier to grow in a range of climatic conditions. Plant cells are highly compartmented; in most cases the bulk of the volume is vacuolar space, which can be filled with quite acid solutions, proteases, and a variety of other detrimental compounds. There is also a large amount of cell wall (cellulose), and the chloroplasts, starch granules, and other organelles occupy much of the cytoplasmic space. Indeed, the cytoplasm (which, together with the internal space of the chloroplasts, contains most of the enzymes) may often be no more than 1–2% of the total cell volume in plants. Consequently, plant extracts may be very low in protein even if very little fluid is added when making the extract.

Microorganisms again present a different range of problems. Whereas the animal source may be available at the local abattoir or at least in the institution's animal house, and a plant source may be at the supermarket, microorganisms (with the notable exception of yeast) will have to be specially grown in controlled conditions on a fairly large scale. Algae, fungi, yeasts, and bacteria each have special requirements for growth conditions, harvesting, and extracting. The particular problem of marked changes in enzyme composition during different growth phases must be carefully studied. Collection of bacteria and other unicellular organisms during the log phase of growth is usually desirable (Figure 2.1), though an enzyme being studied might not be at a maximum activity at this time; preliminary investigations on the organism to determine what physiological state contains the most of the enzyme required should be carried out. *Saccharomyces cerevisiae*, baker's yeast, is so readily available in large quantities that, if this can be used, the whole problem of raw

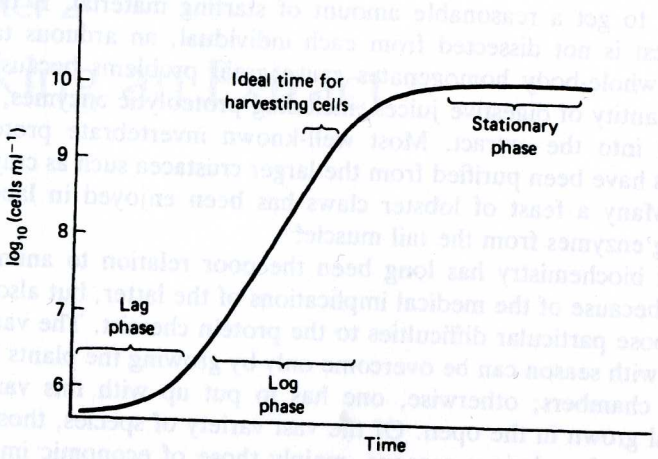

Figure 2.1. Growth of microorganisms in nutrient-rich medium. The ideal time for harvesting is toward the end of the log phase before growth rate slows, giving a high yield of cells. But specific enzymes may be maximum at an earlier or later stage, so some trials at different times are desirable.

material is solved. Compressed yeast cakes are sold that represent nearly 100% pure yeast cells at the end of their growth phase. Grown on a variety of nutrients, the main carbon source is usually molasses or ethanol. Yeast cakes remain viable (and so the enzymes remain active) for some weeks at 0°C, and if frozen will be usable months later. Also very popular with bakers and biochemists are the packs of dried yeast which reconstitute on adding water. These are convenient sources for many proteins and enzymes, though the drying process may tend to partly inactivate a few of the more sensitive ones. Sealed under vacuum or in nitrogen and stored at 0–4°C, these can be used years later, and represent perhaps the most convenient raw material source there is—provided that you can break open the cells (see below).

Molecular biology techniques have so much dominated biochemical and genetic research during the past decade and more, that many people commencing a protein isolation project will be starting with a raw material that contains the protein expressed as a recombinant form in a foreign host. Moreover, the protein may not be in its native form; it may be insoluble; it may have been genetically fused to another protein or peptide fragment, or it may differ in some other way from the protein as found naturally. In almost all cases, it will be present in the host cells at a higher concentration than found naturally (as that is one of the principal reasons for using a recombinant form), and so a purification procedure already established for the natural source may be quite inappropriate for a recombinant source—in addition, the unwanted proteins are different. Further discussion of recombinant protein purification is reserved for Chapter 9.

Freshness and Storage

Usually, the sooner the raw material is used, the better and more physiologically relevant the preparation will be, for if the tissue has been "dead" for some time, natural degradative processes will have commenced. Yet there are occasions when absolute freshness can be a nuisance. For example, skeletal muscle contains a high level of ATP, with phosphocreatine and glycogen present which maintain that level for some hours after death. The structural proteins myosin and actin are normally insoluble at ionic strengths below about 0.25, but the presence of ATP causes the disrupted myofibrils to swell, and a portion of these proteins go into solution. Even at physiological ionic strength (~0.16) some myosin can be solubilized. This can subsequently upset enzyme preparations by precipitating and mixing into fractions that should not contain it. A particular example is the preparation of AMP deaminase [3]. Extraction with a buffer of ionic strength 0.25 is recommended—with really fresh muscle much actin and myosin go into quasi-solution and upset the next step, adsorption on phosphocellulose. But if the muscle is allowed to go into *rigor mortis* first, indicating a loss of ATP, a much better preparation is obtained. This is a rare example; freshness should always be the prime aim unless there is some good reason for delay.

The availability of fresh material does not always coincide with one's ability to use it. Frozen storage, either of the material as received or of an extract from it, must be considered. During freezing a great many events occur. First, the free water freezes and ice crystals grow. The ice crystals are very destructive for membranous layers and organelles, but do not normally upset proteins directly. As the temperature decreases further, the remaining liquid becomes increasingly concentrated in salts as well as protein, until their solubility is exceeded. The less soluble of a pair of salts comprising a buffer will come out of solution first; consequently, the pH can change drastically before complete solidification takes place [4,5]. If the storage is at a temperature of between −15°C and −25°C (typical of the temperatures in domestic refrigerator freezing compartments and simple freezers), the remaining concentrated solution, perhaps at a quite different pH from the original solution, remains unfrozen. Proteases liberated from lysosomes during the freezing can go to work (albeit slowly at such low temperature) in the concentrated protein soup, and a few weeks of storage may do a lot of harm. Thus, freezing should aim at reaching a temperature below −25°C quickly, and storage should be at even lower temperatures if possible. Commercial freezers operating at temperatures down to −80°C are available.

Freezing of extracts is sometimes preferable, because the composition of the medium can be manipulated to optimize storage conditions. Proteolytic enzyme inhibitors can be added after making the extract, and the pH adjusted with a suitable buffer to the best value for stability of the enzymes. Also, large amounts of raw material can all be processed into one extract, so that subsequent samples taken from frozen storage are identical except for their storage time; variation in behavior cannot then be blamed on biological variation of the frozen material.

Remember that thawing speed can be important—the faster, the better, provided that local overheating does not occur. The best way is to immerse the container in warm (40–50°C) water and agitate frequently. It is unlikely that in these conditions the melted solution would rise above 10°C, despite the outside temperature, as long as ice remains inside.

Now that we have the raw material, either fresh or thawed, it is time to homogenize it and obtain an extract containing the enzyme in solution, the first step in the majority of enzyme purification procedures. Cases where preparation of subcellular particles or other insoluble material precedes the release of the enzymes into solutions are described briefly later (Section 2.4).

2.2 Cell Disintegration and Extraction

Most of the proteins and enzymes studied in the early days of protein chemistry were isolated from extracellular fluids. The reason for this is not just because it was easy to obtain the raw material, but because extracellular proteins are for the most part more stable, often as a result of disulfide cross-links, and they tend to be small molecules; early studies on protein structure naturally concentrated on such small proteins. Thus lysozyme, ribonuclease, and chymotrypsin were among the earliest proteins studied in detail, and all are from extracellular sources. But most enzymes are found inside cells, and are very often much less stable; disulfides are generally absent because of the more reducing intracellular environment. The purpose of the present section is to describe methods of disrupting the cells and releasing the enzyme into an aqueous "extract" which is the first stage of enzyme purification techniques.

There are many methods of cellular disintegration, for there are many types of cell. Most cells have particular characteristics which need special attention during disintegration. Animal tissues vary from the very easily broken erythrocytes to tough collagenous material such as found in blood vessels and other smooth-muscle-containing tissue. Plant cells are generally more difficult to disrupt than animal cells because of the cellulosic cell walls. Bacteria vary from fairly fragile organisms that can be broken up by digestive enzymes or osmotic shock, to more resilient species with thick cell walls, needing vigorous mechanical treatment for disintegration.

Table 2.1. Cell Disintegration Techniques

Technique	Example	Principle
Gentle		
Cell lysis	Erythrocytes	Osmotic disruption of cell membrane
Enzyme digestion	Lysozyme treatment of bacteria	Cell wall digested, leading to osmotic disruption of cell membrane
Chemical solubilization/ autolysis	Toluene extraction of yeast	Cell wall (membrane) partially solubilized chemically; lytic enzymes released complete the process
Hand homogenizer	Liver tissue	Cells forced through narrow gap, rips off cell membrane
Mincing (grinding)	Muscle etc.	Cells disrupted during mincing process by shear force
Moderate		
Blade homogenizer (Waring type)	Muscle tissue, most animal tissues, plant tissues	Chopping action breaks up large cells, shears apart smaller ones
Grinding with abrasive (e.g., sand, alumina)	Plant tissues, bacteria	Microroughness rips off cell walls
Vigorous		
French press	Bacteria, plant cells	Cells forced through small orifice at very high pressure; shear forces disrupt cells
Ultrasonication	Cell suspensions	Microscale high-pressure sound waves cause disruption by shear forces and cavitation
Bead mill	Cell suspensions	Rapid vibration with glass beads rips cell walls off
Manton-Gaulin homogenizer	Cell suspensions	As for French press above, but on a larger scale

It is generally not advisable to use a disruption treatment more vigorous than necessary, since labile enzymes may be inactivated once liberated into solution. Table 2.1 gives a list of techniques that can be used, with illustrations in Figure 2.2.

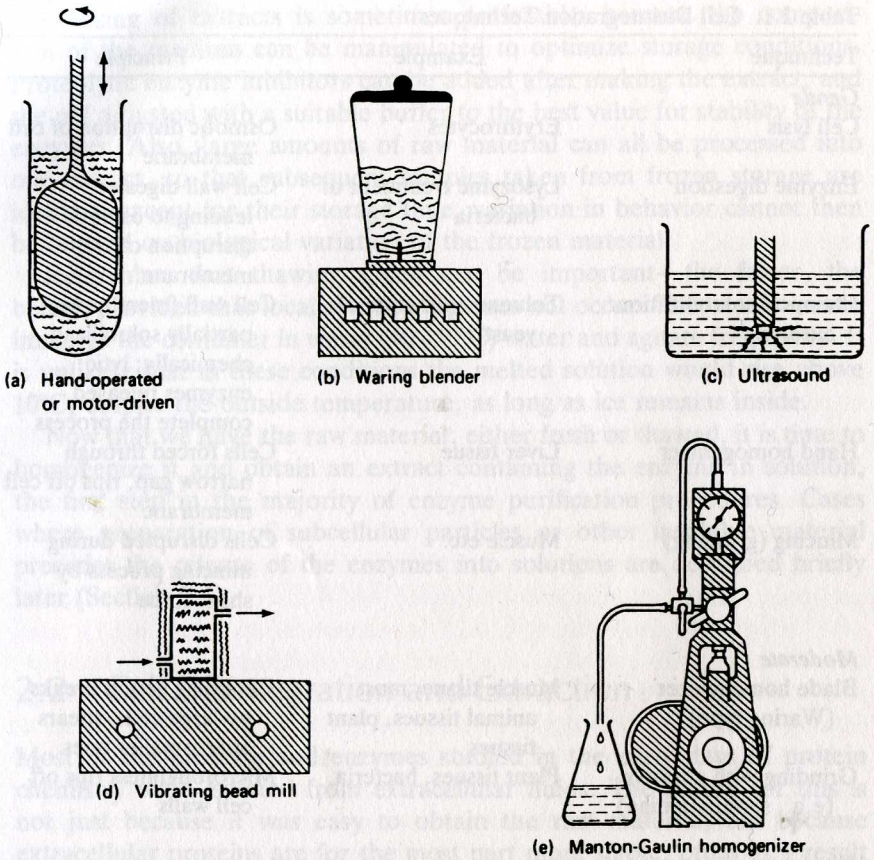

Figure 2.2. Equipment used for breaking up cells to obtain an extract. (a) Hand-operated or motor-driven glass homogenizer. (b) Waring blade-blender (food processor). (c) Ultrasonic probe. (d) Vibrating glass bead mill. (e) Manton-Gaulin cell disintegrator.

These methods are for disrupting cells and releasing proteins into solution. On occasions when an enzyme is present in an organelle, the methods may still be suitable in that they also disrupt the organelles. On the other hand, it may be desirable to isolate the organelles themselves first, so disposing of contaminating cytoplasmic proteins, before extracting the enzyme from the organelle. Less vigorous techniques are needed; preliminary digestion of collagenous or cellulosic extracellular structures with digestive enzyme preparations enables the cells to be broken subsequently by a gentler treatment while maintaining the integrity of the organelles. Preparation of mitochondria from tissues such as skeletal or cardiac muscle using proteinase treatment is an example of such a procedure [6]. However, this is generally only applicable on a small scale,

more suited for metabolic studies on the organelles than enzyme purification. Yields of purified organelles may be very low, and it is often a better procedure to do a complete tissue disruption and then approach the problem of isolating the protein required from the complex mixture of proteins in the extract. Thus, mitochondrial and chloroplast enzymes would often be prepared from a complete tissue homogenate rather than from the isolated organelles.

Finally, the protein may not be soluble in the extraction buffer. In this case special techniques are required and are discussed briefly in Section 2.4.

The "extract" is prepared, after cell disintegration, by centrifuging off insoluble material. Before centrifuging, the mixture is usually described as a homogenate; after centrifugation as much as possible of the desired protein should be present in the liquid layer. Liquid is trapped within the precipitated residue, and the total loss will be related to the proportion of residue to liquid. Most animal tissues have large amounts of insoluble cell material which bind a lot of water. They give a volume of residue about as much as the original tissue (dependent somewhat on the amount of centrifuging). Thus, when making a homogenate of, say, liver, *at least* 2 vol of a suitable extractant buffer should be added when making the homogenate. The more that is added, the larger proportion of the soluble fraction will be extracted, but the extract will be more dilute; its greater volume may create difficulties when working on a large scale. Two-and-one-half volumes of extractant is typical for liver, heart, or skeletal muscle homogenates. Sometimes, with valuable raw material, a reextraction of the residue can be carried out.

Plant tissues are quite different; as mentioned in the previous section, only a small fraction of the volume of plant tissue is truly intracellular; large vacuoles (being regarded here as extracellular) and intercellular spaces mean that on disruption much liquid is released, making additional extractant liquid almost unnecessary. The residue after centrifugation may occupy only 20–40% of the volume of the original plant tissue. Nevertheless, it may be important to use some added extractant liquid so as to control undesirable processes during homogenization. These include acidification and oxidation of susceptible compounds. A particular problem with many plants is their content of phenolic compounds, which oxidize—mainly under the influence of endogenous phenol oxidases—to form dark pigments. These pigments attach themselves to proteins and react covalently to inactivate many enzymes. Two approaches to this problem are useful. First, the inclusion of a thiol compound such as β-mercaptoethanol minimizes the action of phenol oxidases. Second, addition of powdered polyvinylpyrrolidone is often beneficial as it adsorbs the phenolic compounds.

Microorganisms are more like animal tissue with respect to the volume of residue after centrifuging; yeasts and similar fungi with thick cell walls

give a residue which nearly equals the volume of cells initially. With microorganisms, the first step is to harvest the cells by centrifugation, flocculation, or ultrafiltration; On a large scale this will need special equipment (Chapter 10). Even on a laboratory scale, with organisms which grow poorly, it may be necessary to grow batches of tens of liters, which must be harvested. Centrifuges capable of dealing with up to 6 liters at a time are useable for this purpose; alternatively, cross-flow or hollow fiber filtration with 0.2–0.4 μm membranes (Amicon Corp Inc., Beverly MA; Millipore, Millford, MA) can concentrate the culture. It may be possible to either develop a flocculant strain, which allows decantation of the bulk of the spent medium, or to cause flocculation by addition of polyelectrolytes (cationic) such as chitosan [7]. Breakage of the cells can vary from very easy to nearly impossible; a summary of methods is given below. They include mechanical breakage of cells through to gentler lysis techniques, and combinations of these may be most suitable for particular purposes [8].

Cell debris may centrifuge down easily, particularly the coarse residues from animal and plant tissues, although fine intracellular particulates will remain in suspension after, say, 10,000 *g* for 15 min. This cloudiness need not be of any concern, as fractionation steps will remove it. But use of the more vigorous disruption techniques such as bead-milling can break down cell wall material into fine particles which may have a relatively low density, since lipids are usually present. This can be difficult to sediment at the available *g* force for large volumes, and the extract may be very turbid indeed. Methods for clarification of the extract are described in Section 2.3.

The following outlines some procedures for extracting various tissues.

Mammalian Tissues

Dice tissues and cut away connective tissue and fat as far as possible. Place in Waring blender with 2–3 vol cold extraction buffer per gram of tissue. Blend for 30 sec, then repeat if still lumpy. Stir the homogenate for 10–15 min, checking that its pH is suitable, then transfer to centrifuge buckets. Centrifuge at 5,000–10,000 *g* for up to 60 min ($2–3 \times 10^5$ *g* min). Decant extract through Miracloth (Calbiochem., San Diego, CA), cheese-cloth, or glass wool to trap fat particles.

Very fatty tissues may not be manageable this way. A traditional, but still useful way of preparing such tissues is to make what is known as an acetone powder. The homogenization takes place in the presence of large quantities of acetone that have been chilled to a temperature well below 0°C (hazard warning: do *not* place acetone in deep freezers—use an ice–salt bath or dry ice). The acetone solubilizes most of the fat, whereas the proteins are precipitated, but remain in the native conformations

provided the temperature remains below zero. The powder precipitate is washed with cold acetone, then dried, and can be stored before use. The extract is made by stirring the acetone powder with an appropriate buffer in the warm, solubilizing the required proteins. Membrane proteins that would otherwise be insoluble may now go into solution, as the fatty membrane has been dissolved.

Erythrocytes

Red blood cells are easy to extract after collection by centrifugation, and a rinsing step in isotonic NaCl (0.9%, 0.15 *M*) followed by centrifugation again. The cells are osmotically lysed with water, e.g., 2 vol water to 1 vol packed cells. However, approximately 90% of the protein going into solution is hemoglobin, and unless this is what you are purifying, a method for selectively removing it is more useful. Ethanol-chloroform has been used for over 60 years to denature hemoglobin [9].

Soft Plant Tissues

Using only 0.5–1 vol of cold extraction buffer containing 20–30 m*M* mercaptoethanol, homogenize in a blender for 30 sec. Alternatively, pass material through domestic juicer, washing through with the buffer. Centrifuge as soon as possible to minimize oxidative browning ($2–3 \times 10^5$ *g* min). Decant carefully from soft material on surface of precipitate. Addition of powdered polyvinylpyrrolidone can be beneficial in adsorbing phenols.

Yeasts

1. *Mechanical disruption*. Complete disruption can be obtained with a Manton-Gaulin homogenizer (Gaulin Corp., MA, USA) or a Vibrogen Cell Mill (Bühler, Tübingen, Germany), using 2–5 vols buffer per gram wet weight.
2. *Toluene autolysate*. A variety of toluene methods have been used [10,11,12]; certain of them are not fully successful with all qualities of commercial cake yeast. The principle is to treat the yeasts with toluene, usually at a temperature of 35–40°C, when after 20–30 min the yeast "liquefies" due to extraction of cell wall components. Buffer is then added and the slurry stirred in the warm for a few hours or left overnight in the cold. As the method is autolytic, in which cell wall structure is degraded by enzymic action, some cellular enzymes become degraded during the treatment. Ethyl acetate has been used in lieu of toluene [13,14] but does not work with tougher yeast strains.

3. *Ammonia cytolysis* [15]. This method is best suited to dried yeast; it is simple, but some enzymes unstable at pH 10 are lost completely. "Active dried" yeast is stirred with 0.5 *M* NH_4OH, 2 vol per g dry weight, at room temperature, and stirring is continued for 16–20 h. A little toluene, 1–2% of the total volume, sometimes improves the overall yield of soluble proteins. A further 1–2 vol of water plus enough acetic acid to bring the pH down to an appropriate value is stirred in before centrifuging off the cell debris.
4. *Bead mill.* Equipment for shaking cell suspensions with glass beads is available. A slurry of yeast cells (ca. 1 g wet weight to 3 ml buffer) is mixed with an approximately equal volume of glass beads of 0.5–1.0 mm diameter, placed in the container, which is usually of stainless steel, and this is then vigorously shaken while cooling water circulates around it. Within a few minutes, the cells have been sheared apart and the contents released. Continuous flow adaptations of the equipment enable larger volumes to be processed.
5. *Manual beading* [16]. This method is surprisingly effective, rapid, and requires no special apparatus other than glass beads of diameter 0.5 or, preferably, 1.0 mm. A suspension of yeast cells is put into a suitable bottle, and beads are added so that almost the whole suspension in full of beads. The bottle is sealed and shaken manually for 5–10 min. The beads are filtered under suction and washed, and the homogenate centrifuged.
6. *Enzymic lysis* [17,18]. A number of enzymes that act on yeast cell walls have been described; in fact, a mixture containing mannanases, glucanases, and chitinases is needed. Such enzymes are produced by organisms that feed on yeasts. Preparations from *Arthrobacter, Oerskovia*, and *Cytophaga* (Lyticase) have been used; these are useful for small-scale work, but may be uneconomical on scale-up.

Bacteria

Vigorous treatments with sonication, bead-milling, or the French press successfully disrupt bacteria, though not all are convenient for large-scale extraction. Grinding with alumina is often successful on a small to medium scale. Gram-positive species are mostly susceptible to lysozyme; stirring a suspension of cells in buffer plus 0.2 mg ml^{-1} egg white lysozyme at 37°C for 15 min may be sufficient to release the cytoplasmic components. Inclusion of deoxyribonuclease I (10 $\mu g\,ml^{-1}$) improves the quality of the extract obtained by reducing its viscosity. A combination of enzymatic lysis with mechanical disruption may be the optimal process in many cases [19].

Gram-negative species are less susceptible to lysozyme without prior treatment. A combined nonionic detergent–osmotic shock–lysozyme

treatment has been described [20]. Inclusion of nonionic detergent to solubilize cell membranes greatly improves the cell lysis. We routinely use a lysozyme–nonionic detergent method to extract the gram-negative bacterium *Zymomonas mobilis*; this also works quite well with *Escherichia coli* and many other gram-negative bacteria [21], as well as gram-positives. The method extracts most of the soluble cytoplasmic proteins, but avoids total disruption and release of cell wall material, so a relatively clear extract can be obtained without high-speed centrifugation.

To 10 g wet weight of cells, 40–60 ml of extraction buffer, preferably at a pH above 7, containing 0.1% Triton X-100 or Nonidet P-40, 0.2 mg ml^{-1} lysozyme, and 10 $\mu g\,ml^{-1}$ DNase, is added. The mixture is stirred for 1–2 h and the effectiveness of extraction tested on a small sample. We often leave the extraction to continue overnight. Proteolytic inhibitors may be added (see Chapter 12). A clear extract is obtained after centrifugation at 20,000 *g* for 20 min. The effectiveness of this procedure depends greatly on the growth conditions of the cells. Better extraction may be achieved by the inclusion of mercaptoethanol up to 0.1%, or of certain partially miscible organic solvents such as toluene, *n*-butanol, or ethyl acetate at about 1% v/v.

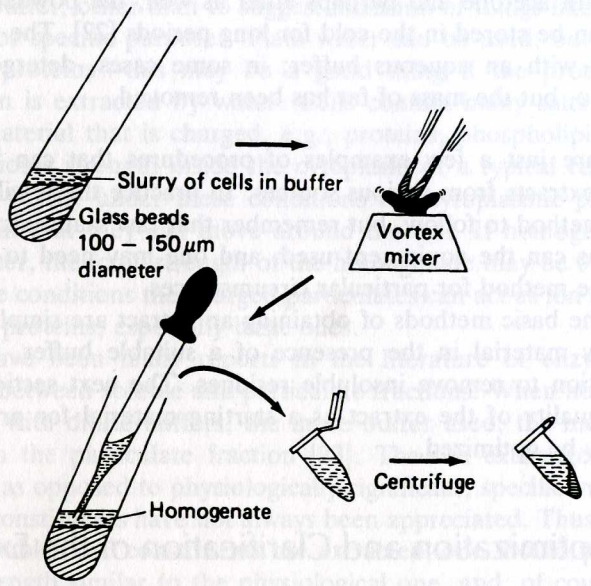

Figure 2.3. Small-scale disintegration of bacteria. Glass beads are added to a small test tube (e.g., 15 mm diameter) to a depth of about 10 mm. A slurry of cells in buffer (e.g., 1 g wet weight in 5 ml of buffer) is added until up to 5 mm above the glass beads. The mixture is vortex-mixed for 2–5 min. The extract is sampled from the surface of the beads and centrifuged in a microcentrifuge tube.

There are other lytic enzymes than the commonly used hen egg-white lysozyme. Culture supernatant fractions from several microorganisms such as *Cytophaga* and *Streptomyces* produce enzyme mixtures that successfully lyse certain cells that are otherwise difficult to break open [17].

Small-scale extraction of bacterial cells can be achieved with all but the hardiest organisms using glass beads of a smaller size than described above for yeast extraction, preferably around 0.1 mm (100 μm). The method is illustrated in Figure 2.3.

Fatty Tissues

Many animal and some plant materials contain a lot of fat which can make homogenization difficult, and the extract may contain a lot of finely dispersed lipid which complicates the subsequent purification steps. There are two ways of overcoming this. First is the use of quite large amounts of detergent to solubilize the fats and at the same time release proteins associated with them (see also under extraction of membrane proteins). The major problems with this are the need for large amounts of detergent, and the difficulty of removing it from the aqueous extract so obtained. Second is the classical technique which still finds use both in the laboratory and commercially, the preparation of an "acetone powder." After washing with acetone and perhaps ether as well, the powder is dried in air, and can be stored in the cold for long periods [22]. The proteins are solubilized with an aqueous buffer; in some cases, detergent may be needed also, but the mass of fat has been removed.

These are just a few examples of procedures that can be used for obtaining extracts from various sources. In practice there will often be a reported method to follow, but remember that raw materials can be very variable, as can the equipment used, and one may need to modify and develop the method for particular circumstances.

Thus, the basic methods of obtaining an extract are simply disruption of the raw material in the presence of a suitable buffer, followed by centrifugation to remove insoluble residues. The next section describes how the quality of the extract as a starting material for protein purification may be optimized.

2.3 Optimization and Clarification of the Extract

At this stage we will assume that a suitable method for cell disruption has been found and an extract made. It is important for reproducibility that each time an extract is made, it is as close to identical with previous extracts as possible. Often the properties of proteins in fractionation procedures depend not only on their own particular characteristics, but

also on the composition of the solution, including other proteins present. Things that can go wrong include suboptimum operation of the homogenization apparatus—low pressure in a pressure cell, worn beads in a bead mill, or blunt blades on a Waring blender, resulting in less than complete cell disintegration. As a result, the extract would be more dilute and may have a different proportional composition since some components may be selectively extracted by suboptimum disruption. It is important to know exactly how much of an enzyme is present in a gram of raw material to evaluate a particular extract. Probably the best method is to make a small-scale extract using a large volume (say 10 ml g^{-1}) of extractant, and measure the amount of activity after different times of treatment. Extended times may eventually result in less activity because the treatment is denaturing proteins, either by heating or by the vigorous shearing nature of the disintegration method. It is very often necessary to take measures to cool the system during cell disruption; ice-cold buffer and short bursts of treatment followed by a cooling period may be needed. As mentioned in the preceding section, the volume of extractant per gram of material is an important consideration; on a large scale the amount used will tend to be a compromise between maximum extraction and minimum volume of extract.

Up to this stage no mention has been made of the nature of the extractant buffer, other than to suggest inclusion of things like β-mercaptoethanol for specific purposes. Plain water can be used, but it will not extract all proteins—this may be a good thing if the protein one is interested in is extracted by water. Cells contain many salts and much insoluble material that is charged, e.g., proteins, phospholipids, nucleic acids. The ionic strength inside the cytoplasm of a typical cell is in the range 0.15–0.2 *M*; under these conditions the cytoplasmic proteins are "soluble," in that they can move around the cell. If homogenized with 2–3 vol water, the ionic strength of the homogenate may be 0.05 or less. Under these conditions the charged particulates can act as ion exchangers and adsorb proteins, especially basic ones.

There have been many reports in the literature of enzymes being distributed between soluble and particulate fractions. When homogenates were made with dilute buffers, the more buffer used, the more protein appeared in the particulate fraction [23]. The ion exchange adsorbent properties (as opposed to physiologically significant, specific interactions) of cellular constituents have not always been appreciated. Thus, to ensure that all "soluble" cell constituents are extracted, one should use a buffer of ionic strength similar to the physiological one, and, of course, at the appropriate pH. If this leads to too much extraction of unwanted compounds, a compromise may be better. Typical buffers would be 20–50 m*M* phosphate, pH 7–7.5; 0.1 *M* Tris-Cl, pH 7.5; 0.1 *M* KCl with a little buffer in it; and for isolating organelles, isoosmotic buffers containing sucrose, mannitol, or sorbitol, as well as salts and buffer ions would

be appropriate. Also included may be EDTA (1–5 m*M*), β-mercaptoethanol, or cysteine (5–20 m*M*), and specific stabilizing agents for particular proteins, e.g., Zn^{2+} for zinc-containing protein, or pyridoxal phosphate for enzymes using this as cofactor.

After the cells have been disrupted in the appropriate buffer, it may be desirable to check the pH of the homogenate. Although really fresh material, homogenized in the appropriate buffer, will give a homogenate of consistent pH, that value may well change downward due to metabolic processes leading to acidification. The best example is that of skeletal muscle, in which glycogen can be rapidly converted to lactic acid, and the pH of a homogenate could drop from 7.0 to 6.0 in 30–60 min (including during centrifugation). In such circumstances the pH can be raised *above* the desired value (e.g., with *M* Tris) before centrifuging. Alternatively, inclusion of a glycolytic inhibitor can arrest the process; fluoride (10–30 m*M*) is sometimes used.

These general comments refer to maximizing extraction conditions of all soluble components. For a particular protein it may be better to use other conditions, purposely preventing a complete solubilization of all components provided that the protein required is totally extracted.

Often, the extract obtained is turbid, perhaps with fat particles floating on the surface after centrifugation. The fat can be removed by coarse filtration through a plug of glass wool or a fine mesh cloth. The particulate material in suspension will include organelles and membrane fragments that may sediment fully only at about 100,000 *g*, an impractical procedure for liters of extract. Filtration will usually be useless, since if the filter is fine enough to trap the particles, it will rapidly clog. However, if the cloudiness is relatively slight and a clear extract is needed—for instance, for passing straight on to an adsorption column—filtration can be carried out with a filter aid such as Celite (Section 1.2).

Clarification of cell extracts on a large scale can be carried out by membrane ultrafiltration, using a membrane with a pore-size of about 0.2 μm.

Let us consider the different types of extract. Sometimes the particulates will not constitute a large proportion of the extract and can be ignored; they will aggregate in, say, the first cut of an ammonium sulfate fractionation and be discarded then. On the other hand, many animal tissues contain fats and membranous structures which end up in suspension in the extract. Acidification to a pH between 6.0 and 5.0 will usually cause aggregation so that some of the particulate material can be centrifuged off at relatively low speed. Ribosomal and other nucleoprotein material can be removed by this acidification, which can be regarded as a form of isoelectric precipitation (Section 4.2); the phosphate groups protonate to neutralize, or at least lessen, the charge on the particulate suspension. Provided that the protein wanted (1) does not also isoelectrically precipitate at the pH used, (2) does not adsorb to the precipitate

forming, and (3) remains stable at the subphysiological pH, this treatment can be most beneficial. The extract should be kept cold, and the pH lowered with a suitable acid (e.g., 1 *M* acetic acid). After stirring for 10–20 min, the precipitate is centrifuged off and the supernatant pH readjusted, if required, before commencing the first fractionation step proper. Plant tissues are more acidic in the first place, and particulate material such as chloroplast fragments, which are more fatty, will not aggregate so readily. Otherwise, there is a relatively small amount of particulate material in plant extract (apart from coarse particles like starch, which sediment down easily after making the extract).

Microorganisms cause a variety of problems with their extracts. First, there is the large amount of nucleic acid material extracted from rapidly proliferating cells. Second, during cell disruption the cell wall and extracellular material may either be finely dispersed to give a turbid extract, or partially solubilized so that, as well as protein and nucleic acids, there is a large amount of gumlike polysaccharide in solution. This can cause considerable problems in the early steps of fractionation.

A common procedure is to treat with substances that cause precipitation of the nucleic acids and associated compounds. These include:

Streptomycin. An antibiotic which acts by interacting with ribosomes. Addition of a solution of streptomycin sulfate to the extract precipitates ribonuclear proteins and clarifies the extract.

Protamine. A natural DNA-binding protein from sperm. Addition of a neutralized solution of protamine sulphate to the extract precipitates most DNA and RNA complexes. A number of proteins bind biospecifically (e.g., nucleic-acid binding proteins) or by chance, and so may be lost from the extract, but can be reextracted from the precipitate (Section 4.5).

Polyethyleneimine. A synthetic, positively charged polymer that interacts with nucleic acids and causes precipitation of aggregated nucleoproteins; similar to protamine in its effect, and only a very small amount (e.g., 0.1% w/v) is needed (see also Section 6.6).

Yeast extraction presents several problems, the main one being that the cell walls of fungi are mostly very thick and difficult to break open. Consequently, the more vigorous methods are needed to obtain the extract; if this is to be achieved in a short time, use of a glass bead vibrating mill or a Manton-Gaulin homogenizer is the only reliable method that can be used on a large scale. These methods cause a substantial amount of low-density particulate material to be liberated, and centrifugation does not remove it all. The turbidity can upset the efficiency of precipitation by ammonium sulfate, and may cause clogging of chromatographic columns. Toluene autolysis of yeasts generally gives clearer extracts, but the conditions needed can result in proteolysis.

Strong ammonia was originally reported as a cytolysis method for preparing the very stable enzyme phosphoglycerate mutase [15]. This procedure has been adapted and used extensively on "active dried" yeast, to give a clear extract containing most, but not all, glycolytic enzymes [24]; but some are totally lost because of the high pH. Proteolysis does not seem to be a big problem with this method, as the proteases have little activity at pH 9.5–10, the pH of the mixture. The efficiency of extraction has tended to vary from one batch of yeast to another.

At this stage, one should be ready to go ahead with the purification method; an extract containing as much of the enzyme as possible has been made, and, where appropriate, particulate and most nonprotein materials have been removed. But before concluding this chapter, there is one important class of enzymes that the preceding sections have not dealt with, that is, the insoluble, membrane-associated enzymes which by definition would not be in the clarified extract.

2.4 Extraction of Membrane Proteins

Many proteins and enzymes are not naturally present in an aqueous phase either inside or outside of cells. In prokaryotes, there are cell membrane proteins and, in gram-negative organisms, periplasmic proteins which are partly immobilized between the outer and inner membranes of the cell envelope. In multicellular eukaryotes, as well as individual cell membranes, there are many organelles within cells that are membranous and have proteins associated with them. These include mitochondria, nuclei, endoplasmic reticulum, Golgi, vacuolar, and lysosomal membranes. When attempting to purify a protein from a membranous structure, the first thing to consider is whether the trouble involved in isolating the organelle/membrane is worth the substantial degree of purification it achieves. Usually it is, for although membrane isolation can often involve considerable losses, this need be no great concern at the first step if the raw material is readily available. On the other hand, the time, equipment, and labor costs involved in first isolating the membranous structure may sometimes be better employed on dealing with the more impure "total extract" made on the whole material. There is no general advice here; each circumstance must be taken individually. But if the ultimate objective is a scale-up process, steps to isolate an organelle, such as differential centrifugation, easily carried out on a laboratory scale, may not be possible on a production line.

If the decision is to isolate the organelle, then there are standard ways of going about this, depending on the nature of the organelle and the tissue being used. Some form of homogenization that does not disrupt the organelle (or if it does, keeps the desired proteins on the membrane fragments) must be used. This is followed by a centrifugation, resuspension of the particulate matter in a suitable buffer, and recentrifugation for washing off remaining soluble proteins. Differential centrifugation is commonly carried out, the heaviest fragments such as nuclei being removed at low g force, large but low-density organelles such as mitochondria sedimenting next, and the smallest particles such as ribosomes and other microsomal fractions sedimenting only at $100{,}000\,g$ or more. The supernatant fraction after $100{,}000\,g$ contains soluble proteins only. But some proteins which were membrane-associated may have been released during the processing of the tissue and so be present in this fraction; it is important to analyze each fraction carefully for the desired protein.

Membrane proteins are categorized as either peripheral or integral. Peripheral membrane proteins are only loosely associated on the surface of the membrane, and can normally be released by mild treatments that do not involve solubilization of the membrane itself. These are the type which might be partially released during the membrane isolation, in which case a whole extraction process may be more suitable. Having obtained the organelle or membrane fragment preparation enriched in the desired protein, release as a soluble form can be achieved by one or more of a combination of the following treatments [25]:

1. Sonication;
2. Metal chelators such as EDTA, EGTA at 1–10 mM;
3. Mild alkaline conditions (pH 8–11) at low ionic strength;
4. Dilute non-ionic detergent;
5. Low concentrations of partially miscible organic solvents such as n-butanol;
6. High ionic strength, e.g., 1 M NaCl;
7. Phopholipase treatment.

The protein, once released into solution, should remain soluble during further processing even without the extractant present, since the process has been to disrupt the weak salt or hydrophobic interactions with the membrane. Removing the extracted membrane by centrifugation is the first step after extraction.

Integral membrane proteins present a much greater problem. Since they are embedded within and often right across the membrane, it is normally necessary to solubilize the membrane itself in order to release the protein. And once in solution, it will be necessary to retain a lipophilic component (usually a detergent) together with the solubilized protein to prevent it from aggregating. For the surfaces of an integral membrane protein are hydrophobic in so far as they interact with the lipids themselves, and in isolation, such hydrophobic patches will mutually attract, resulting in aggregation. Solubilization requires detergent, and this will need to be retained throughout the purification. The presence of detergents causes further problems as described below.

Table 2.2. Some detergents commonly used for extraction of membrane proteins (many of the names are trade names). In general, the ionic detergents are more solubilizing, but also are more likely to denature the solubilized proteins

Nonionic:
Tween 80
Triton X-100
Triton X-114
Emulgens
Lubrol
Digitonin
Octyl glucoside
Zwitterionic:
Lysolecithin
CHAPS
CHAPSO
Zwittergents
Ionic:
Cholate
Deoxycholate
Cetyl trimethylammonium bromide
Dodecyl sulfate

Whereas virtually all proteins can be solubilized with ionic detergents such as sodium dodecyl sulfate (SDS) or cetyl trimethylammonium bromide (CTAB), they will also mostly be denatured, often irreversibly. Even if a denatured product is acceptable, normal separation procedures are not suitable for use with denatured proteins. So the initial extraction of the membranes must be with a gentler detergent that retains the native conformation of the protein, even if bioactivity is lost as a result of its extraction. Many and varied detergents are available and described in greater detail than here in several sources [26,27,28]. Some of the more commonly used detergents for solubilizing membranes are listed in Table 2.2. The structure of one of the most frequently used nonionic detergents, Triton X-100, is shown in Figure 2.4.

By definition, the "gentler" detergents are less efficient at solubilizing, and higher concentrations are required than with the more "vigorous" types. It is unfortunate that the gentlest and most refined detergents not only need to be used at higher concentrations, but are also the most expensive. Solubilization is not as clear-cut as simply centrifuging at 30,000 *g* and seeing if the desired protein is in the supernatant. Detergent treatment of membranes may cause them to fragment into vesicles, with the protein still in an insoluble form, but not sedimenting except at high *g* force. If the desired protein is still in the supernatant after a 100,000 *g* spin for 60 min, then it can be considered solubilized. Nevertheless, it

could still be associated with detergent micelles (see below), or partially aggregated with other membrane proteins.

When considering what concentration of detergent to use, some account must be taken of the *amount* of detergent relative to the membrane fraction. If, for example, the membrane suspension is 5 mg ml^{-1}, then 0.1% detergent is only one-fifth of the amount of membrane in milligrams, and will certainly not be enough to solubilize it. As a rule-of-thumb, 1 mg of membrane requires a minimum of 2 mg of detergent. This is approximately 1 mg of detergent for each mg of protein and 1 mg for each mg of lipid. So, a 5 mg ml^{-1} suspension requires at least 1% (10 mg ml^{-1}) of detergent. On the other hand, at a later stage in purification, when there is no lipid present, and the protein concentration is less than 1 mg ml^{-1}, 0.1% detergent may be more than adequate to retain solubility and bioactivity.

All detergents form micelles. These are enclosed clusters of detergent molecules with the hydrophilic portion on the outside, and the lipophilic portion inside (Figure 2.5). (In organic solvents, reversed micelles form, with the lipophilic portion outside.) But at very low concentration, there is not enough detergent for the micelles to form. Above a certain value, called the critical micelle concentration, the detergent molecules cluster together into micelles, and can cause complications in protein purification protocols. The micelle clusters have a size comparable with proteins; typically, micellar molecular weights are in the range 30,000 to 100,000 [27]. Consequently, they behave in a similar fashion in size-fractionation procedures, and by associating with the proteins can cause very poor resolution in other classical methods.

Extraction of membranous fractions with detergent is likely to result in a mixture of solubilized lipid, free protein, protein tightly associated with detergent, and free detergent, both as single molecules and in micellar form. The first step in fractionation (see Chapter 9) must be able to deal with this, remove excess lipid, and remove some unwanted protein components.

Some membrane components are so intractable that in order to get them into a soluble form, they must be denatured using a "vigorous" ionic detergent. Purification of proteins in a denatured state is not an easy process, but one bonus is that preextraction in milder conditions solubilizes and so removes most of the other proteins. It may be sufficient to extract with sodium dodecyl sulphate and run an electrophoretic sepa-

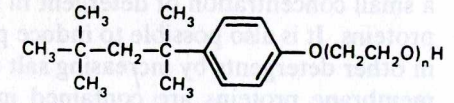

Figure 2.4. Structure of Triton X-100.

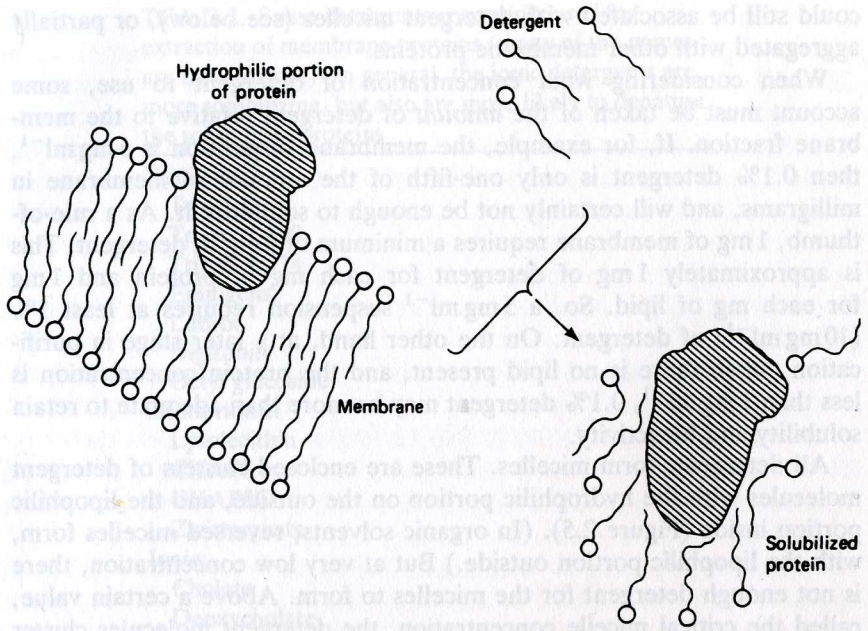

Figure 2.5. Action of detergent in solubilizing membrane-located proteins. The solubilized protein may be as illustrated, or bound in a more complex detergent-micelle, depending on the relative proportions of proteins and detergents.

ration to purify the desired component—provided that the latter can be identified.

A useful method of extraction of membrane proteins which should be included is the use of Triton X-114 [29]. Many detergents have limited water-solubility at elevated temperatures, and on warming a solution, separation into an aqueous phase containing little detergent, and a heavier, detergent-rich phase will occur. This can be a quite abrupt phenomenon with a critical temperature characteristic of the detergent. With Triton X-114 this occurs as low as 20°C. Extraction close to 0°C using 1–3% Triton X-114 should solubilize all peripheral and most integral membrane proteins. After centrifuging off insoluble residue, the extract is warmed to 25°C, at which point phase separation takes place; a brief, gentle centrifugation results in an oily, detergent layer containing most of the integral membrane proteins, and an upper, aqueous layer, which has only a small concentration of detergent in it, containing most of the peripheral proteins. It is also possible to induce phase separation at low temperatures in other detergents by increasing salt concentrations. Because the integral membrane proteins are contained in a phase consisting of a very high detergent concentration, they may be inactivated in the process.

There have been a number of attempts at purifying proteins in non-aqueous solvents, especially membrane proteins which are particularly apolar. Included in these is the use of 100% aqueous w/v choral hydrate [30]. (This is not totally nonaqueous; its use may become restricted due to the stratospheric ozone-destroying nature of chloral hydrate.) Purification of proteins with detergent into reverse micelles has been reported [31,32], and manipulation of some proteins which are soluble in non-aqueous solvents has been successfully achieved [33,34,35].