Chapter 7

Immunochemical techniques

7.1 INTRODUCTION

7.1.1 The immune system

The immune system of animals is responsible for mounting immune responses against molecules recognised as being foreign (non-self). The science of immunology studies such responses and the immune system responsible for them.

The immune system provides protection for animals against infectious microorganisms (viruses, bacteria, mycoplasmas, fungi and protozoa) and also helps in the elimination of parasites and toxins. It combats tumours and neoplastic cells and can reject transfused cells and transplanted organs from genetically non-identical animals. Its physiological role is to ensure that the animal is free from life-threatening life forms and biological substances (the derivation of the word 'immunology' is from the Latin *immunitas* = freedom from). Inappropriate (i.e. undesirable) immune responses can cause clinical problems such as allergies, graft-versus-host disease and autoimmune disorders. Immune responses can be classified as either innate or aquired. Innate immunity does not require prior exposure to the foreign substance and is mediated mainly by cells of the monocytic lineage (e.g. macrophages) and polymorphonuclear leukocytes. Innate immunity is relatively non-specific, although it clearly normally distinguishes between self and non-self. It constitutes a potent, rapid-reacting, first-line defence against invasion and unwanted infection. Laboratory procedures based on innate immunity are limited in usefulness in general application to biochemical methodology.

Acquired immunity requires exposure ('priming') to the non-self material. It is mediated primarily by lymphocytes and may be further divided into cell-mediated and humoral immune responses. Cell-mediated immunity can be attributed mainly to the activity of T lymphocytes, which interact with foreign substances (antigens) in a specific manner and mediate a diverse array of immunobiological processes; for example, cytotoxic T cells specifically 'kill' unwanted cells or microorganisms. Methodology based on cell-mediated immune mechanisms can be useful for the study of cellular immunology and some aspects of clinical immunology, but its application to biochemistry is neither easy nor (usually) useful.

Humoral immunity is mediated primarily by soluble proteins known as antibodies, which circulate in blood and permeate most body organs. They can also be present on cell surfaces, where they function as antigen receptors and binding proteins. Antibodies are produced and secreted by B lymphocytes, but this is influenced by the activity of cells of other types (especially lymphocytes of the T-helper type). Terminally differentiated B cells known as plasma cells are the most potent natural secretors of antibodies. The study of antibodies (and some other immunologically important molecules such as complement components) is known as immunochemistry. Such antibodies can show exquisite specificity and sensitivity for antigens, although this is not always the case, and many procedures and methods have been devised that exploit these properties. Such methods are known as immunochemical techniques. They are obviously very important for studying aspects of immunology itself but are invaluable methods for carrying out investigations in just about every biological science (especially biochemistry). Definitions of some commonly used immunochemical terms are given in Table 7.1.

7.1.2 Antibodies

Antibodies are a group of globular proteins known as immunoglobulins. These consist of monomers or multimers of a basic four-chain, bilaterally symmetrical structure containing two light and two heavy chains (Fig. 7.1). Five varieties of heavy chain (known as γ, μ, α, δ and ϵ chains) occur in higher vertebrates and these determine the class of the immunoglobulin (known as IgG, IgM, IgA, IgD and IgE, respectively; Table 7.2). All classes can bind antigen but the immunobiological functions mediated by the immunoglobulin molecules vary. In mammals, the IgG and IgA classes are divided into subclasses, which reflect different heavy chain amino acid sequences (but less different than the sequences of the different classes). The number of subclasses found differs between species. The light chains do not mediate significant immunobiological activity, but do contribute to antigen binding and the stability and higher structure of the immunoglobulin molecule. IgG, IgE and IgD are predominantly monomers of the basic four-chain structure, but IgA is often dimeric and IgM is pentameric, at least in mammals. Multimeric IgA and IgM contain an additional small protein known as the J chain, which is necessary for polymerisation, and IgA present in secretions also contains a protein known as the secretory component.

Immunoglobulin molecules can be cleaved by some proteolytic enzymes to yield fragments that are useful for immunochemical procedures and also reveal important aspects of antibody structure and function. The plant protease papain cleaves human IgG to yield three fragments of approximately the same size (about 50 000 M_r). Two of the fragments are identical and one is different; the former can be separated from the latter using ion-exchange chromatography. The two identical fragments are able to bind antigen in a monovalent manner, but cannot precipitate antigen from solution or in gel. They are known as fragment–antigen binding or Fab in immunochemical terminology. The third

Table 7.1 Glossary of immunochemical terms

Antigen
A substance that is recognised and bound by an antibody.

Antigenic determinant
See Epitope.

Antiserum
Serum from an animal containing antibodies reacting with particular antigens. Sometimes known as an immune serum.

Autoantibodies
Antibodies that react with self antigen(s). These are not normally present in blood or body fluids, but, if present, are often associated with pathological conditions known as autoimmune diseases, e.g. rheumatoid arthritis, systemic lupus erythematosus, primary biliary cirrhosis, insulin-dependent diabetes mellitus.

Clone
A growing population of cells, derived from a single progenitor cell. The clone is derived asexually by continued division and all cells are genetically identical unless mutation occurs during growth.

Divalent
Able to bind two molecules of ligand. A divalent antibody is thus able to bind two molecules of antigen.

Epitope
A site on the antigen that is recognised and bound by an antibody. It is normally about six amino acid or carbohydrate residues in size. Epitopes on protein antigens may not be continuous in structure. Sometimes also called an antigenic determinant.

Lymphocytes
Cells associated with functions responsible for development and maintenance of specific immunity. They are a subdivision of leukocytes and are the main constituents of lymphoid tissues.

Immunogen
A substance or mixture of substances used to induce an immune response.

Microtitre plate
Plastic plate containing many (usually 96) wells in which many types of imunoassay may be carried out more conveniently than in individual tubes. The wells may be flat-bottomed for use in ELISA, U-shaped for use in RIA or V-shaped for haemagglutination tests. They may be flexible or rigid.

Monovalent
Able to bind only one molecule of ligand.

Multivalent
Able to bind more than one molecule of ligand.

Myeloma cells
Tumour cells of plasma cell lineage. Sometimes also called plasmacytoma cells.

Paraprotein
Monoclonal immunoglobulin secreted by myeloma cells (see above). Such molecules closely resemble immunoglobulins produced by 'normal' plasma cells/lymphocytes. They are sometimes called myeloma proteins.

Peptide
A molecule consisting of a number of amino acid residues linked by peptide bonds. Large peptides are sometimes called polypeptides and/or proteins.

Plasma
Fluid obtained from uncoagulated blood after removal of cellular components. Differs from serum (see below) in containing all components of the coagulation system. Its preparation necessitates the use of an anticoagulant, e.g. heparin, citrate.

Serum
Fluid derived from coagulated blood after removal of the clot and cell components.

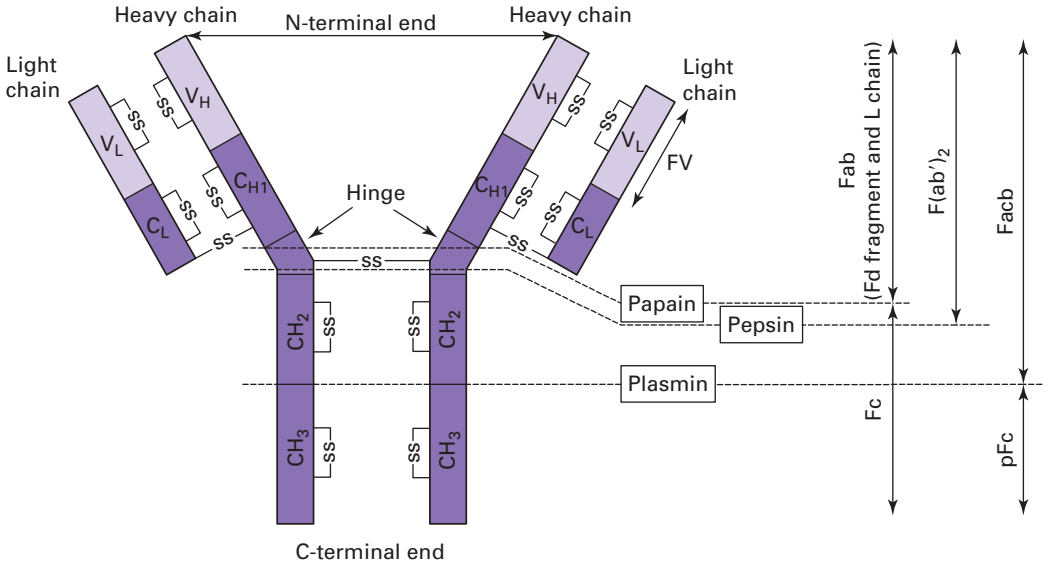


Fig. 7.1. General structure of IgG showing enzyme cleavage sites and resulting fragments. Note that the number of -S—S- bonds in the hinge area varies according to subclass and species.

fragment does not bind antigen, but early biochemical studies showed it to be more easy to crystallize than the Fab fragments (this reflects a greater homogeneity in structure). It is known as fragment crystallisable or Fc by immunochemists. The mammalian protease pepsin cleaves human IgG at low pH to produce one large fragment, a smaller fragment and some small peptides. The large fragment (100 000 M_r) can bind antigen divalently (as long as the antigen is relatively small), can cross-link antigen and can form antigen complexes that precipitate. It is structurally very similar to a dimer of the Fab fragment produced by papain digestion and is known as $F(ab')_2$. Similarly, the smaller fragment is a truncated version of the Fc fragment and is known as Fc′. Both papain and pepsin cleave the IgG molecule in the hinge region, but the former enzyme cuts next to the hinge on the N-terminal side whereas the latter enzyme acts on the C-terminal side of the hinge (see Fig. 7.1). This explains the production of monovalent Fab fragments by papain, but divalent dimeric antigen-binding $F(ab')_2$ fragments by pepsin; the two Fab′ components comprising $F(ab')_2$ are linked via the disulphide bridges that make up the hinge region. Other enzymes can also be used to derive immunoglobulin fragments, but papain and pepsin are the most commonly used. Similar fragments can be produced from non-human IgG, but there is considerable species variation in susceptibility to digestion. Enzyme-derived fragments of other immunoglobulin classes can also be produced (see Suggestions for further reading, Section 7.11).

For most immunochemical procedures, IgG is by far the most useful immunoglobulin reagent.

Table 7.2 Polypeptide chain composition of human immunoglobulins

Class/ subclass	Normal serum concentration (mg ml^{-1})	M_r ($\times 10^{-3}$)	H chains domains	Chain compositions	M_r ($\times 10^{-3}$) Total	M_r ($\times 10^{-3}$) Peptide	% Carbohydrate of chains (no. of groups)	Hinge amino acids	Intra-heavy chain disulphide bonds
IgG1	5–10	146	4	2γ1	51	49	3–4 (1)	15	2
				2L(κ:λ 2:1)	23	23	< 0.5		
IgG2	1.8–3.5	146	4	2γ2	51	49	3–4 (1)	12	4
				2L(κ:λ 2:1)	23	23	< 0.5		
IgG3	0.6–1.2	170	4	2γ3	60	57	3–4 (1)	62	11
				2L(κ:λ 1:1)	23	23	< 0.5		
IgG4	0.3–0.6	146	4	2γ4	51	49	3–4 (1)	12	2
				2L(κ:λ 1:1)	23	23	< 0.5		
IgM	0.5–2.0	900	5	10μ	67	57	12–16 (5)	0	1[a]
				2L(κ:λ 3:1)	23	23	< 0.5		
				1J[f]	15	14	7.5		
IgA1[b]	0.8–3.4	160	4	2α1	56	50	10–15 (5)	20	2[c]
				2L(κ:λ 1:1)	23	23	< 0.5		
IgA2[b]	0.2–0.6	160	4	2α2	53	48	10–15 (2)	7	2[c]
				2L(κ:λ 1:1)	23	23	< 0.5		
Secretory IgA1 or IgA2[d]	–	390	4	4α	53–56	48–50	10–15		
				4L	23	23	< 0.5		
				1J	15	14	7.5		
				ISC[e]	75	63	15–16		
IgD	0.003–0.3	165	4	2δ	60	51	14–71 (3)	64	1
				2L(κ:λ 1:10)	23	23	< 0.5		
IgE	0.0001–0.0007	185	5	2ε	70	59	13–16 (6)	0	2
				2L	23	23	< 0.5		

[a] There is one intra-heavy chain disulphide bond linking cysteines between C_H2 and C_H3 domains; there are further disulphide bonds between monomer units.
[b] Data are given for the monomeric form, which predominates in human sera. Polymeric forms also exist (up to pentamer); these contain one molecule of J chain per polymer in addition to α and L chains.
[c] There is an additional disulphide bond from the cysteine in the tailpiece to either the corresponding cysteine in the other heavy chain or to a cysteine in J chain or secretory component in secretory IgA.
[d] Data are given for the dimer; a tetrameric form is also common in humans.
[e] SC, secretory component, derived from a membrane-bound poly(Ig)receptor used to transport the IgA through epithelial cells during its secretion.
Source: Reproduced from M. A. Kerr and R. Thorpe, *Immunochemistry Labfax* (1994), Bios Scientific, Oxford.

7.1.3 Immunoglobulin structure, immunoglobulin genes and the generation of antibody diversity

Immunoglobulin chains of particular classes or types contain distinct regions that are either very constant in sequence or more variable. The chains are built up of domains which exhibit a similar three-dimensional structure (Fig. 7.1). The heavy chains are made up of three (γ-, α- and δ-chains) or four (μ- and ϵ-chains) constant domains and one variable domain, whereas light chains have one constant and one variable domain (Fig. 7.1 and Table 7.2). The constant regions of antibodies are important for immunoglobulin three-dimensional structure, are responsible for most immunobiological functions such as complement fixation and interact with the immunoglobulin receptors found on many types of cells. The variable regions are responsible for binding antigen. Within the variable regions are relatively short sequences that show particularly high variability in sequence and these are responsible for direct interaction with the antigen. These are known as hypervariable regions or complementarity-determining regions (CDRs), and there are three of these per heavy or light chain. The less variable portions of the variable regions are important for maintenance of the appropriate structure of the antibody molecule, especially for efficient antigen binding.

Immunoglobulins are clear exceptions to the normal rule that one gene codes for one protein chain, and several germline genes are involved in coding for each of the heavy and light chains. The constant regions of heavy chains are coded for by single genes, one for each class or subclass, known as C_H genes, which are distinct in the germline from genes for the heavy chain variable region (V_H genes). Relatively large numbers of variable region genes occur in clusters (for human heavy chains there are about 100 V_H genes) and between these and the constant region genes there are additional short genes that code for joining (J; six for human heavy chain genes) and diversity-associated (D; four for human heavy chain genes) sequences. A similar gene organization exists for the light chains (known as V_L, J_L and C_L gene segments), except that D sequence light chain genes do not occur; there are about 70 V_L and 4 J genes for human κ light chains. Early in B cell maturation, translocation of the genes occurs such that a linear assembly of a particular V gene with a J gene, a D gene (only for heavy chains) and a C gene occurs; this codes for a complete immunoglobulin chain. This results in the ability of an animal to produce a range of immunoglobulin molecules that differ particularly in their hypervariable region sequences and thus the part of the molecule that binds antigen. This diversity can be increased by use of variable amounts of the D region (for heavy chains) and by somatic mutation during maturation of the immune response. All of these combinations are responsible for the ability of at least higher vertebrates to produce a very large number of different antibodies (theoretical considerations suggest that humans can produce an estimated 10^8 different antibodies), and accounts for the differing specificity and affinity of immunoglobulin molecules for antigen. It also allows production of antibodies that recognise virtually *any* foreign antigen of appropriate size. These properties of antibodies are exploited in immunochemical methods.

7.2 PRODUCTION OF ANTIBODIES

Virtually all immunochemical techniques rely on the use of antibodies and their effectiveness is dependent on the quality of the antibody or antibodies employed. The nature of the antibody affects both the specificity of the methods (i.e. the ability to discriminate between the desired analyte and other substances that may be present) and the sensitivity of the procedure (i.e. its ability to detect/measure low concentrations of the analyte). The avidity of the antibody for antigen is important for the latter (see Section 7.9). Although some antibodies that can be useful in immunochemical methods occur 'naturally', for example some autoantibodies, it is normally necessary to stimulate their production by immunising animals (but see Section 7.2.6 for an an important exception to this). Many different procedures for this have been developed.

7.2.1 The immune response; polyclonal and monoclonal antibodies

All vertebrates can produce antibodies against foreign antigens. However, responses in lower vertebrates are very limited (although even the most primitive animals, such as hagfish and lampreys, can mount immune responses against some non-self proteins) and normally antibodies derived from such species are not useful for immunochemical methods. Mammals mount the most useful humoral immune responses and mammalian antibodies are normally used in immunochemical techniques. However, avian antibodies can also be employed in special cases. In most cases IgG antibodies are the most useful for immunochemical techniques, but IgM and IgA can be used for some procedures. IgE is limited to studies relating to allergic and anaphylactic phenomena and IgD is not normally useful. No specific functions have been identified for secreted IgD.

It is now clear that each mature B lymphocyte secretes an antibody with a single immunoglobulin sequence. The humoral response to antigen results in activation of a heterogeneous population of B cells that secretes different immunoglobulins. Maturation of the response results in clonal expansion of these initially primed cells to derive populations of plasma cells that secrete an array of antigen-binding immunoglobulins, often of different classes and subclasses. In the antigen-binding fraction of antibodies, immunoglobulins showing variable specificity and avidity for the immunogen will be present. Usually, many different antibodies, recognising several different epitopes on each antigen are present. Such a response is described as polyclonal, as antibody is derived from more than one clone of B lymphocytes and shows heterogeneity in the amino acid sequences of the antigen-binding immunoglobulins present. Preparations of such polyclonal antibodies, either unpurified as immune sera or purified (see Section 7.3) are often used for immunochemical techniques. However, more recently, methods have been developed for deriving monoclonal antibodies, which are derived from a single B cell clone and show identical amino acid sequence. Monoclonal antibody preparations show homogeneous

characteristics (including specificity and avidity for antigen, i.e. they recognise a single epitope) and can be advantageous for immunochemical purposes, if carefully selected and characterised. Production of monoclonal antibodies is considered in Section 7.2.3.

7.2.2 Production of polyclonal antibodies (antisera)

It is possible to produce antibodies that bind to proteins, peptides, carbohydrates and nucleic acids, but the latter show little if any specificity for sequence and so are usually of little use for immunochemical techniques. Antibodies against carbohydrate can be used for analytical work, but can show limited specificity, except in some special cases. In general, most immunochemical methods are devised for use with antibodies that recognise proteins and peptides.

Most higher vertebrates will produce a humoral immune response against a 'foreign' protein. However, the magnitude of the antibody response depends on a number of variables. Of particular importance are the size of the protein/peptide and the phylogenetic distance between the source of the antigen and the animal used to produce antibody. For the latter it is generally the case that the greater the phylogenetic difference, the better. However, choice of species for antibody production also depends on the amount of antigen available, the amount of antiserum required and the quality of antiserum desired. In some cases use of closely related species or even different strains of a single species for derivation of antigen and production of antibodies can provide antibodies with particular properties/specificities. The most important consideration for immunogenicity is the difference in amino acid sequence (and therefore structure) of the antigen used as immunogen and the equivalent antigen (if present) in the animal used to produce antibody. It is generally the case that the greater this difference, the more immunogenic the antigen will be. In some cases, particular parts of the antigen produce very potent immune responses and such epitopes are known as immunodominant.

Peptides with an M_r of less than about 2000 are normally poorly immunogenic or non-immunogenic. Immunogenicity tends to increase with size; proteins with $M_r > 10\,000$ are usually immunogenic as long as they are recognised as foreign in responding animals.

Antibodies that bind small peptides (and other small, non-immunogenic molecules such as steroids and drugs) can be produced by linking (conjugating) these substances to larger proteins (known as carrier proteins). The antisera produced will contain antibodies that recognise the carrier as well as others that bind to the small molecule. Some proteins are particularly effective as carriers (such as keyhole limpet haemocyanin and thyroglobulin) and some produce a restricted anti-carrier humoral response, such as purified protein derivative (PPD) from Bacille, Calmette, Guérin (BCG). Substances that are not immunogenic alone, but are when conjugated, are known as haptens.

For production of potent antibodies that perform well in immunochemical techniques, it is usually necessary to use an adjuvant as part of the immunogen.

Table 7.3 Some commonly used adjuvants

Adjuvant	Composition and use
Freund's complete adjuvant (FCA)	Mineral oil containing heat-killed mycobacteria (*Mycobacterium tuberculosis* or *M. butyricum*) Used as emulsion with aqueous antigen
Freund's incomplete adjuvant (FIA)	Mineral oil Used as emulsion with aqueous antigen
Alum	Complex aluminium salts. There are various versions of the adjuvant: some can be purchased ready for use (e.g. Alhydrogel); others can be prepared in the laboratory by mixing various salts, e.g. $NaHCO_3$, and aluminium potassium sulphate. Aqueous antigen is absorbed to gel
Bentonite	Wyoming sodium bentonite (Montmorillonite) as gel. Aqueous antigen adsorbed to surface
Quil A	Saponin derived from *Quillaja saponana* Molina (South American tree). Mixed to form a complex with aqueous antigen
Muramyl dipeptide (MDP)	*N*-Acetylmuramyl-L-alanyl-D-isoglutamine. Mixed with aqueous antigen. Various derivations of MDP are also used as adjuvants
Monophosphoryl lipid A (MPL)	Used in various formulations, often as an emulsion with oils. Antigen included in emulsion
Bacillus pertussis	Killed organisms mixed with aqueous antigen

FCA is probably the most potent adjuvant but may be inappropriate for some purposes.
Source: Reproduced from M. A. Kerr and R. Thorpe, *Immunochemistry Labfax* (1994), Bios Scientific, Oxford.

Such substances potentiate the immune reponse by forming a slow-release depot of antigen, by stimulating T cell help or by aiding antigen presentation. Some adjuvants function by more than one of these effects (see Table 7.3 for some adjuvants commonly used for immunochemical purposes). Although a single immunisation with antigen will usually result in production of antibodies, such antisera are usually suboptimal, containing antibodies of low avidity and a high proportion of IgM, which can be of limited use for immunochemical methods. It is usual practice to use several subsequent immunisations, spaced such that the immune response is boosted to produce a hyperimmune animal, with a high concentration of avid antibodies specific for antigen in its blood. Such 'hyperimmune sera' (really antisera from hyperimmunised animals) are usually the polyclonal reagents of choice for immunochemical techniques. Precise details of amount of antigen used and spacing of 'boosting' immunisations vary enormously according to antigen and species used; for some general principles see Table 7.4. In general, the larger the animal, the more antigen is required; however, larger animals

Table 7.4 **Examples of immunisation protocols that have been used successfully**

Species	Priming	Rest period (weeks)	First boost	Rest period (weeks)	Subsequent boosts
Mice, rats and guinea pigs	5–100 μg antigen in FCA (or other adjuvant), s.c. or i.m.	2–3	50–100 μg antigen in FIA, other adjuvant or PBS, i.m. or s.c.	3	5–100 μg antigen in FIA or PBS, i.p., s.c., i.m. or i.v.
Rabbits	50–250 μg antigen in FCA or other adjuvant, i.d., i.m. or s.c.	3–4	50–250 μg antigen in FIA or other adjuvant or PBS, i.m., or s.c.	4 or longer	50–250 μg antigen in FIA or PBS, i.m., s.c. or i.v.
Sheep and goats	250 μg–10 mg antigen in FCA or other adjuvant, i.m., s.c. or i.d.	4	250 μg–10 mg antigen in FIA or other adjuvant, i.m. or s.c..	4–8 or longer	250 μg–10 mg antigen in FIA or other adjuvant, i.m., s.c. or i.v.
Horses and donkeys	250 μg–50 mg antigen in FCA or other adjuvant, i.m., s.c. or i.d.	4	250 μg–50 mg antigen in FIA or other adjuvant, i.m. or s.c.	4–8 or longer	250 μg–50 mg antigen in FIA or other adjuvant, i.m. or s.c.
Primates	50 μg–1 mg antigen in adjuvant, i.m. or s.c.		50 μg–1 mg antigen in adjuvant, or PBS, i.m., s.c. or i.v.	4–8 or longer	50 μg–1 mg antigen in adjuvant or PBS, i.m., s.c. or i.d.
Chickens	30–200 μg antigen in FCA or other adjuvant, i.m.	2–3	30–200 μg antigen in FIA or other adjuvant, i.m.	3 or longer	30–200 μg in FIA or other adjuvant or PBS, i.m.

FCA Freund's complete adjuvant; FIA, Freund's incomplete adjuvant; PBS, phosphate-buffered saline; i.p., intraperitoneally; s.c., subcutaneously; i.m., intramuscularly; i.v., intravenously; i.d., intradermally.

Source: Reproduced from M. A. Kerr and R. Thorpe, *Imunochemistry Labfax* (1994), Bios Scientific, Oxford.

contain more blood (and therefore serum/plasma). Thus larger animals will require the availability of greater quantities of immunogen for antibody production but will generate larger amounts of antiserum (mice will produce only a few millilitres whereas sheep and horses can yield several litres).

7.2.3 Monoclonal antibodies

Monoclonal antibodies can be especially useful for immunochemical methods. Such antibodies are secreted by cloned, i.e. monoclonal cells. Mature, antibody-secreting lymphocytes from immunised animals can be cloned, but these survive for only a very short period in culture, and therefore do not provide useful amounts of antibody. However, procedures have been developed to allow production of large quantities of monoclonal antibodies by producing continuously growing (immortal) cell lines that secrete antibody efficiently. These involve generation of hybrid cells, transformation of lymphocytes with a virus, or recombinant DNA procedures.

7.2.4 Hybridoma production

In 1975, Köhler and Milstein devised a procedure for producing hybrid cells that secrete antibody and grow continuously in culture (Fig. 7.2). This involves fusing lymphocytes from immune mice with mouse myeloma cells. Such hybrid fused cells, known originally as fusomas but later as hybridomas inherit the ability to secrete antibody from the lymphocyte parent and the ability to grow continuously from the myeloma cell. Myeloma cell lines that no longer secrete immunoglobulin paraprotein have been derived and are advantageous for hybridoma technology (the hybridomas derived do not secrete the paraprotein).

Lymphocytes and myeloma cells are mixed together at high density and treated with a fusing agent (nowadays polyethylene glycol, although Sendai virus was originally used). Under such conditions, fused cells are produced but unfused lymphocytes and myeloma cells predominate and the latter will overgrow and overwhelm the hydridomas if they are not removed. This is normally achieved by the use of a selective medium in which the myeloma cells die, but hybridomas survive. The most widely used selective system involves the inclusion of the antibiotic aminopterin in growth medium. This inhibits the *de novo* nucleotide synthesis pathway, in which nucleotides (and thus eventually nucleic acids) are produced from small molecules. Normal cells survive in this medium as they are able to use the salvage pathway for nucleic acid synthesis, in which nucleotides produced by breakdown of nucleic acid are recycled. But, if cells are unable to produce the enzyme hypoxanthine–guanine phosphoribosyl transferase (HGPRT), they are unable to utilise the salvage pathway and therefore die in aminopterin-containing medium. Such cells can be produced by culture in 8-azaguanine and numerous HGPRT-negative mouse myeloma cell lines have been established.

After fusion of lymphocytes with HGPRT-negative myeloma cells, aminopterin-containing medium, supplemented with hypoxanthine and thymidine to ensure

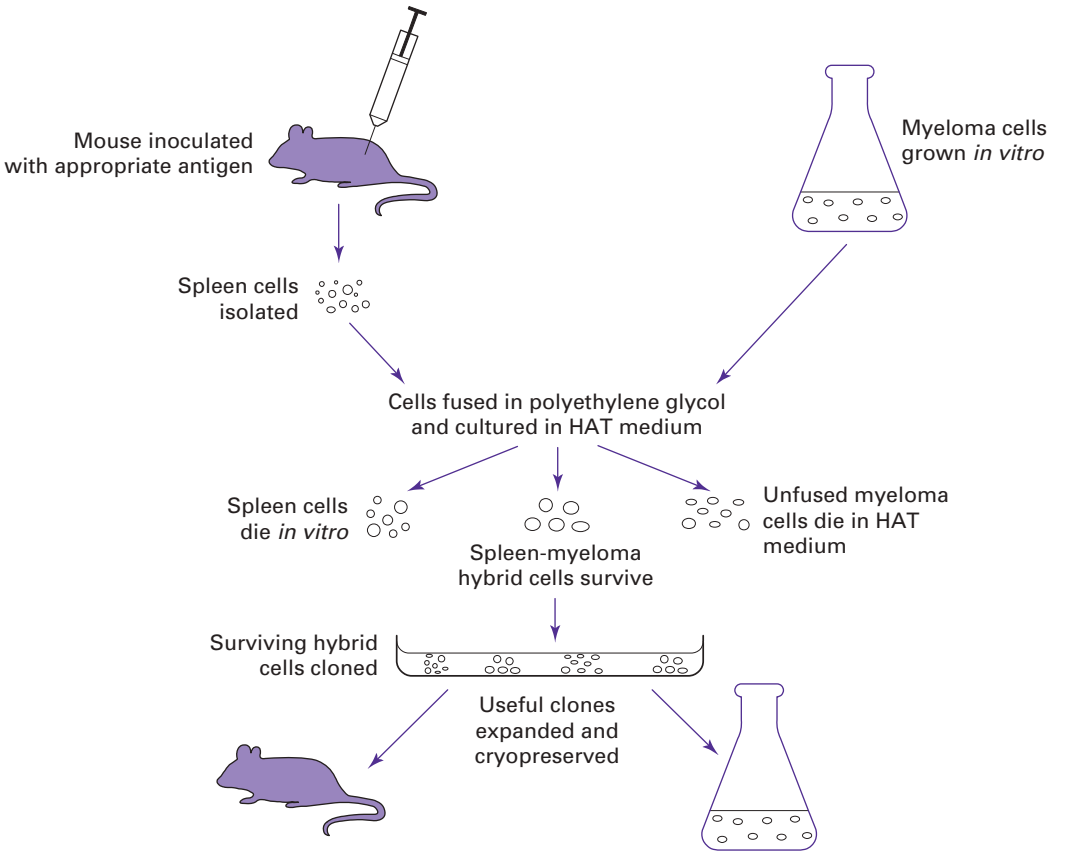


Fig. 7.2. A schematic representation of a typical procedure for production of murine monoclonal antibodies.

an adequate supply of substrates for the salvage pathway (HAT medium) is added, which kills myeloma cells but allows hybridomas to survive as they inherit HGPRT from the lymphocyte parent. Residual unfused lymphocytes die after a short period in culture, which results in a pure preparation of hybridomas that can be cloned using one of three procedures:

- *Limiting dilution cloning:* A single hybridoma cell suspension is diluted and dispensed into culture plate wells to approximate one cell per well (and numbers surrounding this). Wells are inspected to assess clonality and, after culture, cell supernatant is assessed for the presence of appropriate antibody. This procedure is normally repeated (often more than once) to obtain a monoclonal hybridoma line.
- *Soft agar cloning:* A single cell suspension is diluted in approximately 0.25% molten agar, overlayed on solid 0.5% agar and allowed to set. Clones grow in culture as foci that can be located visually (using an inverted microscope).

When of appropriate size, the resulting clumps of cells are 'picked' from the agar using a fine Pasteur pipette, cultured in medium and the supernatant assessed for antibody content.

- *Cloning using fluorescence-activated cell sorting:* Cells are fluorescently labelled for appropriate antibody secretion (e.g. using fluorescein isothiocyanate-labelled antigen) and then individually isolated using flow cytometry via a fluorescence-activated cell sorting (FACS) machine. Appropriate cells are cultured and supernatants screened for antibody content.

All cloning methods are designed to produce cultures of monoclonal hybridomas but do not guarantee this. Repeating the cloning techniques will obviously increase the chance of cells being monoclonal, but cloning checks need to be used if assurance of monoclonality is to be obtained. For this, 'subclones' of the hybridoma lines are prepared (as above) and the percentage of cells secreting antibody of appropriate defined specificity assessed. If the parental line is monoclonal, this should be close to 100%. If not, the hybridoma line either is not monoclonal or is unstable in respect to secretion of monoclonal antibody.

Such monoclonal cell lines can be grown in culture to produce supernatant containing monoclonal antibody (industrial-scale fermenters or hollow fibre culture supports can be used that yield kilograms of antibody) or grown in the peritoneal cavities of mice to produce ascitic fluid containing high concentrations (about 5–10 mg cm^{-3}) of antibody. The cell lines can be cryopreserved to provide an everlasting source of monoclonal antibody.

A similar approach can be used for the production of rat monoclonal antibodies, except that *O*-diazoacetyl-L-serine (azaserine) is sometimes substituted for aminopterin in the selective medium. This inhibits a range of amination reactions, some of which are essential for *de novo* purine synthesis. Hamster heterohybridomas can also be produced by fusing hamster lymphocytes with mouse HGPRT-deficient myeloma cells. Hybridoma technology is generally less successful with higher mammals, owing to the lack of suitable myeloma cell lines for fusion and the instability of mouse/higher species lymphocyte heterohybridomas. A rabbit myeloma cell line suitable for production of rabbit hybridomas has been described.

7.2.5 Transformation of lymphocytes with virus; production of human monoclonal antibodies

In some cases, it it desirable or even essential to use human rather than rodent monoclonal antibodies. For example, human antibodies are generally better for *in vivo* therapeutic use in humans as they are much less immunogenic and mediate immunobiological functions, and it can be very difficult if not impossible to produce rodent monoclonal antibodies against some antigens, for example the human Rh D blood group antigen. A few hybridoma-derived human monoclonal antibodies have been produced (almost always from heterohybridomas), but this approach is difficult and inefficient. However, infection of human B lymphocytes with Epstein–Barr virus results in transformation of a subpopulation of cells

and allows the production of continuously growing cell lines that can be cloned. Some of these clones secrete monoclonal antibody, which can be used for therapeutic and immunochemical purposes. A relatively high proportion of such cell lines secrete IgM antibody, but lines secreting immunoglobulin of all classes and subclasses can be produced. Some cell lines show instability and low level immunoglobulin secretion that can sometimes be resolved by fusion with stable non-antibody-secreting heterohybridomas or myeloma lines.

7.2.6 Engineered antibodies

Although mouse or rat monoclonal antibodies with the desired antigen-binding properties can be produced from deliberately immunised laboratory animals, a problem with their clinical use in humans, for example in cancer patients, is that they can elicit an immune response in the recipient as they are recognised as being 'foreign'. Also, some potential applications of monoclonal antibodies may require particular effector functions (mediated by the Fc portion of particular subclasses) combined with a particular specificity (determined by the variable portion), which may not be readily produced simply by immunisation. Small antibody fragments rather than the relatively large intact molecule may be preferable for other clinical applications, for example where tissue penetration is desired. Genetic engineering methods have therefore been developed to attempt to overcome these limitations. Genes encoding antibody heavy and light chains can be amplified using the polymerase chain reaction (PCR) and cloned into suitable vectors for expression and manipulation, for example splicing the variable region from one antibody to the constant region of another. Mammalian cells are usually used for expression of whole antibodies (to ensure glycosylation and correct chain folding and assembly) whereas antibody fragments can be expressed in *Escherichia coli*. It has also become possible to mimic the *in vivo* antibody response *in vitro* by expressing antibody fragments derived from gene repertoires on the surface of bacteriophage (phage display) to allow selection of particular specificities.

Polymerase chain reaction amplification of antibody genes

Antibody genes may be readily amplified by PCR. This amplification involves repeated cycles of extension between two oligonucleotide primers that hybridise to the 5′ and 3′ ends of the gene sequence. The steps involve:

- preparation of a cell lysate and extraction of the RNA (this fraction will contain the mRNA which encodes the antibody heavy and light chains);
- synthesis of single-stranded complementary DNA (cDNA) using the enzyme reverse transcriptase, the RNA/cDNA hybrid then being used as a template for the PCR;
- specific amplification of the antibody gene(s) present in the RNA/cDNA template using oligonucleotide primers that anneal to sequences outside the region for which sequence information is required (usually the variable region; Fig. 7.3).

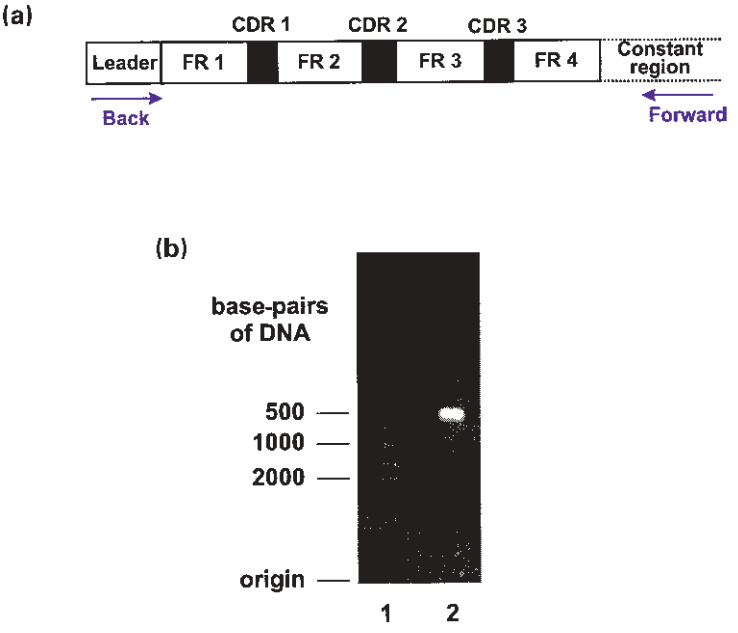


Fig. 7.3. PCR amplification of a heavy chain variable region gene. (a) The position of the forward and back primers relative to the variable region. FR, framework region; CDR, complementarity-determining region. (b) Photograph showing agarose gel electrophoretic analysis of the product of PCR amplification of the heavy chain variable region gene (track 2). Track 1 shows DNA markers. The gel was stained with ethidium bromide (a fluorescent dye that binds to nucleic acid) and viewed under ultraviolet illumination.

Primers can be designed for PCR amplification of most families of variable region genes as the nucleotide sequences flanking the variable region genes are relatively conserved. The incorporation of restriction endonuclease sites in the primers allows subsequent cloning of the amplified gene(s).

The starting material may be a hybridoma secreting a monoclonal antibody to allow, for example, genetic manipulations. Alternatively, RNA prepared from human peripheral blood lymphocytes can be used to prepare repertoires of heavy and light chain variable region genes to allow the creation of antibody fragment gene repertoires and phage display (see below).

Gene repertoires and phage display

Amplified heavy and light variable region (V_H and V_L, respectively) gene repertoires can be spliced together using a stretch of synthetic DNA that encodes a peptide 'linker' to form single-chain (sc) Fv antibody fragment gene repertoires (Fv fragments are the smallest antibody fragments that still contain the intact antigen-binding site; Fig. 7.4b). The use of a linker prevents the otherwise non-covalently attached V_H and V_L portions from dissociating. The scFv repertoire is then reamplified with flanking primers containing appropriate restriction endonuclease

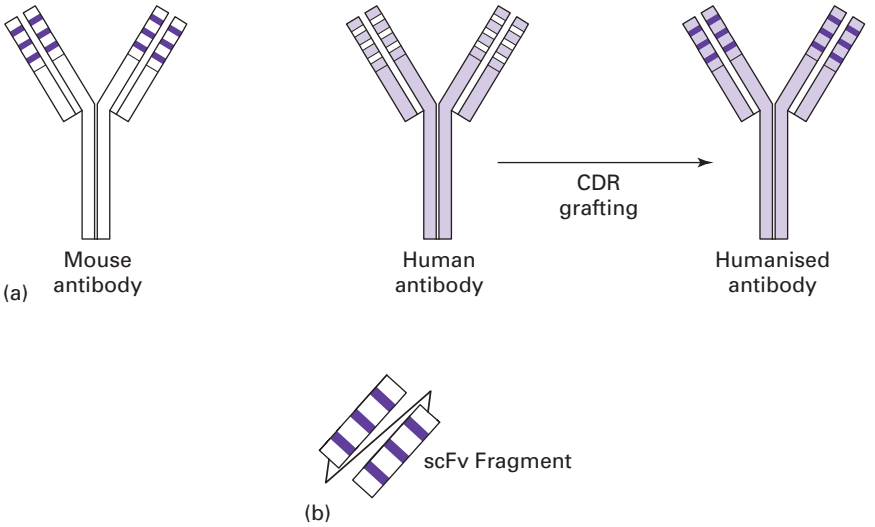


Fig. 7.4. Genetically engineered antibodies and fragments. (a) CDR (complementarity-determining region) grafting to produce humanised antibody; (b) single-chain Fv fragment.

sites that allow subsequent digestion with the appropriate enzymes and ligation into a suitable phage display vector, for example pHEN-1, which is then expressed in *E. coli*. Thus a phage antibody 'library' can be created. The phage display vector allows the scFv fragments to be expressed at the surface of the phage as a fusion product with a phage coat protein. This allows phage-encoding fragments to be selected by screening against antigen bound to a solid support. Alternatively, soluble scFv fragments can be expressed (depending on the strain of *E. coli* used as host). A potential advantage of the above technology is the production of antibodies from non-immunised individuals. This depends on the creation of a vast number of antibody gene repertoires, phage display, and selection using the antigen of interest.

The use of antibody fragments may be advantageous for certain clinical applications, for example their small size should increase tumour penetration. However, their use in the laboratory as reagents can be problematical as they tend to have relatively low functional affinity.

Strategies for reducing immunogenicity

Several approaches have been developed to render antibodies less immunogenic, whilst retaining their antigen-binding properties. In chimeric antibodies, the constant domains of the mouse or rat antibody are substituted with those of human sequence. This also confers effective Fc-mediated properties for *in vivo* use in humans and human-based *in vitro* methods. The constant domains may even be substituted with a non-antibody protein such as a toxin or enzyme. An alternative approach is to humanise the mouse or rat antibody by grafting the mouse CDR into human variable region genes (Fig. 7.4a). These antibodies need to be expressed in

mammalian cells. Amino acid residues may also have to be changed or added to ensure correct conformation of the variable region.

It is now possible to produce monoclonal antibodies that are entirely human sequence with a range of desirable specificities and immunological functions. For this, genes coding for antigen-binding fragments with appropriate specificities, usually derived using phage display, are spliced onto genes coding for appropriate immunoglobulin heavy and light chains. The constructed genes are expressed (normally in mammalian myeloma cells) to provide complete immunoglobulin molecules that are entirely derived from human gene sequences. As for 'conventional' phage display production of Fv fragments, the antigen-binding moieties can be derived from Fv libraries constructed from 'naïve' i.e. non-immunised individuals.

Sequencing monoclonal antibodies

PCR cloning of antibody variable region genes has greatly facilitated elucidation of the encoded amino acid sequences, since it is much easier to sequence the nucleic acid encoding the antibody, and then translate this into protein sequence, than to carry out amino acid sequencing by Edman degradation (see Section 8.4.3) of immunoglobulin protein. The latter method of sequencing requires relatively large amounts of pure antibody preparations and is limited to relatively short stretches of sequence, which necessitates sequencing many overlapping peptides to obtain a complete sequence. Determining the amino acid sequence of antibodies allows their structure to be correlated with their immunological properties, for example antigen-binding properties, and prediction of their three-dimensional shape. The nucleotide sequence also gives information on the use of germline genes in a given antibody response (remember that the antibody-encoding genes are formed from the joining of different germline gene segments during lymphocyte maturation; Section 7.1) and the process of affinity maturation (somatic mutation).

Amplified antibody genes can be sequenced directly; alternatively, to avoid any spurious sequences present in the PCR product, the amplified material can be cloned into a bacteriophage vector and used to transform *E. coli* to provide more reliable and reproducible sequence data. The chain termination method of sequencing is described in Section 5.11.

7.3 PURIFICATION AND FRAGMENTATION OF IMMUNOGLOBULINS

Many immunochemical techniques can be carried out using unpurified antibody in the form of antisera, monoclonal antibody-containing culture supernatant or rodent ascites, for example agar gel methods or immunohistochemistry (Sections 7.4 and 7.8). However, other methods require partial or complete purification of specific antibodies or at least isolation of the total immunoglobulin fraction, for example if they are to be labelled (Section 7.5), immobilised for use in immunoaffinity chromatography (Section 7.3.4), or analysed by isoelectric focusing (Section 10.3.4) or high performance liquid chromatography (HPLC)

(Section 11.3.2). There are a variety of procedures available for purifying immunoglobulins, the optimal method and experimental details depend on the class/subclass, the species in which the antibody was produced, the intended use, and the type of starting material, for example serum in the case of polyclonal antibodies or culture supernatant or ascites in the case of monoclonal antibodies. Methods of immunoglobulin purification from the mixtures of proteins found in serum, culture supernatant and ascites include precipitation techniques that exploit differential solubility characteristics of antibodies and other proteins: ion-exchange chromatography (exploits charge differences between immunoglobulins and other proteins), gel filtration (separates proteins according to size) and affinity chromatography (exploits a specific interaction between antibody and a molecule which it binds – termed the 'ligand' from the Latin *ligare* = to tie or bind). It may be necessary to combine two or more of these procedures to achieve the required purity. However, only affinity chromatography using immobilised antigen is normally capable of purifying antibodies of a single specificity unless the starting material contains a monoclonal antibody.

7.3.1 Precipitation techniques

Certain salts, organic solvents and organic polymers cause immunoglobulin molecules to precipitate from solution to form visible, insoluble aggregates that can be collected by centrifugation, and then resolubilised in an appropriate buffer (see Section 8.3.4). Immunoglobulin molecules in solution are surrounded by a tightly bound hydration shell, and precipitation techniques work by perturbing this hydration layer. Immunoglobulins are soluble within a certain salt concentration range, but become insoluble at both high and low extremes. High concentrations of salts attract the hydration layer away from the protein as the ions become solvated, encouraging hydrophobic areas on the immunoglobulin molcule to interact with similar areas on other molecules causing 'clumping' of molecules. This is called 'salting out' and results in reversible precipitation of the antibody. Multiply charged anions with monovalent cations are most effective at salting out; ammonium and sodium sulphate are most commonly used. However, the solubility of some immunoglobulins also decreases as the salt concentration is lowered because there are insufficient ions to maintain hydration of the immunoglobulin protein. This is called euglobulin precipitation, and is particularly useful for preliminary purification of IgM, although it usually does not work well for IgG. Water-miscible organic solvents can also be used to precipitate immunoglobulins by decreasing the solvating power of water. Ethanol precipitation is used on an industrial scale for fractionation of immunoglobulin from other plasma proteins. Polyethylene glycol is a high molecular weight, water-soluble organic polymer that can be used to separate immunoglobulins from other plasma proteins by precipitation, in a similar way to organic solvents.

Precipitation of immunoglobulin tends to be most effective at its isoelectric point (the pH at which the immunoglobulin has no net charge), since electrostatic

repulsion between molecules is minimised. Precipitation techniques are cheap and easy to carry out but are often used only as a preliminary step in multistep purification schedules as the product is usually not sufficiently pure.

7.3.2 Gel filtration

Gel filtration separates molecules according to size (Section 11.7). Since IgM is considerably larger than other immunoglobulin subclasses and most other serum proteins, conventional or HPLC gel filtration is commonly used for IgM purification. Although gel filtration alone is not very good for IgG purification, it can be used in combination with other methods such as ion-exchange chromatography for IgG purification.

7.3.3 Ion-exchange chromatography

Conventional or HPLC or fast protein liquid chromatography (FPLC) ion-exchange chromatography systems make use of the surface charge of immunoglobulins to separate them from other components (Section 11.7). At neutral pH, most immunoglobulins are negatively charged and will bind to positively charged anion-exchange matrices. Increasing the salt concentration or changing the pH of the buffer will then elute the immunoglobulin from the matrix (Fig. 7.5).

7.3.4 Affinity chromatography

Affinity chromatography exploits a specific but reversible interaction between a ligand that is covalently attached to an inert support and the antibody to be purified (Section 11.8). The ligand may be, for example, an antigen for purification of antibodies of a particular specificity, an anti-immunoglobulin antibody for purification of antibodies from a particular species, class or subclass, or an immunoglobulin-binding protein: several strains of bacteria produce such proteins that bind immunoglobulin with high affinity (Table 7.5). The most commonly used bacterial immunoglobulin-binding proteins are called protein A and protein G; they bind the constant region of IgG (depending on species and subclass). A light-chain-binding protein has also been described (protein L), but this binds only some light chain types. Affinity chromatography using immobilised protein A or G is often the method of choice for purification of IgG monoclonal antibodies from culture supernatants. The inert support can be agarose, sepharose, polyacrylamide, polystyrene or high-pressure stable acrylic polymer for use in HPLC. Alternatively, ligand can be attached to magnetic beads, which can then be isolated using a magnetic attractor. Many different immobilised ligands are available commercially; alternatively, ligands can be coupled, via primary amine, carboxyl or thiol groups, to activated supports in the laboratory (for examples, see Fig. 7.6). When the solution containing the antibody is brought into contact with the immobilised ligand, the antibody binds to the ligand and is thus also immobilised (Fig. 7.7). The

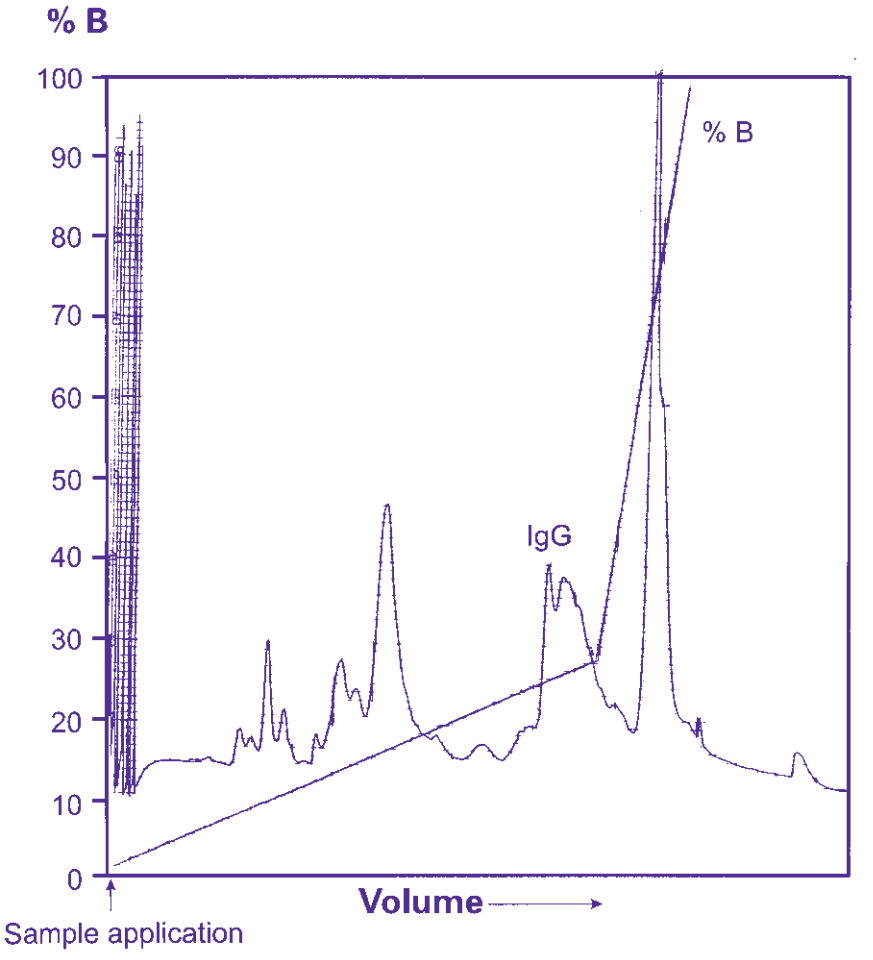


Fig. 7.5. Chromatograph showing purification of mouse IgG from ascites using fast protein liquid chromatography ion-exchange chromatography. A preliminary IgG purification step was carried out using ammonium sulphate precipitation from the ascites (Section 7.3.1). The precipitate was redissolved and equilibrated in 20 mM triethanolamine pH 7.7 (A). The anion-exchanger was also equilibrated in A. Following application of the sample, a salt gradient formed by using increasing amounts of B (A + 1 M NaCl) was used to elute the immunoglobulin. The peak corresponding to IgG is indicated.

non-bound material can then be washed away. The specific interaction between antibody and ligand is then disrupted to elute the antibody (see below), whilst the ligand remains immobilised. Conditions for dissociating the specific ligand–antibody interaction commonly disrupt the electrostatic interactions and/or hydrophobic bonding (due to van der Waals' interactions) involved in antibody–ligand binding (Table 7.6).

Lectins are glycoproteins of non-immune origin, isolated from plants and animals, which bind specific carbohydrates such as galactose or fucose. For

Table 7.5 Ligands for affinity chromatography

Ligand type	Example(s)	Antibody purification
Hapten	DNP	Antibodies that bind hapten
Antigen	Haemoglobin, factor VIII	Antibodies of a single specificity
Bacterial immunoglobulin binding protein	Protein A, protein G Protein L	Most IgG subclasses from many species Some κ chains from many species
Anti-immunoglobulin antibodies	Goat anti-human IgG antibodies	Class and/or species-specific immunoglobulin fraction
Lectins	Jacalin Mannan-binding protein	Human IgA Mouse IgM

DNP, dinitrophenol.

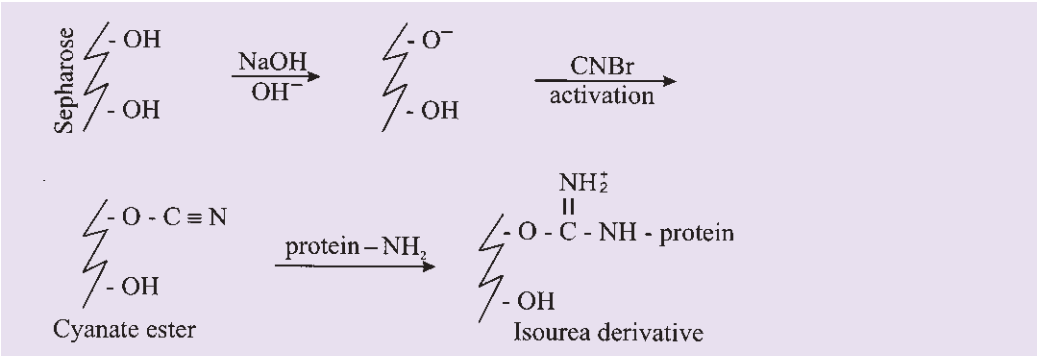
example, mannan-binding protein (MBP) is a mannose- and *N*-acetylglucosamine-binding lectin found in mammalian sera. When coupled to an inert support, it can be used to purify mouse IgM, which contains 12–16% mannose-containing carbohydrate. Jacalin is a galactose-binding lectin that can be used to purify human IgA1.

Affinity chromatography techniques using immobilised antibodies are widely used to purify antigens.

7.3.5 Fragmentation and dissociation of immunoglobulins

Some applications of antibodies require the use of antibody fragments rather than the intact molecule (see Section 7.1.2). For example, removing the antibody Fc portion will prevent binding of antibodies to Fc receptors present on leukocytes and other cells. This may be necessary if cell surface antigens recognised by the antibody are to be evaluated by, for example, immunofluorescence microscopy. The preparation of antibody fragments normally involves the use of proteolytic enzymes to cleave peptide bonds. Different enzymes can be used to cleave the heavy chain in specific places to give rise to different fragments. The most commonly used fragments are those produced by papain, pepsin and plasmin; their cleavage sites on IgG are shown in Fig. 7.1, with fragment nomenclature. These enzymes are available commercially either in soluble form or covalently coupled to Sepharose (an inert support), which facilitates removal of the enzyme when digestion is completed (the Sepharose and immobilised enzyme can be removed simply by centrifugation). Antibodies can also be dissociated into their constitutive chains. The heavy and light chains are joined by disulphide bridges that can be easily broken by a reducing agent such as dithiothreitol under conditions that

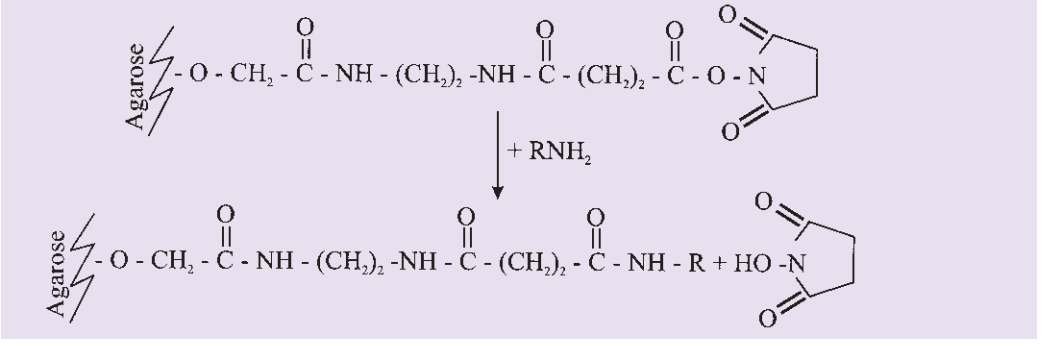
(a) Mechanism of activation of Sepharose by CNBr to allow subsequent coupling of proteins via amine groups



At high pH, CNBr reacts with hydroxyl groups on the Sepharose to form cyanate esters. These react with the amine groups of proteins to form covalent linkages.

(b) Mechanism of ligand immobilisation via commercially available *N*-hydroxy succinamide ester coupling

(i) Affi-Gel 10 (used for basic protein coupling)



(ii) Affi-Gel 15 (used for acidic protein coupling)

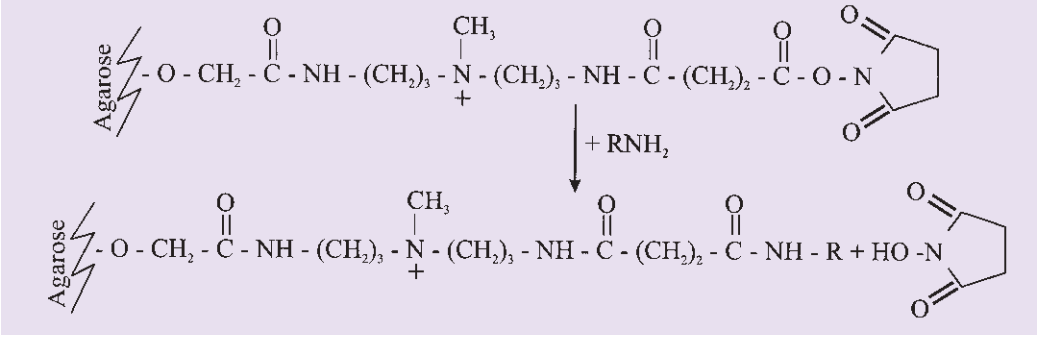


Fig. 7.6. Coupling of ligands to supports to prepare affinity columns.

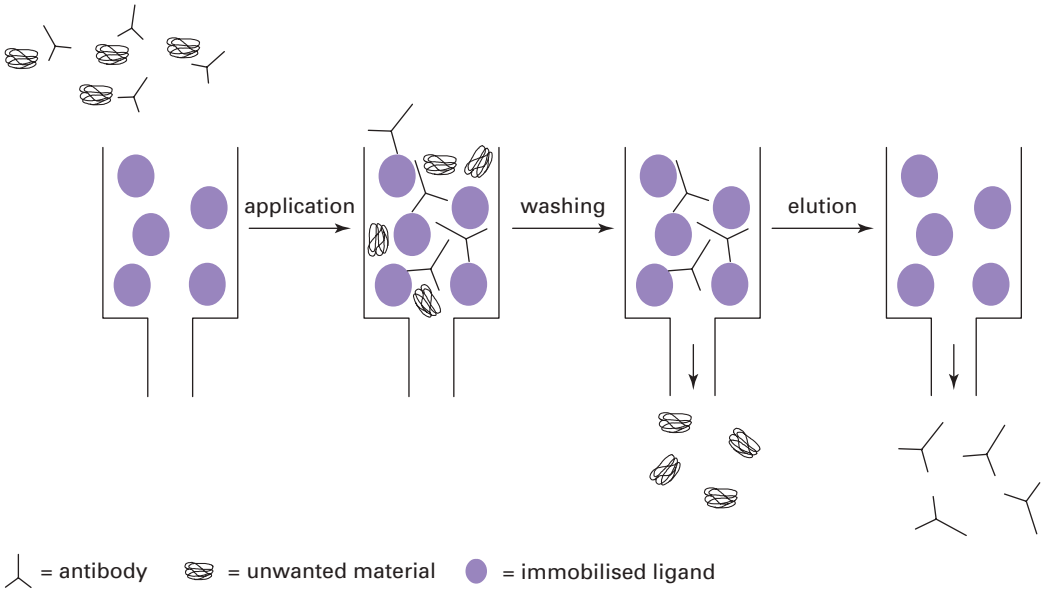


Fig. 7.7. Diagrammatic representation of antibody purification by affinity chromatography.

Table 7.6 Conditions for elution of antibodies from affinity columns

Elution conditions	Mode of action
Glycine-HC1, pH 2.2–2.8 1 M propionic acid 0.05 M diethylamine, pH 11.5 1 M ammonia, pH 11.0	Change conformation and disrupt electrostatic interactions
2–8 M urea 5–6 M guanidine hydrochloride	Strongly denaturing
3.5 M sodium thiocyanate 4 M potassium thiocyanate 2–5 M $MgCl_2$, KI, NaI	Chaotropic agents
50% ethylene glycol, pH 11.5 10% (v/v) dioxane at acid pH	Disrupt hydrophobic interactions

leave the intrachain disulphide bonds intact. An alkylating agent such as iodoacetamide can then be used to ensure that the bonds do not reform. However, the heavy and light chains will still associate non-covalently unless a dissociating agent such as propionic acid is added. The chains can be separated on the basis of differing size by gel filtration (Fig. 7.8). Isolated immunoglobulin chains usually bind antigen less avidly than do the intact molecule and fragments such as Fab and $F(ab')_2$.

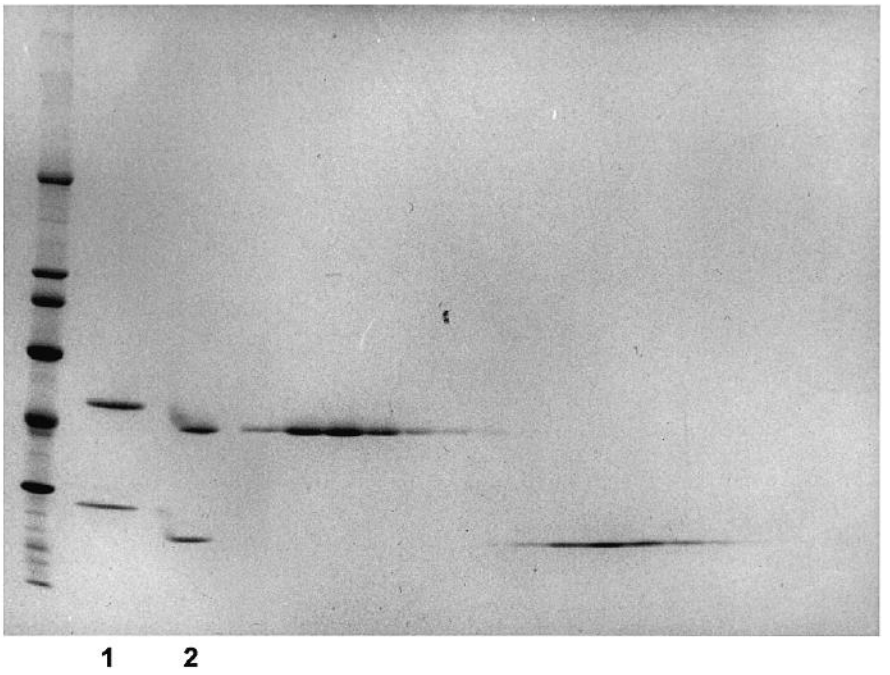


Fig. 7.8. Analysis of separated heavy and light chains of IgG using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). IgG was reduced and alkylated and the heavy and light chains were separated by gel filtration in propionic acid. The fractions were analysed by SDS–PAGE under non-reducing conditions. Lanes 1 and 2 show the reduced and alkylated IgG before gel filtration, analysed under reducing and non-reducing conditions, respectively. Note the apparent increase in size of the chains (lane 1) when analysed under conditions that reduce the intra-chain disulphide bonds. Molecular weight markers are on the left.

7.4 IMMUNOPRECIPITATION

An important property of many antibodies is their ability to precipitate antigens from solution. Antibodies are divalent (IgG) or multivalent (IgM), and if the antigen is also multivalent, the antibody–antigen interactions give rise to a molecular lattice that is too large to remain in solution, so precipitation occurs. The formation of an insoluble antibody–antigen complex is very dependent on antibody and antigen concentrations, and occurs within a narrow concentration range known as the zone of equivalence. This represents the conditions under which macromolecular antigen/antibody complexes are formed that are sufficiently large to be precipitated. Outside the equivalence concentration, conditions known as antigen or antibody excess occur, which result in the formation of small, soluble complexes (see Fig. 7.9). However, precipitation never occurs with some monoclonal antibodies that recognise a single epitope on an antigen (i.e. a monovalent antigen) because a lattice is not formed.

Immunoprecipitation can be exploited in both agar techniques and in solution.

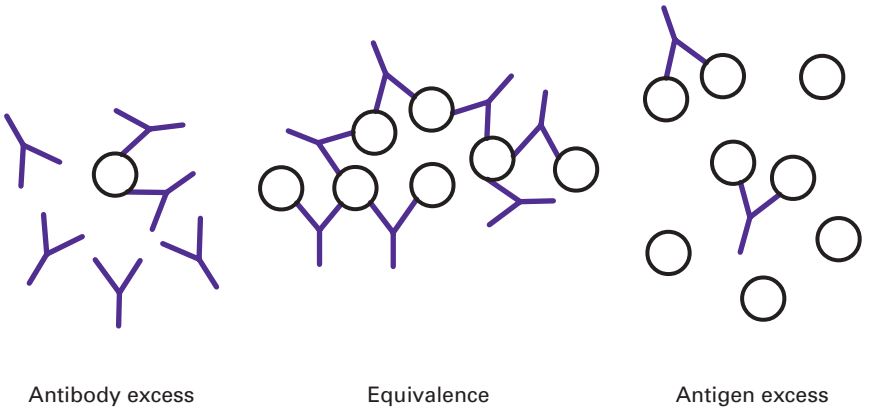


Fig. 7.9. Diagrammatic representation of immune complexes formed at varying antigen : antibody ratios. Immunoprecipitation occurs only when an insoluble antigen–antibody lattice is formed at the equivalence point.

7.4.1 Agar and agarose immunoprecipitation

Agar is a high molecular weight polysaccharide derived from seaweed; agarose is a purified linear galactan hydrocolloid isolated from the same substance. Both dissolve in aqueous solutions upon heating and, upon cooling, form gels with a large pore size that allow most proteins, including antibodies, to diffuse through. If antibody migrating through the gel encounters antigen, an insoluble precipitate is commonly formed at equivalence. The precipitate can often be visualised as an opaque line in the gel (precipitin line); the use of a protein stain such as Coomassie Brilliant Blue allows visualisation of weak (invisible) precipitin lines. Protein antigens that are insoluble in physiological buffers (e.g. membrane or cytoskeletal proteins) can be solubilised in non-ionic detergents for analysis in detergent-containing gels without adversely affecting the formation of the precipitin line.

Precipitation techniques in agar are typically carried out using gels 2–3 mm thick, cast on glass (e.g. microscope) slides (warm agar solution is simply poured onto the slide and allowed to set). Wells are then cut into the gel using, for example, a large-bore pipette. In diffusion techniques, antibody and/or antigen migrate through the gel by simple diffusion. In double diffusion, separate wells cut in the agar are filled with antibody and antigen, respectively (Fig. 7.10). Both diffuse into and through the agar, automatically forming concentration gradients. Provided these cover the equivalence concentrations, a precipitin line is formed at the equivalence point. The technique can be used to give information on antigenic (and hence structural) similarities or differences between antigens (Fig. 7.11).

In single radial immunodiffusion (SRID), antigen is loaded into wells cut in an agar gel containing a fixed concentration of antibody (Fig. 7.10). A precipitin ring around the well is formed at equivalence. The diameter of the ring at equivalence is related to the antigen concentration. Two relationships have been determined:

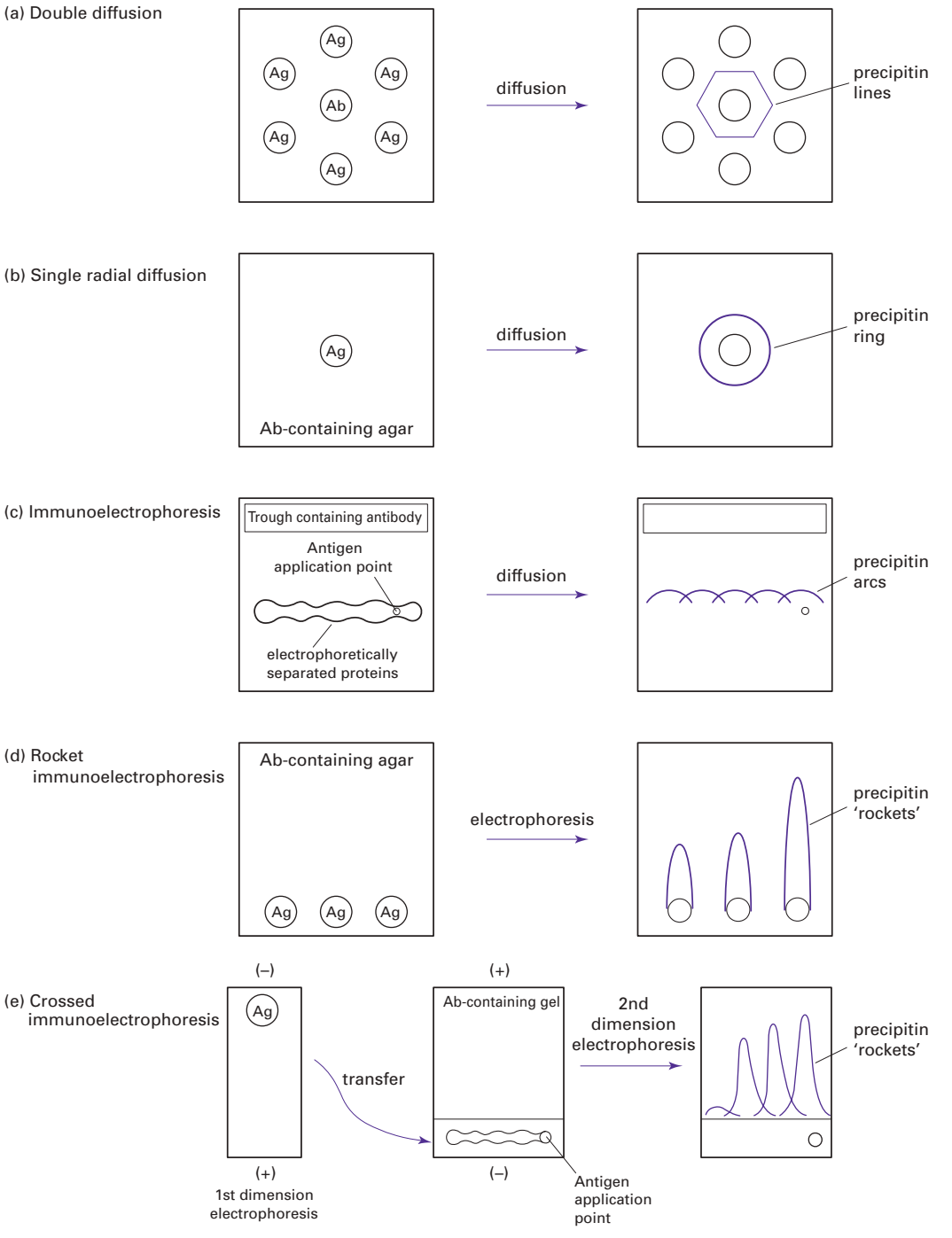


Fig. 7.10. Diagrammatic representation of immunoprecipitation techniques in agar. For details, see the text. Ag, antigen; Ab, antibody.

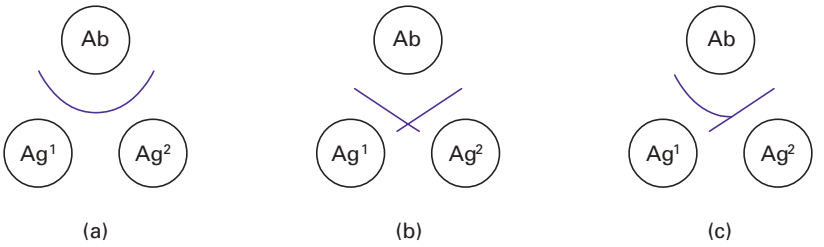


Fig. 7.11. Interpretation of precipitin lines following double immunodiffusion. Fusion of immunoprecipitin lines (a) infers immunochemical identity of antigens (Ag) 1 and 2, whereas crossing of the lines (b) shows their non-identity. Partial fusion or spur formation (c) suggests partial identity, i.e. antigen 2 has some determinants that are not shared by antigen 1, but all the determinants recognised by these antibodies (Ab) that are present on antigen 1 are also present on antigen 2.

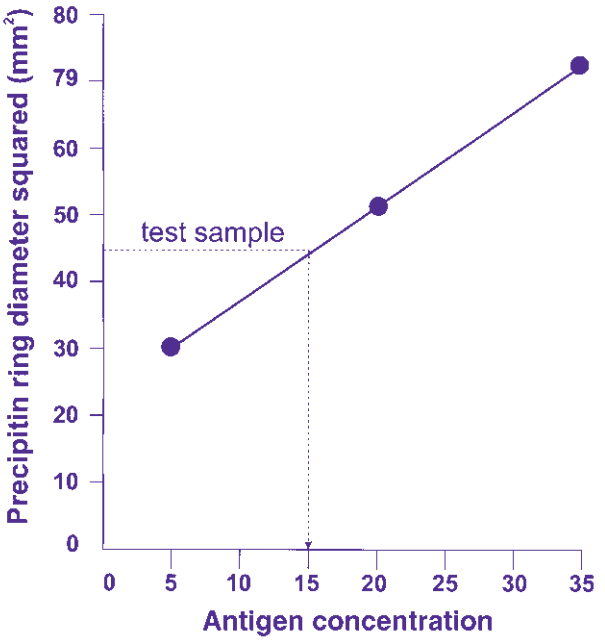


Fig. 7.12. The use of single radial immunodiffusion to measure antigen concentrations in test samples. The standard curve was constructed from the square of the precipitin ring diameters given by calibrator samples containing known amounts of antigen. The square of the precipitin ring diameters of the test samples can then be read from the standard curve to give the antigen concentration in the samples.

(i) the square of the ring diameter is proportional to the antigen concentration (Mancini method; Fig. 7.12); and (ii) the diameter of the ring is proportional to the log of the antigen concentration (Fahey and McKelvey method). This technique is useful for determining concentrations of antigens such as serum proteins. For example, if the agar contains specific antibodies against human IgG, and wells cut into the gel are loaded with test samples containing unknown quantities of

human IgG (e.g. serum samples) together with calibrator samples containing known concentrations of human IgG, precipitin rings will form around the wells at equivalence. By measuring the ring diameters produced by the calibrator samples of known IgG concentration, a standard curve can be constructed by plotting the square of the diameter of the precipitin ring against the IgG concentration. The square of the ring diameters of the test samples can then be read off the standard curve to give the IgG concentration in the test samples (see Fig. 7.12).

In immunoelectrophoresis, a mixture of proteins containing the antigen are first separated by agar gel electrophoresis. Antibody is then allowed to diffuse through the gel from a trough cut in the gel parallel to the direction of electrophoresis (Fig. 7.10). Precipitin arcs are then formed where antibody meets antigen at equivalence. A disadvantage of this technique is the relatively poor resolution of antigen mixtures using agar gel electrophoresis, but it can be useful for detecting precipitating antibodies. Clinically, the technique is carried out on samples of patient's serum, concentrated urine or spinal fluid to detect abnormalities in concentrations of antigens and/or the presence of abnormal proteins relative to normal control samples analysed at the same time.

Rocket immunoelectrophoresis is an adaption of single radial immunodiffusion and involves electrophoretic migration of antigen from wells cut in antibody-containing gel (Fig. 7.10). At equivalence, rocket-shaped precipitin lines are formed, the area under which is proportional to the antigen concentration. The technique can be used to determine antigen concentrations in unknown samples (e.g. serum protein levels) by reference to a standard curve as in RID, or to investigate immunochemical relationships between different samples if these are placed in adjacent wells close together (see Fig. 7.11 for interpretation of precipitin line patterns).

In crossed immunoelectrophoresis, proteins are first separated by agar gel electrophoresis, after which they are electrophoresed into an antibody-containing gel at right angles to the direction of the first electrophoresis (Fig. 7.10). The technique can be used for analysis of serum proteins.

The sensitivity of precipitation techniques in agar varies enormously depending on the antibodies and antigens involved.

7.4.2 Immunoprecipitation in solution

An antibody can be used specifically to immunoprecipitate its antigen from a mixture of proteins in solution, for example a cell lysate. If the immunoprecipitate is insoluble, it can be sedimented by centrifugation for analysis; soluble antibody–antigen complexes can be isolated by precipitation with an immunoglobulin-binding protein such as protein A or G, or an anti-immunoglobulin antibody, or those reagents covalently bound to an insoluble support such as agarose (see Section 7.3.4). This basic method forms the basis of classical radioimmunoassays (RIA) (see Section 7.7.1). It also allows the isolation of an unknown antigen from a mixture of proteins. For the latter, it may be necessary to label the mixture of

Example 1 SINGLE RADIAL DIFFUSION FOR ESTIMATING THE CONCENTRATION OF ANTIGEN

Question

The precipitin ring diameters of an antigen calibrator at three different concentrations were:

Concentration ($\mu g\ cm^{-3}$)	Ring diameter (mm)	Ring diameter squared (mm^2)
10	4	16
60	7	49
100	8.7	76

The ring diameter of the sample of unknown antigen concentration was 6 mm. What was the concentration of the antigen in the unknown?

Answer

To answer this question, a graph of the type shown in Fig. 7.12 must be plotted. The graph of the precipitin ring diameter squared (y-axis) against the antigen concentration (x-axis) is a straight line. Given that the unknown sample gave a ring diameter of 6 mm (i.e. 36 mm^2), the antigen concentration in the sample can be read off the calibration graph and seen to be 40 $\mu g\ cm^{-3}$.

proteins, for example cell lysate, with ^{125}I prior to immunoprecipitation. The immunoprecipitate can then be analysed by SDS–PAGE (Section 10.3.1) and autoradiography (Section 14.2.3) to give information on the antigen. Alternatively, non-radiolabelled immunoprecipitate can be analysed by SDS–PAGE and immunoblotting with antibodies of known specificity (Section 7.6), which can allow positive identification of immunoprecipitated proteins. Immunoprecipitation is commonly carried out on radiolabelled intact cells or cell membranes to give information on cell surface antigens (Fig. 7.13).

Although an antibody can be used to analyse individual proteins in a mixture separated by SDS–PAGE using immunoblotting procedures (Section 7.6), immunoprecipitation in solution has the advantage that the antibody is allowed to react with native rather than partially denatured antigen as is the case in immunoblots. Some antigens lose their immunoreactivity following electrophoresis and immunoblotting, or even (especially for cell surface antigens) solubilisation. This occurs when epitopes are conformation dependent or arise through the interaction of several protein subunits/components.

7.5 LABELLING ANTIBODIES

The specificity of antibodies makes them powerful analytical tools. Although immunoprecipitation techniques in agar (see Section 7.4) result in visible precipitated antibody–antigen complexes, in most immunochemical assays binding of antibody to antigen can be visualised only by labelling the antibody (or

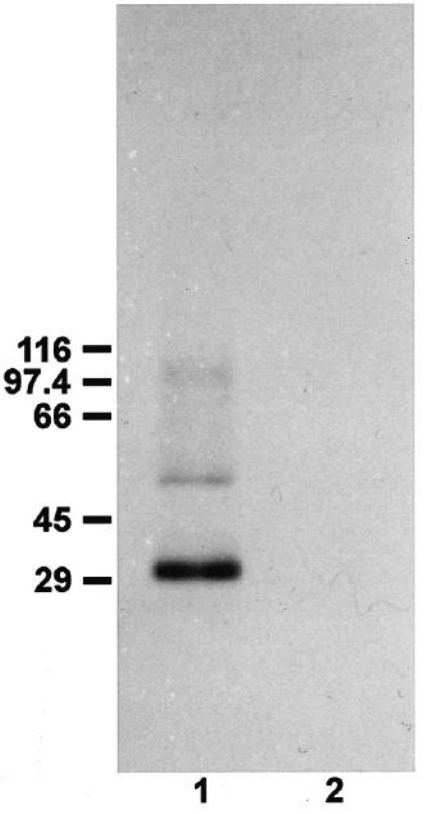


Fig. 7.13. Autoradiograph of monoclonal anti-Rh D immune precipitates from ^{125}I-labelled Rh D-positive and Rh D-negative erythrocyte membranes after analysis by SDS–PAGE. Human erythrocytes positive or negative for the Rh D blood group antigen were surface labelled with^{125} I. The cells were lysed by hypotonic shock and the membranes incubated with monoclonal anti-Rh D. After being washed to remove unbound antibody, the membranes were solubilised and the antibody–antigen complexes isolated using protein A–Sepharose. The antibody–antigen complexes were then analysed by SDS–PAGE and autoradiography. The molecular weight of the major protein immunoprecipitated from Rh D-positive membranes is approximately 31 000 (lane 1) and corresponds to the Rh D polypeptide. No protein was immunoprecipitated from the Rh D-negative membranes (lane 2). The relative positions of the molecular weight markers are shown ($M_r \times 10^{-3}$).

sometimes antigen) or (more commonly) an antibody against immunoglobulin (see Section 7.5.1) with a marker that can be qualitatively and sometimes quantitatively detected. Thus an antibody can be labelled with a radioactive isotope for use in radioimmunoassays, or an enzyme that gives a coloured product for use in enzyme-linked immunosorbent assay (ELISA), or a fluorochrome that emits visible fluorescence for use in immunohistochemistry (Table 7.7). Binding of unlabelled antibody to antigen in these techniques would be undetectable. Antibodies labelled with fluorochromes or enzymes are commonly referred to as conjugates.

Table 7.7 Common antibody labels for immunochemical techniques

Label	Examples	Main use(s)
Fluorochromes	Fluorescein	Immunohisto/cytochemistry; flow cytofluorimetry; fluorimetric assays
	Rhodamine	Immunohisto/cytochemistry; flow cyotfluorimetry
	Phycoerythrin	Flow cytofluorimetry
	Texas Red	Flow cytofluorimetry
	7-Amino-4-methylcoumarin 3-acetate (AMCA)	Flow cytofluorimetry
	[a] BODIPY derivatives	Flow cytofluorimetry
	[a] Cascade Blue	Flow cytofluorimetry
Enzymes	AP	Immunohistochemistry; EIA; immunoblotting
	β-Galactosidase	As above
	HRP	As above; immunoelectron microscopy
	Glucose oxidase	Immunohistochemistry
	Urease	EIA
Radioisotope	^{125}I	Competitive and non-competitive RIA
Electron dense	Gold	Immunoelectron microscopy
	Ferritin	As above

BODIPY, 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene; AP, alkaline phosphatase; EIA, enzyme immunometric assay; HRP, horseradish peroxidase.
[a] Trademark of Molecular Probes Inc.

7.5.1 Direct and indirect immunochemical procedures

In simple, direct immunochemical procedures, the antibody against the antigen of interest (the 'primary' antibody) is conjugated with the label. In the more commonly used indirect procedures, binding of unlabelled primary antibody to the antigen is detected using a labelled antibody against immunoglobulin (or less commonly, a labelled bacterial immunoglobulin-binding protein such as protein A or protein G). This secondary antibody is usually raised against the immunoglobulin from the animal species in which the primary antibody was produced, and may also be class or subclass specific. Direct and indirect procedures are illustrated diagrammatically in Fig. 7.14. Indirect methods utilising labelled anti-immunoglobulin antibodies have several advantages over direct procedures: they are more sensitive, since several labelled anti-immunoglobulin molecules can bind to each unlabelled primary antibody, resulting in a stronger signal; each primary antibody does not have to be labelled individually as, for example, labelled rabbit anti-mouse IgG antibodies will recognise all mouse IgG monoclonal antibodies; and there is no risk of loss of reactivity of the primary antibody as a result of direct labelling. However,

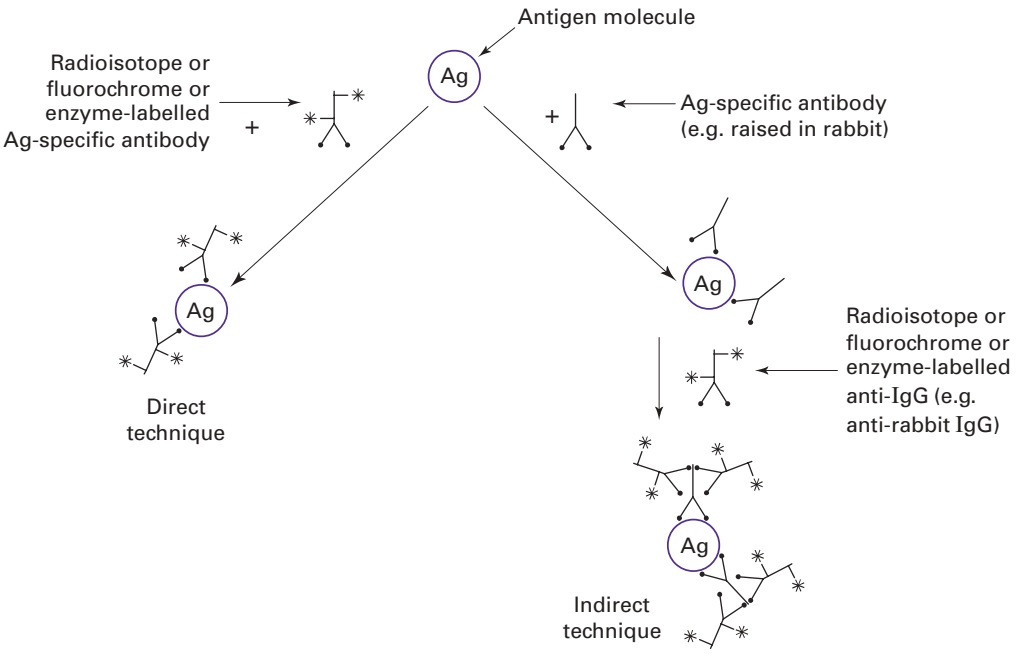


Fig. 7.14. Diagrammatic representation of direct and indirect immunochemical methodology. Ag, antigen.

there are cases that necessitate the use of directly labelled antibodies (see Sections 7.8.3 and 7.8.5). A semi-direct procedure takes advantage of the high-affinity specific interaction between biotin and avidin/streptavidin (see Section 7.5.5) and involves the use of biotinylated primary antibody and labelled avidin/streptavidin preparations.

7.5.2 **Radiolabelling**

Radioiodination

The most commonly used radioisotope for radiolabelling antibodies is ^{125}I. This isotope is readily available as $Na^{125}I$, is relatively inexpensive and has a high specific activity. It emits γ-radiation, which can be easily and directly detected and quantified using a γ-counter, and is good for autoradiography. However, suitable precautions must be taken to minimise exposure of workers to radiation and to prevent contact, contamination and ingestion (the thyroid is particularly susceptible). Covalent labelling of proteins directly with ^{125}I involves oxidative generation of cationic iodine (I^+) and its spontaneous electrophilic addition to tyrosine residues, and, to a lesser extent, to tryptophan and histidine residues. The major substitution is a monoiodotyrosyl residue, although di-iodotyrosyl residues may also be formed (Fig. 7.15). The concentrations of reagents should allow for only one or two tyrosine residues per antibody

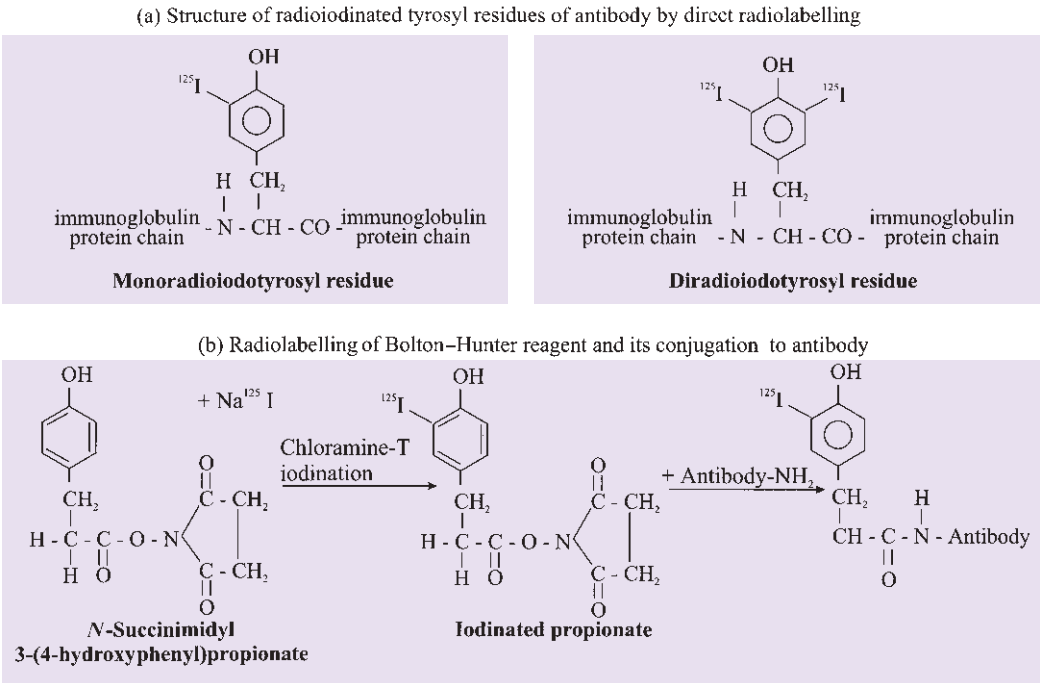


Fig. 7.15. Direct and indirect radiolabelling of antibodies with ^{125}I.

molecule to be ^{125}I-labelled otherwise loss of immunoreactivity and/or radiation damage may occur.

There are several established methods for radioiodination, which differ in the choice of oxidising agent used to generate I^+. Since strong oxidising agents can destroy the immunoreactivity of antibodies, there must be a compromise between efficient generation of I^+ and preservation of antigen-binding capacity.

Chloramine T (*N*-chloro-*p*-toluene sulphonamide) is an aromatic oxidising agent commonly used for high specific activity iodination of antibodies and other proteins. Chloramine T iodination is very rapid (45 s), but it can denature antibodies and lead to loss of antigen-binding capacity. The reaction must be stopped quickly after appropriate iodination, by adding a reducing agent such as sodium metabisulphite or an excess of tyrosine to 'mop up' iodine as iodotyrosine. Following radioiodination, the antibody needs to be separated from free (i.e. non-antibody bound) ^{125}I. This can be achieved either by gel filtration, or if IgG antibody has been radiolabelled, a strongly basic anion exchanger can be used to adsorb iodotyrosine. The use of chloramine T immobilised on beads offers a milder alternative, since the reaction can be stopped simply by removing the solid material.

Another oxidising agent commonly used for radioiodination is iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril), which is milder than chloramine T. Iodogen is insoluble in aqueous solvents, so its use necessitates dissolving it in

chloroform or benzene, usually in a plastic tube, and then allowing the organic solvent to evaporate and leave the iodogen coated on the side of the tube. The radioiodination procedure is then carried out in the iodogen-coated tube and stopped by taking the solution out of the tube.

Radioiodination of antibodies using the enzyme lactoperoxidase is a very mild procedure that does minimal damage to antibodies, but does not produce radiolabelled antibodies of high specific activity. Lactoperoxidase-catalysed iodination is therefore usually used only for iodinating cell surface components, since it minimises diffusion of reactants across the cell membrane to label internal components.

Antibodies can also be conjugated with low molecular weight, previously radioiodinated phenolic compounds. Commonly, *N*-succinimidyl 3-(4-hydroxyphenyl) propionate (the Bolton–Hunter reagent) is radioiodinated using chloramine T and the 5-[^{125}I]iodophenyl derivative is then conjugated to amino groups of the antibody (Fig. 7.15). The advantage of indirectly radiolabelling antibodies in this way is that there is no risk of oxidative damage, but, as with direct labelling, only one or two residues per antibody molecule should be conjugated with the iodophenyl derivative.

7.5.3 Labelling with fluorochromes

Fluorochromes emit fluorescent light under ultraviolet illumination. The fluorescence of fluorochrome-labelled antibody in solution can be quantified using a fluorimeter. However, the greatest use of fluorochrome-labelled antibodies is in immunohisto/cytochemistry where binding of fluorochrome-labelled antibodies to tissue sections or cells is visualised using a microscope equipped with fluorescence optics (Section 7.8.3). Antibodies labelled with fluorochromes are used extensively in flow cytometric techniques (Section 7.8.5).

Many fluorochromes are available with different excitation and emission spectra, but the ones most commonly used for microscopy are fluorescein, which emits green fluorescence, and tetramethylrhodamine, which emits red fluorescence. For conjugation, fluorescein and tetramethylrhodamine isothiocyanate are usually used, they readily form covalent linkages with primary amine groups on lysine residues in the antibody molecule (Fig. 7.16), although iodoacetamido derivatives of fluorescein and rhodamine are also available for coupling via sulphydryl groups. Other fluorochromes, mostly used in flow cytofluorimetry, and which can be coupled to amine (or sulphydryl groups) include Texas Red (sulphorhodamine; Fig. 7.16), 7-amino-4-methylcoumarin 3-acetate (AMCA; fluoresces blue) and BODIPY (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene).

Phycobiliproteins are a group of intensely fluorescent proteins found in algae and cyanobacteria and widely used in flow cytofluorimetry. They include B- and R-phycoerythrin (M_r 240 000), C-phycocyanin (M_r 72 000) and allophycocyanin (M_r 110 000). Phycobiliproteins can be attached via their amine groups to the thiol groups of the antibody, using chemical heterobifunctional cross-linking agents (Section 7.5.4).

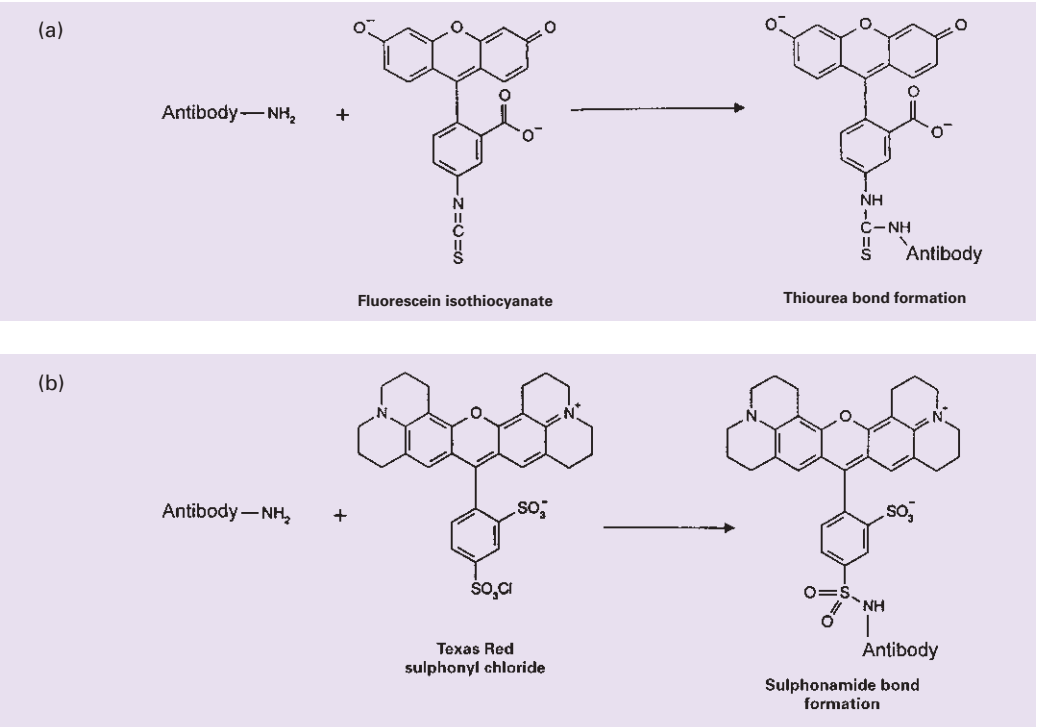


Fig. 7.16. Conjugation of (a) fluorescein isothiocyanate and (b) Texas Red (sulphorhodamine) to amine groups of antibody.

7.5.4 Labelling with enzymes

Enzyme-labelled antibodies are widely used in immunoassays (e.g. ELISA), immunoblotting and immunohisto/cytochemistry. In each case, direct or indirect binding (Section 7.5.1) of the enzyme-labelled antibody to antigen (which may be in tissue sections or on blots or on the wells of a microtitre plate) is visualised by carrying out the enzyme reaction in which a colourless substrate is converted to a coloured product. In enzyme immunoassays, the product needs to be soluble to allow spectrophotometric quantification; in immunoblotting and immunohistochemical procedures, the product must be insoluble to allow precise localisation of the initial antigen–antibody interaction and visible either directly by eye or using microscopy. The use of enzyme-labelled antibodies allows catalytic amplification of the signal, since each enzyme molecule can convert many substrate molecules into coloured product. Properties of enzymes commonly used for conjugation are listed in Table 7.8. Since enzymes are proteins, they have to be conjugated to antibody using chemical cross-linking reagents. There are two types of cross-linker: homobifunctional reagents, which react with the same chemical group on both the enzyme and the antibody, and heterobifunctional reagents, which react with different chemical groups on each protein.

Table 7.8 **Properties of enzymes used for conjugation to antibodies**

Enzyme	Source	Structure	Reaction catalysed
Peroxidase	Horseradish	Monomeric glycoprotein M_r 40 000	H_2O_2 + oxidisable substrate → oxidised product + $2H_2O$
Alkaline phosphatase	Calf intestine (usually)	Zn^{2+}-containing glycoprotein	R-O-P + H_2O → R-OH + P_i orthophosphoric monoester substrate; alcohol; inorganic phosphate
β-Galactosidase	*E. coli*	Multimeric protein (4 subunits) M_r 540 000	β-D-Galactoside + H_2O → galactose + alcohol
Glucose oxidase	*Aspergillus niger*	Flavo-glycoprotein	β-D-Glucose + O_2 → H_2O_2 + gluconic acid
Urease	Jack bean	M_r 480 000	$(NH_2)_2CO + 3H_2O \rightarrow CO_2 + 2NH_4OH$

Glutaraldehyde is a simple homobifunctional cross-linker that cross-links the amine groups (e.g. of lysine; Fig. 7.17) of proteins. Conjugation can be carried out either in a one-step procedure in which the glutaraldehyde is added to a mixture of enzyme and protein (works for horseradish peroxidase (HRP), alkaline phosphatase and β-galactosidase), or, for HRP conjugation, in a two-step procedure in which glutaraldehyde is first reacted with enzyme, and the glutaraldehyde-coupled enzyme is then reacted with antibody. There is a wide range of heterobifunctional reagents available, consisting typically of an amine-reactive group and a thiol-reactive group separated by a spacer arm, which cross-links the amine groups of antibodies to the sulphydryl groups of enzymes. An example is succinimidyl-4-(*N*-maleimidomethyl)cyclohexane 1-carboxylate (SMCC; Fig. 7.17). Another method of conjugation involves the use of periodate to generate active aldehyde groups by cleavage of carbohydrate chains of glycoprotein enzymes. These groups then react with primary amine groups in the antibody to form Schiff bases, which are then reduced to produce stable bonds.

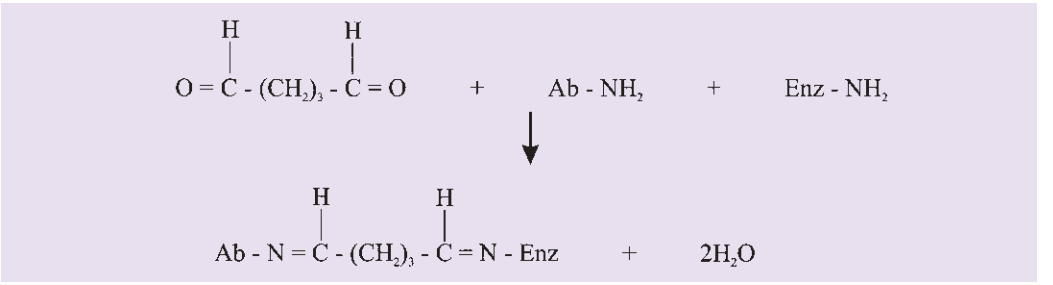
7.5.5 Biotinylation of antibodies

The very high affinity interaction (affinity constant $> 10^{15}$ M^{-1}) between biotin (vitamin H; M_r 244) and avidin (a protein from egg white) or streptavidin (a protein from the bacterium *Streptomyces avidinii*) can be exploited in immunochemical techniques by conjugating biotin to antibodies for use with fluorochrome- or enzyme-conjugated or radiolabelled avidin/streptavidin (Fig. 7.18). Antibodies can be conjugated easily with biotin derivatives, most commonly *N*-hydroxysuccinimidobiotin or analogues incorporating a spacer arm. The latter reduces steric hindrance by increasing the distance between the biotin and the antibody. Most of these biotin derivatives react with primary amine groups, although there are some that are reactive with thiol groups or carbohydrate residues on the immunoglobulin.

7.6 IMMUNOBLOTTING

In many cases it is informative to establish the specificity of antibodies by investigating their ability to recognise components present in complex mixtures. Specific antibodies can also be used to identify such components and establish cross-reactivities that may occur with immunochemically related molecular species. For such methods it is clearly necessary to separate the antigenic components using an analytical method. In theory, any biochemical technique can be used for this, but the high resolving power of polyacrylamide gel electrophoretic methods (especially SDS–PAGE and isoelectric focusing; see Sections 10.3.1 and 10.3.4) makes these ideal for such purposes. The most commonly used technique involves transferring separated proteins from polyacrylamide gels to a porous membrane and probing this 'blot' with antibody (antibody can be applied directly to the gel, but the very limited permeability of polyacrylamide gel makes this inefficient and time consuming). Antibody–antigen complexes are then detected either by the use of labelled anti-immunoglobulin reagent or by labelling the antibody directly

(a) Glutaraldehyde is a homobifunctional reagent which cross-links amino groups



(b) SMCC is a heterobifunctional reagent which cross-links amino to sulphydryl groups

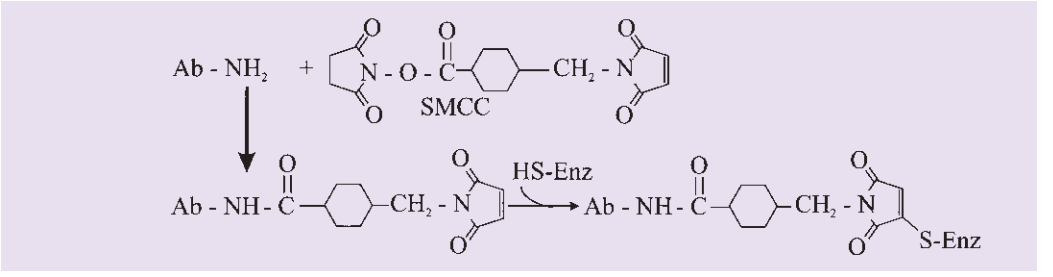


Fig. 7.17. (a and b) Structure and mode of action of cross-linking reagents. Ab, antibody; Enz, enzyme.

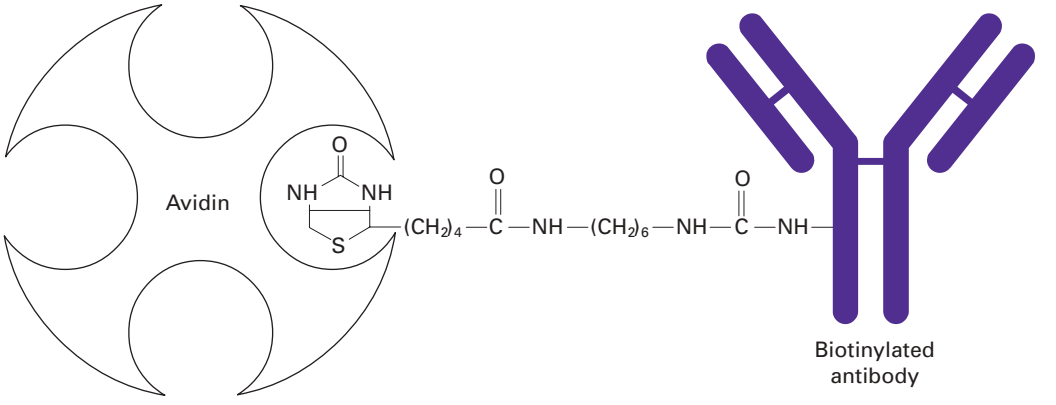


Fig. 7.18. The interaction of biotin-conjugated IgG with avidin. The binding is very strong and each avidin molecule has four biotin binding sites. For the structure shown, coupling of biotin to immunoglobulin has been carried out using biotinamidocaproate *N*-hydroxysuccinimide ester.

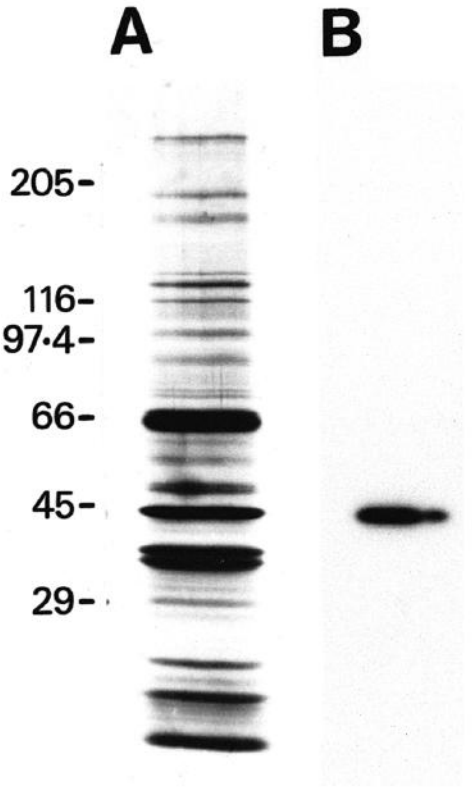


Fig. 7.19. Use of immunoblotting to show how specific antibodies can be used to identify their antigens in complex mixtures. Components of smooth muscle homogenate were separated using SDS-PAGE in duplicate tracks. One track was protein-stained to allow visualisation of all the components present in the homogenate (lane A); components in the second track were electrophoretically transferred to nitrocellulose and incubated with mouse monoclonal antibody against smooth muscle α-actin followed by ^{125}I-labelled anti-mouse IgG. After autoradiography, one protein band is immunostained (lane B) corresponding to smooth muscle α-actin. The relative positions of molecular weight markers ($\times 10^{-3}$) are shown.

with radioisotope (^{125}I is usually employed for this) or enzyme (see Section 7.5). Antigens recognised by antibody thus appear as 'bands' on autoradiographs or substrate-developed blots and comparison of these with protein-stained gels allows identification of antigens recognised by antibody (Fig. 7.19). This process shows some similarity to Southern and northern blotting used with nucleic acid and is known as immunoblotting or 'western' blotting (see also Section 10.3.8).

Nitrocellulose is normally employed as the porous membrane for immunoblotting, but other materials are also occasionally used. Increased sensitivity with immunoblotting can be obtained by the use of enhanced chemiluminescence (ECL) development. In ECL-linked immunoblotting, peroxidase-conjugated anti-immunoglobulin is used to detect antigen–antibody complexes (cf. ELISA; Section 7.7.3). This enzyme is used to generate a peracid by cleavage of hydrogen peroxide

and this in turn oxidises a substrate to yield light, which is detected using X-ray film (as for autoradiography). The substrate is normally luminol, and phenolic 'enhancer' compounds are included to increase photon yield. Other ECL systems have been developed for use with alkaline phosphatase conjugates. ECL detection is sensitive and fast compared to direct use of enzyme or radioisotope conjugates. Other chemiluminescence amplification systems suitable for use in immunoblotting involving different enzymes, substrates and enhancers are also available.

7.7 IMMUNOASSAYS

Many immunochemical techniques provide quantitative assessment of the concentration of analyte in pure solutions or complex mixtures, for example single radial immunodiffusion and rocket immunoelectrophoresis (Section 7.4.1). However, the great potential of the application of immunochemistry to sensitive and specific assay of a diverse range of chemical and biological molecules has led to very considerable effort being focused in this area. Many versions of basic immunochemical assay principles now exist; such methodologies are termed immunoassays to emphasise their quantitative aspect.

Refinements to immunoassay methodology for research purposes have been driven mainly by the need for ever greater specificity and particularly sensitivity. Immunoassays are widely used for routine diagnostic/prognostic purposes and other applications (e.g. measuring levels of enviromental contaminants such as pesticides and toxic by-products of industrial processes); techniques that allow high sample throughput, ease of automation (robotic processing is often used), economy, robustness, precision and accuracy have been developed and are being sought. A thorough description of all alternative immunoassay techniques and formats would occupy several volumes, but the general principles and some of the more frequently employed options are described below.

7.7.1 Competitive binding immunoassays

In competitive binding immunoassays, antigen present in the samples to be assayed competes with a fixed amount of labelled antigen in the presence of a limiting quantity of antibody. When the system has reached equilibrium, free antigen is separated from antibody-bound antigen and the amount of labelled antigen present in the latter determined by scintillation (for β emission) or γ counting. This is inversely proportional to the concentration of antigen present in the samples (see Fig. 7.20). Inclusion of a number of dilutions of a standard solution of known antigen content allows the construction of a dose–response curve (often known as a standard curve), which can be used, by comparison, to derive antigen concentrations in samples. The earliest immunoassays were of this type, and the most common label used was a radioisotope (^{3}H, ^{125}I). Such assays are normally known as radioimmunoassays (RIAs), although they should be called competitive binding radioimmunoassays to distinguish them from radiobinding assays (Section 7.7.3). The first RIA described was for insulin, and similar approaches using binding

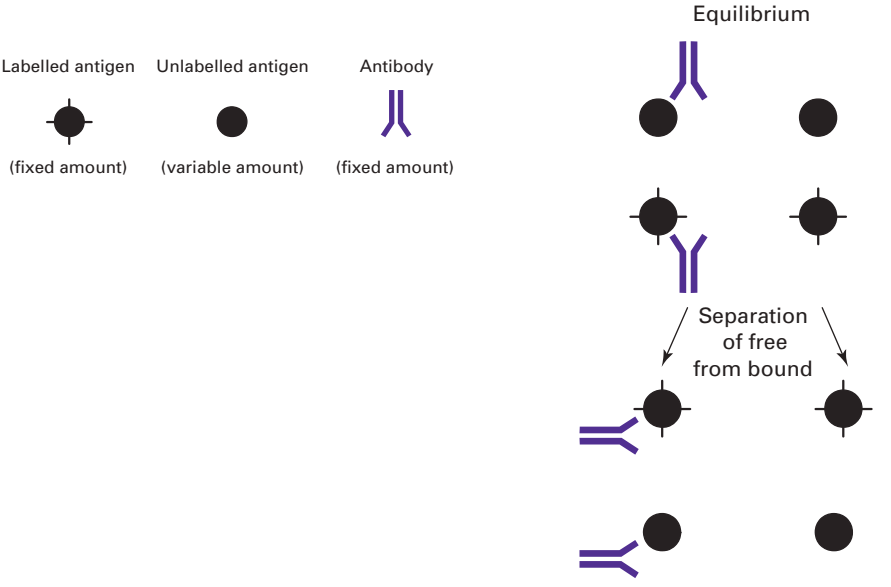


Fig. 7.20. Diagrammatic representation of competitive binding (inhibition) immunoassay.

proteins rather than antibodies were also developed at this time, for example for vitamin B12. The sensitivity of such assays varies considerably and depends on several factors, such as the label used. RIAs using ^{125}I labels can theoretically reach a sensitivity of 10^{-14} M, but in practice this is usually $\leq 10^{-12}$ M. In developing an RIA, it is initially necessary to derive an antibody versus labelled antigen binding curve to select appropriate antibody and labelled antigen concentrations for the assay. Normally, conditions at which 50–70% of the labelled antigen is bound by antibody are selected for the competitive RIA. Numerous procedures have been developed for separating free from bound antigen (see Table 7.9).

RIAs have been produced for a wide range of analytes from small molecules (e.g. steroids, peptide hormones in human or animal serum/plasma) to large proteins (e.g. serum levels of α_2-macroglobulin, immunoglobulins). They can be precise, accurate and economical (very small amounts of antibody are required). However, they are difficult to automate, take a relatively long time and the dose–response curve usually covers only a relatively narrow range of analyte concentration. They can be less sensitive than some other immunoassays. It is also necessary to establish systems for containment and disposal of radioactive reagents and for medical surveillance of staff involved in use of RIA methods. This bureaucracy has resulted in a reduction in use of RIA technology.

As the antibody concentrations used are limiting, competitive binding assays are sometimes called reagent (antigen) excess immunoassays (cf. immunometric assays; Section 7.7.2). Competitive immunoassays using non-radioisotope labels have been developed, but this approach is rather disappointing. Use of enzyme labels often results in insensitive assays and other labelling options can produce

Table 7.9 Methods for separating bound and free labelled ligand in radioimmunoassays

Method	Principle
Coated charcoal	Adsorption of bound or free fraction
Florisil	
S. aureus – protein A	
Polyethylene glycol	Fractional precipitation of bound fraction
Ethanol	
Ammonium sulphate	
Second antibody	Precipitation of bound fraction
soluble	
solid phase – cellulose	
– magnetic particles	
First antibody	Precipitation of bound fraction
solid phase – coated disks and tubes	
– cellulose	
– magnetic particles	

non-robust, imprecise and insensitive assays. The reason for this is not always clear, but labelling antigen with relatively bulky enzyme molecules (rather than the small atoms used in RIA) can alter antibody recognition of labelled compared with unlabelled antigen species, causing assay problems. This limitation has resulted in decreasing use of competitive immunoassay technology.

7.7.2 Immunometric assays

Immunometric assays differ from competitive immunoassays in several ways, although they provide similar quantitative information concerning antigen concentration. In the first-described immunometric assays, a fixed amount of labelled antibody was allowed to react with variable amounts of antigen. Unbound labelled antibody was then removed by washing and the labelled antibody remaining measured to provide an estimate of the antigen content (see Fig. 7.21). This approach is rarely used nowadays, but the concept of using an excess of labelled antibody, i.e. antibody excess immunoassays, rather than excess of labelled antigen (antigen excess immunoassays, i.e. competitive immunoassays) has numerous advantages and a few disadvantages (see below).

In most recently devised immunometric assays, antigen is allowed to react with insoluble or immobilised antibody, i.e. is 'captured' from solution, and the bound antigen is then detected using an excess of another (or in some special cases the same) antibody specific for the antigen. Captured antibody can be immobilised by covalent attachment to agarose microbeads, or by electrostatic binding to plastic or glass beads or the surfaces of plastic tubes or microtitre plates (see Fig. 7.22). The latter option is most often used, and special plates are available that have been treated to optimise antigen binding. Some methods use immobilised anti-Fc

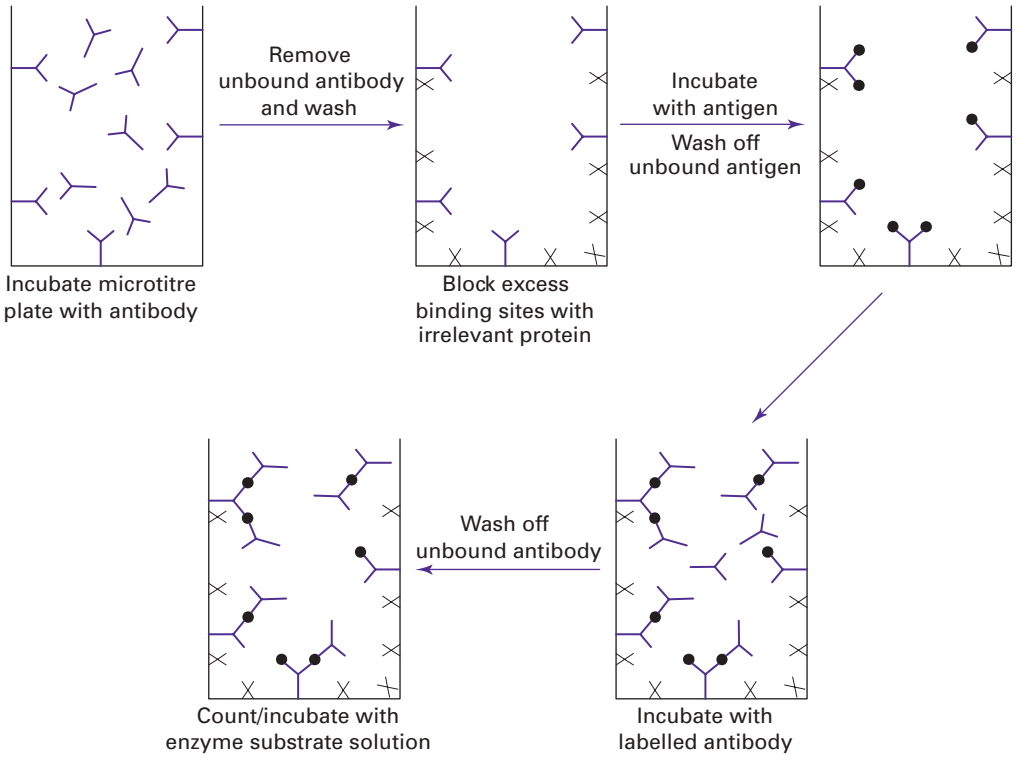


Fig. 7.21. Diagrammatic representation of a two-site immunometric assay (IRMA or two-site ELISA) for estimation of antigens. This particular format uses 96-well microtitre plates.

immunoglobulin to ensure that the captured antibody is immobilised in the correct orientation to interact optimally with antigen.

The detecting antibody can be directly labelled or can be indirectly measured using labelled anti-immunoglobulin reagent or other approaches, for example the avidin–biotin interaction (Section 7.5.5). Immunometric assays are usually relatively fast to carry out, can be very sensitive, and cover a wider range of analyte concentration than competitive assays. However, they require more antibody than competitive assays and normally two antibodies that recognise different determinants on the antigen are needed. Some immunometric assay formats (especially those employing microtitre plate layouts; see Fig. 7.21) can be automated and performed and controlled robotically to enable very high sample throughput.

Immunometric assays using radiolabelled antibody have been developed for a wide range of analytes and the first such assays were of this type. They are known as immunoradiometric assays (IRMAs). Even more immunometric assays using enzyme-labelled antibodies have been produced and these should be referred to as enzyme immunometric assays (EIAs or EIMAs). However, they are often unfortunately called ELISAs, which confuses them with enzyme-linked

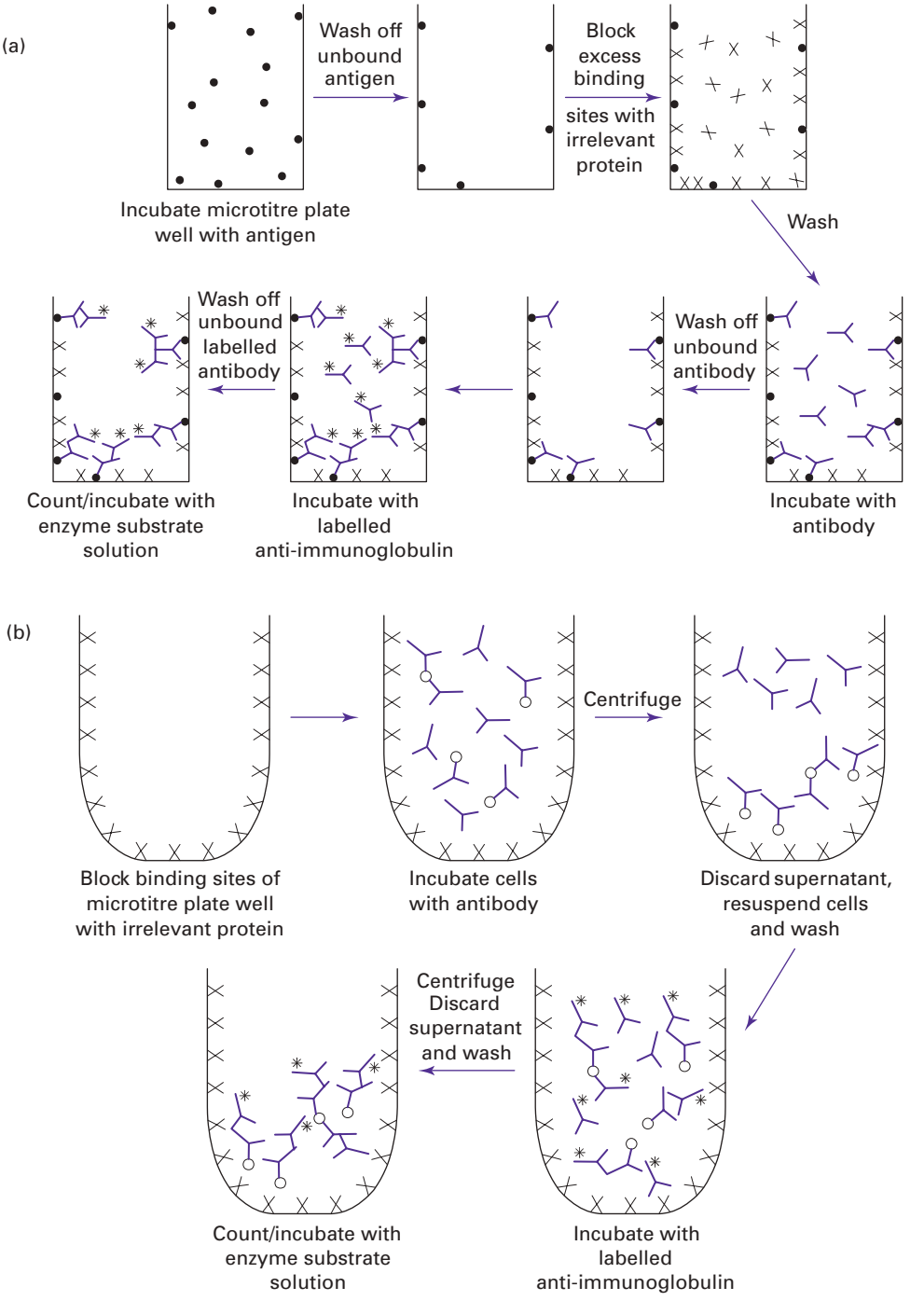


Fig. 7.22. Diagrammatic representation of microtitre plate format solid-phase binding immunoassays: (a) for antibodies directed against soluble antigens; (b) for antibodies directed against cell surface antigens.

immunobinding assays (Section 7.7.3). To try to distinguish them from 'real' ELISAs, they are often called sandwich ELISAs or two-site ELISAs. Most enzyme immunometric assays use a colorimetric substrate that is measured spectrophotometrically to determine antigen concentration. However, fluorescent substrates can be used for some enzymes (such as β-galactosidase) that have been claimed to increase assay sensitivity. An ever-increasing range of other labelling systems have been devised for immunometric assays and some of these are described in Section 7.7.4. All immunometric assays require calibration using dilutions of a standard solution of analyte of known concentration (cf. competitive immunoassays). The requirement that immunometric assay theory requires antibodies recognising different epitopes on antigen limited their application until the advent of monoclonal antibody technology (Section 7.2). Polyclonal antibodies can be used for immunometric assays, but unless the antigen is polymeric this approach is of limited use owing to occupation, by the capture antibody, of the antibody-binding sites recognised by the detecting antibody. The use of two monoclonal antibodies recognising different antigenic epitopes that do not display steric hindrance for binding of either antibody allows optimisation of immunometric assays. Combination of the use of a monoclonal antibody (for capture) with polyclonal detecting antibody can provide sensitive and specific immunoassays. Selection of appropriate antibodies and producing antibody preparations of appropriate quality are crucial for successful immunometric assays.

7.7.3 Solid-phase immunobinding assays (for estimation of antibody)

Immunobinding assays are solid-phase assays using immobilised antigen for assessing the antibody content of samples. They are often regarded as immunoassays, although their value for accurate quantification of antibody concentration can be questioned (if accuracy is important it is usually better to use an immunometric or competitive binding assay; Sections 7.7.1 and 7.7.2). However, immunobinding assays are very easy, quick, cheap and simple, and are ideal for checking the comparative antibody content of sera and other biological fluids and especially for screening sera from immunised animals, hybridoma culture supernatants, ascitic fluid and pathological samples. Antigen-containing solution is simply incubated in plastic tubes or (more often) in the wells of plastic microtitre plates, which allows a (small) proportion of the protein to coat the surfaces of the tubes or wells. After unbound antigen(s) has been washed away, the samples of known or unknown antibody content are incubated in the antigen-coated tubes/wells. Antibody (if present) binds to the immobilised antigen(s) and, after washing, can be detected using labelled anti-immunoglobulin or immunoglobulin-binding protein (see Fig. 7.22). Such assays, which use radiolabelled antibody or antibody-binding protein are nomally called solid-phase radiobinding assays, but most assays used nowadays employ enzyme-labelled detecting reagent. They are usually called enzyme-linked immunosorbent assays (ELISAs) but can be referred to as solid-phase enzyme immunobinding assays.

Unfortunately, enzyme immunometric assays are also often called ELISAs (Section 7.7.2) and to try to avoid confusion, the immunometric version is sometimes called two-site ELISA whereas the binding assay type is known as one-site ELISA. As antigen is simply captured onto tube or well surfaces by non-specific binding, such assays are occasionally known as sticky plate assays or (especially when complex impure antigen solution is used) as dirty plate techniques.

Quantification can be achieved by comparison with a standard solution of known antibody content, but this can be difficult largely due to the heterogeneity of immunoglobulin molecules present. It is very common to express comparative results as a titre derived from dose–response curves generated using different samples. Mid-point titres, i.e. the dilution at which 50% maximal binding is achieved, are the usual way of calculating the titre, but this requires the maximal value of the dose–response curve to be the same for all samples. The use of end-point titres, i.e. the minimal dilution at which no signal is generated, is unreliable and very often invalid.

A variant of conventional solid-phase immunobinding assays, usually known as dot blot assays are occasionally encountered. For these techniques, antigen-containing solution is spotted, dried onto nitrocellulose filters and then incubated with samples with suspected antibody content. Any antigen-specific antibodies are then detected by using an enzyme-labelled or radiolabelled anti-immunoglobulin or antibody-binding protein. Advantages of the method are that antigen can be concentrated by repeat spotting at a single location on the filter, and that many antigen samples can be incubated with a single antibody sample. A major disadvantage is that valid quantification of results is virtually impossible. The name of the technique is misleading as no blotting actually occurs at any stage of the assay; it probably relates to many technical similarities with immunoblotting (see Section 7.6). A possibly better name is dot immunobinding assay, but this is not often used.

7.7.4 Enhanced immunoassays

The quest for evermore sensitive immunoassays has resulted in the design of amplification systems to enhance the signal derived from the immunoassay (cf. immunoblotting; Section 7.6). Most of these are based on the enzyme immunometric assay (two-site ELISA) format and are carried out in microtitre plates. Several different amplification systems have been developed but a commonly encountered system is enzyme-linked, and is added as a 'cassette' to a conventional alkaline phosphatase-based two-site immunometric assay. In this, the alkaline phosphatase is used to dephosphorylate $NADP^+$ to produce NAD^+. The NAD^+ comprises the limiting concentration reagent of an alcohol dehydrogenase catalysed loop in which the NAD^+ is reduced to NADH and this in turn generates a coloured formazan by reduction from the oxidised leukoformazan (see Fig. 7.23). The additional enzyme-catalysed loop amplifies the original signal considerably as compared with that which could be produced from the alkaline phosphatase conjugate alone. Another popular amplification system, known

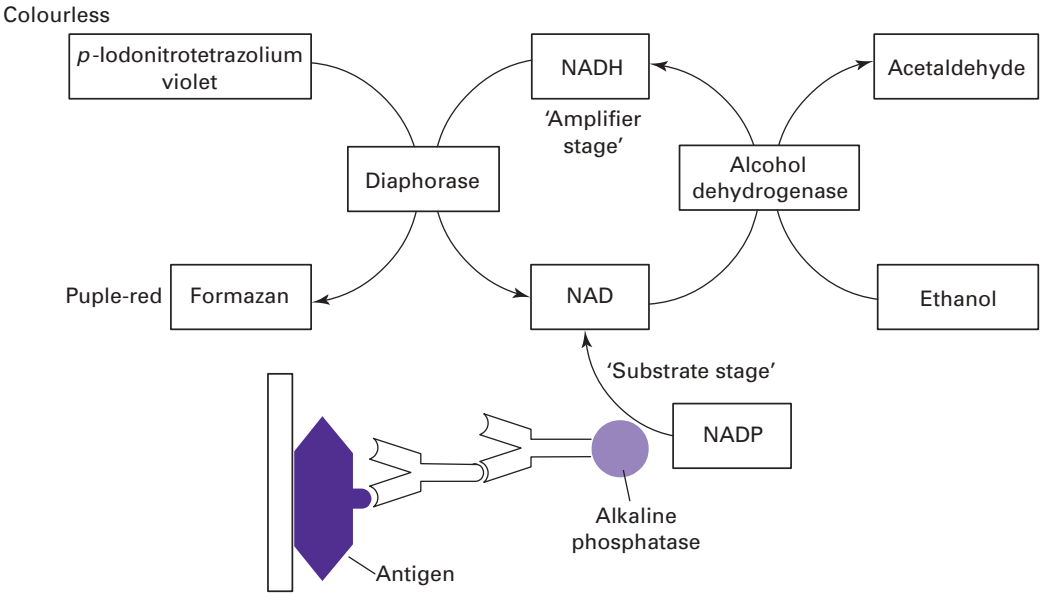


Fig. 7.23. Substrate amplification system for the detection of alkaline phosphatase.

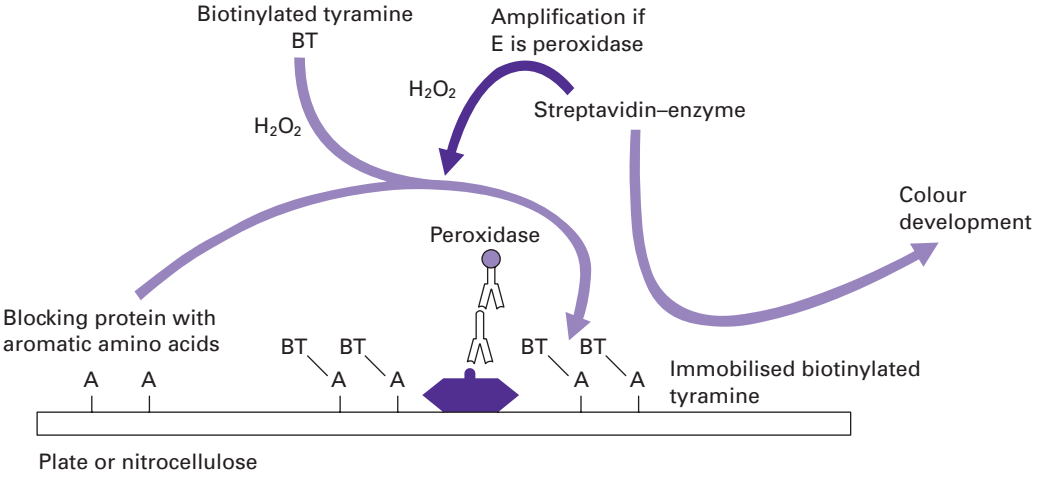


Fig. 7.24. Principles of the ELAST amplification system.

as the ELISA amplification system or ELAST (see Fig. 7.24) uses a standard peroxidase-based immunometric assay, but involves incorporation of a biotinylated tyramine reagent that, when oxidised (by the peroxidase conjugate), binds covalently to tyrosine or tryptophan residues present on the excess of blocking protein used to coat antigen-unoccupied sites on the microtitre plate surface.

The immobilised biotin is then detected using labelled streptavidin (Section 7.5.5). The amplification system results in a far greater number of immobilised biotin molecules than would arise from the use of a simple biotin-labelled antibody.

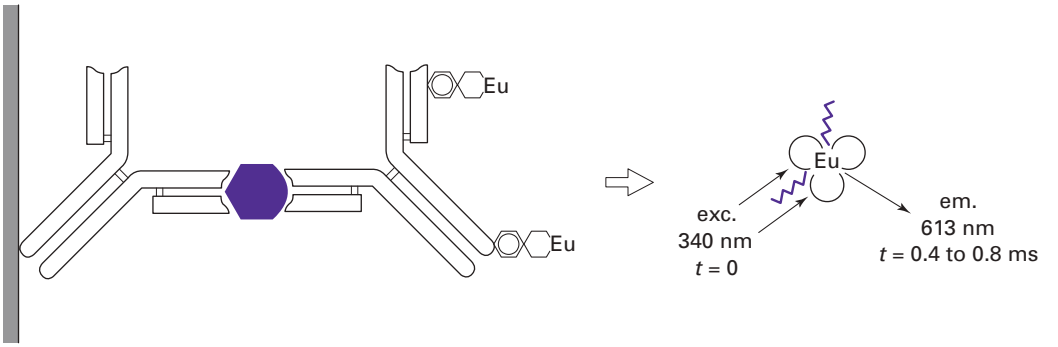
Amplification systems almost always result in enhanced signal, but this often affects both the 'real' analyte-derived output and the assay background. At worst, this can simply increase the total assay signal but not improve sensitivity. Unless care is shown, use of potent amplification systems can result in less robust assays, with increased chance of assay artefacts.

7.7.5 Peptide-based immunobinding assays (peptide mapping, epitope mapping)

Synthetic peptides can be substituted for antigen in solid-phase immunobinding assays. This allows detection of antibodies with known epitope specificity and also the determination of the epitope specificity of new antibodies (peptide mapping or epitope mapping). For the latter, a series of overlapping sequence peptides are made, covering the entire primary structure of the antigen and used sequentially to coat the wells of microtitre plates. Incubation with either labelled antibody or antibody followed by labelled anti-immunoglobulin allows the identification of peptides recognised by the antibody and therefore elucidation of the epitope(s) recognised by the antibody(s). This approach is especially useful with monoclonal antibodies. Usually peptides of 10 to 18 residues length are used, with an overlap of about 5 to 8 residues. The procedure is simple and valuable, but is limited to detection of 'linear' antigenic determinants and care must be taken to ensure that all the peptides bind efficiently to microtitre plate well surfaces. Coating the wells with polylysine can improve peptide binding (it produces a relatively strong positively charged surface) or they can be synthesised with biotin end-residues and captured with streptavidin-coated plates (this also optimises orientation on binding). It is also possible to synthesise peptides on 'pins' formed in the wells of special plates and use these for mapping (the pepscan procedure). The 'pin' heads are chemically activated to ensure binding of activated amino acid residues to be added sequentially thus building the required peptide sequence.

7.7.6 Fluorescence- and photoluminescence-based immunoassays

In attempts to increase assay sensitivity and ease, a variety of adaptions of the basic competitive and especially immunometric immunoassay methods have been developed and involve fluorescence or luminescence readouts. At their simplest, these substitute fluorescent or luminescent substrates for the colorimetric substrates normally used for two-site ELISAs. These are called fluorimetric EIAs or enzyme-linked fluorescence immunoassays (ELFIAs) and luminoimmunoassays (LIAs) or enzyme-linked chemiluminescence immunoassays (ECLIAs),



1	2	3	4
Solid-phase immunoassay	Eu-labelled immunoreagents	Dissociative fluorescence enhancement	Time-related fluorimetry

Fig. 7.25. The dissociative fluorescence enhancement principle exemplified in a two-site sandwich assay. exc., excitation; em., emission.

respectively. However, some assays use sufficiently different approaches to be considered separately (see below).

7.7.7 Delayed enhanced lanthanide fluorescence immunoassay

Most delayed enhanced lanthanide fluorescence immunoassays (DELFIAs) (see Section 12.5.3) use the standard two-site immunometric assay principle and are carried out in microtitre plates. The detecting antibody (see Fig. 7.25) is labelled directly with a lanthanide (these are 4f transition metals – europium is usually used in DELFIA) that is non-covalently coupled via a chelating agent such as diethylenetriaminepenta-acetic anhydride (DTPA) or diethylenetriaminetetra-acetic anhydride (DTTA). After the immunometric assay has been carried out, the lanthanide is released from the antibody by lowering the pH to about 3.2 (the chelates are unstable at this pH) and free lanthanide is then captured using a soluble diketone. This is complexed into micelles, which prevents subsequent quenching of fluorescence (the so-called enhancer step). The antibody–lanthanide chelates are not fluorescent, but the captured, micelle-bound lanthanide ion is, and this permits its detection. The peak fluorescence emission of miscelle-complexed lanthanides is relatively slow and this allows delayed measurement of light output after addition of the enhancing reagents. By this means, artefactual immediate autofluorescence due to sample components etc. can be distinguished from the later 'real' lanthanide signal. Because of this, such assays are often called time-resolved immunofluorimetric assays (TRIFMA). In such assays, the fluorescence is proportional to the amount of antigen present in samples. DELFIAs can be sensitive, fast, accurate, robust, cover a relatively wide analyte concentration range and run using robots to allow very high sample throughput.

7.7.8 Homogeneous substrate-labelled fluorescence immunoassay

Substrate-labelled fluorescence immunoassays (SLFIAs) use principles similar to those of competitive immunoassays, but do not require separation of free from bound antigen. They require the synthesis of antigen conjugates containing a chemical structure that is not fluorescent per se, but is cleaved by an enzyme to yield an intensely fluorescent compound. They also need an antibody that binds the antigen conjugate such that the enzyme is prevented from cleaving it and liberating the fluorochrome. In SLFIA, samples containing known and unknown amounts of antigen are incubated with a fixed amount of antigen conjugate in the presence of a limiting concentration of antibody. Under such conditions, the antigen in the samples competes for antibody with the conjugated antigen. After equilibrium has been reached, enzyme is added to liberate the fluorochrome from non-antibody-bound antigen conjugate. The fluorescence measured is therefore proportional to the amount of antigen in the samples. A combination of the enzyme β-galactosidase and conjugates prepared by coupling antigen to a galactosyl 4-methylumbelliferyl residue is often employed for SLFIA. The β-galactosidase hydrolyses the conjugate to liberate free 4-methylumbelliferone, which is readily measured using fluorimetry. A major difficulty encountered with the general application of this type of assay is producing the appropriate antibody, i.e. an antibody that effectively inhibits the enzyme-catalysed reaction. It is usually successful only for relatively small analytes, for example morphine and other opiates.

7.8 IMMUNOHISTO/CYTOCHEMISTRY

To understand cell structure, organisation and function, and cell or tissue development and differentiation in health and disease, it is often necessary to be able to determine the distribution of an antigen *in situ*. Immunohistochemical and immunocytochemical techniques exploit the specific interaction of an antibody with its antigen to locate or to determine the distribution of the antigen *in situ* in tissues or cells, respectively. Alternatively, these procedures can be carried out using tissues or cells known to contain a particular antigen to investigate the specificity of antibodies or antisera. The principle of immunohisto/cytochemistry is analagous to those of solid-phase immunobinding (Section 7.7.3) and immunoblotting (Section 7.6), except that the antibody is incubated with thin sections of solid tissue mounted on glass slides or cell preparations containing the antigen rather than with antigen immobilised on microtitre plates or nitrocellulose membranes. In immunohisto/cytochemistry, the antibody (or anti-immunoglobulin antibody; Section 7.5.1) must be conjugated with a fluorescent or enzyme label that gives an intense signal to allow visualisation when the sections or cells are examined using immunoenzyme microscopy. The location of the label reveals the site of the antibody–antigen interaction, which can be localised to, for example, particular cell types or cellular organelles (an example is shown in Fig. 7.26).

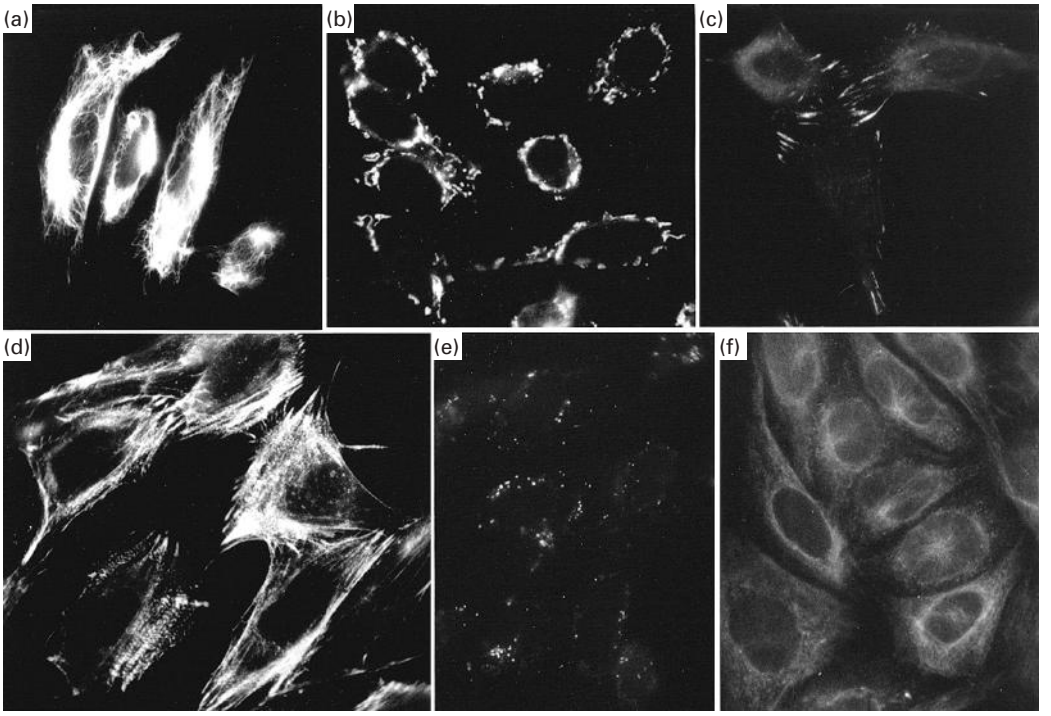


Fig. 7.26. Immunofluorescence photomicrographs showing immunostaining of cellular organelles: (a) intermediate filaments; (b) mitochondria; (c) microspikes; (d) stress fibres; (e) granules; (f) cytoplasmic component(s). Monolayers of the rat glioma cell line C6 were incubated with human IgM monoclonal antibodies and then fluorescein-labelled anti-human IgM.

All immunohisto/cytochemical procedures need stringent positive and negative control antibodies for comparison with the test antibody to ensure that the immunostaining is specific. Although it is common for the binding of labelled antibody to a specific tissue or cellular antigen to be referred to as immunostaining, this term should not be confused with the differential staining of tissue constituents by routine chemical stains such as haematoxylin and eosin for histology and pathology.

7.8.1 Immunoenzyme microscopy

The main advantage of enzyme labels (usually horseradish peroxidase (HRP) or alkaline phosphatase (AP); Section 7.5.4) for immunohisto/cytochemical procedures is that an ordinary white light microscope can be used for viewing the sections or cells, and the use of chemical counterstains such as haematoxylin (stains nuclei blue) aids in identification of morphology. The main disadvantages are the presence of endogenous tissue enzymes (which can give high background staining) and the extra step involved in carrying out the enzyme reaction.

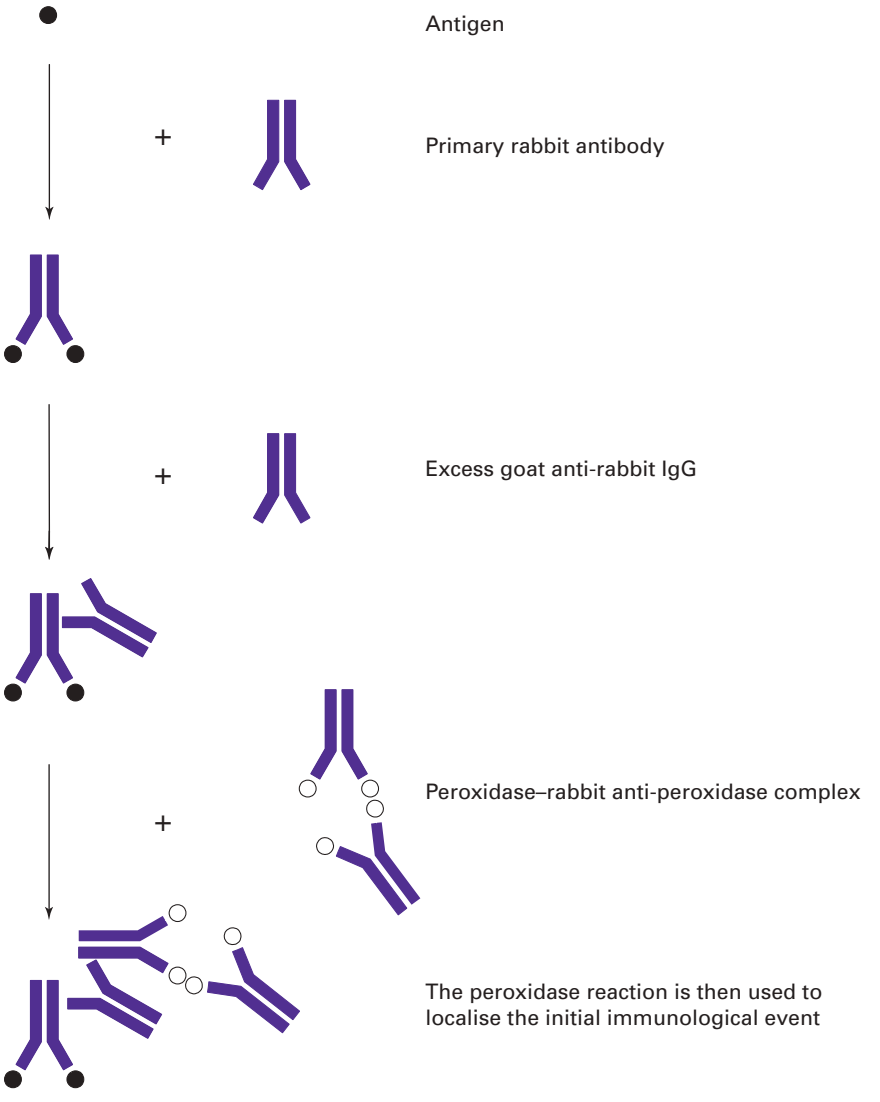


Fig. 7.27. Diagrammatic representation of the peroxidase–anti-peroxidase technique. ●, antigen; ○, peroxidase.

7.8.2 The peroxidase–anti-peroxidase technique

The PAP (peroxidase–anti-peroxidase) technique is a modification of the indirect enzyme immunohisto/cytochemical procedure for amplifying the signal (Fig. 7.27). In this method, unlabelled anti-rabbit or anti-mouse immunoglobulin forms a bridge between rabbit or mouse primary antibody, respectively, and a peroxidase–(rabbit or mouse)–anti-peroxidase complex. Alkaline phosphatase–anti-alkaline phosphatase complexes are also available. An advantage of this procedure is that no possible destruction of antibody and/or enzyme can occur during

chemical coupling. Specially developed commercial systems that exploit both anti-immunoglobulin and biotin–avidin reactions are also available. Such amplification systems are many times more sensitive than standard indirect procedures.

7.8.3 Immunofluorescence techniques

Immunohistochemistry was originally developed using fluorochrome-labelled antibodies (see Section 7.5.3). Their use has dramatically increased in recent years with the expansion of flow cytofluorimetric techniques (Section 16.3.2), which allow analysis of single cells in suspension according to the expression of cell surface antigens (Section 7.8.5), and the advent of confocal microscropy in which laser light sources replace the standard light sources to allow analysis of images at different depths through the tissue section or cell to build up a three-dimensional picture. A unique application of fluorochrome-labelled antibodies for immunohisto/cytochemistry is that, because different fluorochromes emit different wavelengths of ultraviolet light, the use of appropriate filters on a fluorescence microscope allows the same tissue section or cell sample to be immunostained with two, three or even four different antibodies, each of which is used in conjunction with a different fluorochrome. Such double or triple staining procedures sometimes necessitate the use of directly conjugated primary antibodies, as, for example, fluorescein conjugated rabbit anti-mouse immunoglobulin antibodies will not distinguish between primary mouse monoclonal antibodies of differing specificity.

The choice of fluorochrome depends on the light source, detection system available and personal preference. Light sources include tungsten, quartz-halogen or mercury arc lamps for fluorescence microscopes, argon ion or krypton–argon lasers for flow cytometry.

Fluorochrome-labelled antibodies are quick and easy to use, and offer good sensitivity. Their main disadvantage is the requirement for a microscope equipped with fluorescence optics, and that the fluorescence, particularly that of fluorescein, fades during prolonged viewing of individual microscopic fields unless a powerful reducing agent such as 1,4-bicyclo-2,2,2-octane is included in the mountant. Such agents are thought to suppress a destructive reaction of the fluorescein in its excited state with protein. Autofluorescence of some tissue components, i.e. their intrinsic fluorescence, can also be a problem.

7.8.4 Capping

An important feature of immunostaining viable cells, particularly lymphocytes, is the aggregation of cell surface antigens due to cross-linkage by intact antibodies. Such patches eventually form a cap over one pole of the cell, which is subsequently shed or endocytosed. Capping can be visualised using fluorochrome-labelled antibodies and provides information on the association of molecules carrying different antigenic markers if double or triple immunofluorescence labelling is carried out. It can be prevented by immunostaining at 4 °C or in the

presence of sodium azide, which prevents modulation of cell surface molecules, or by the use of monovalent Fab fragments, in order to study the initial binding of antibody to cell surface antigen.

7.8.5 Flow cytometry

A disadvantage of assessment of immunocytochemical staining using conventional microscopy is that it is very difficult to quantify the intensity of the immunostaining. Although it is possible to estimate the proportion of cells that are immunostained in a population by performing manual cell counts, the accuracy of the estimate is dependent on the total number of cells that are counted and the proportion of positive cells.

These limitations are overcome in flow cytofluorimetric analysis. In this technique, cells that have been labelled with fluorochrome-conjugated antibody in suspension are introduced into a liquid jet and passed individually through the beam of a laser. As each cell passes through it emits a flash of fluorescence and scattered light. The signals are collected and converted by the flow cytofluorimeter to give quantitative information on the intensity of fluorescence and the light-scattering properties of each cell. Forward light scatter is related to cell size; the amount of side scatter is related to the granularity of the cell (presence of intracellular granules, pronounced organelles and/or nucleus). Examples of data displays are shown in Fig. 7.28. Many thousands of cells can be quickly analysed in this way, for example with respect to the proportion of cells that are immunostained or the intensity of fluorescence. Flow cytofluorimeters are also able to sort cells according to specified parameters such as intensity of fluorescence and size (cells usually remain viable after immunostaining with fluorochrome-labelled antibody). Many fluorochromes are available for flow cytofluorimetric analysis that allow simultaneous monitoring for several colours, provided the emission spectra do not overlap. This allows the use of several antibodies, conjugated with different fluorochromes, at the same time. Directly conjugated monoclonal antibodies are often used in flow cytofluorimetric techniques. This permits simultaneous labelling with several fluorochromes (see Sections 7.8.3 and 7.5.3). Also the anti-immunoglobulin conjugates used in indirect immunocytochemical techniques can either bind directly to lymphocytes that express cell surface immunoglobulin or bring about agglutination of primary antibody-coated cells. (IgG is divalent and can bind two primary antibody molecules, each of which is bound to a separate cell, thus bringing the cells together.) A wide range of directly conjugated monoclonal antibodies against different cell surface markers such as the CD (cluster of differentiation) series of antigens, which are characteristic of different populations of blood cells, are available commercially. These allow analysis of cell sets and subsets involved in biological systems to be evaluated, for example T and B lymphocyte populations. If immunostaining of surface antigens of viable cells for flow cytometric analysis is being carried out, steps may have to be taken to prevent non-antigen-specific binding of antibody to Fc receptors that are present on, for example, leukocytes. This can be achieved

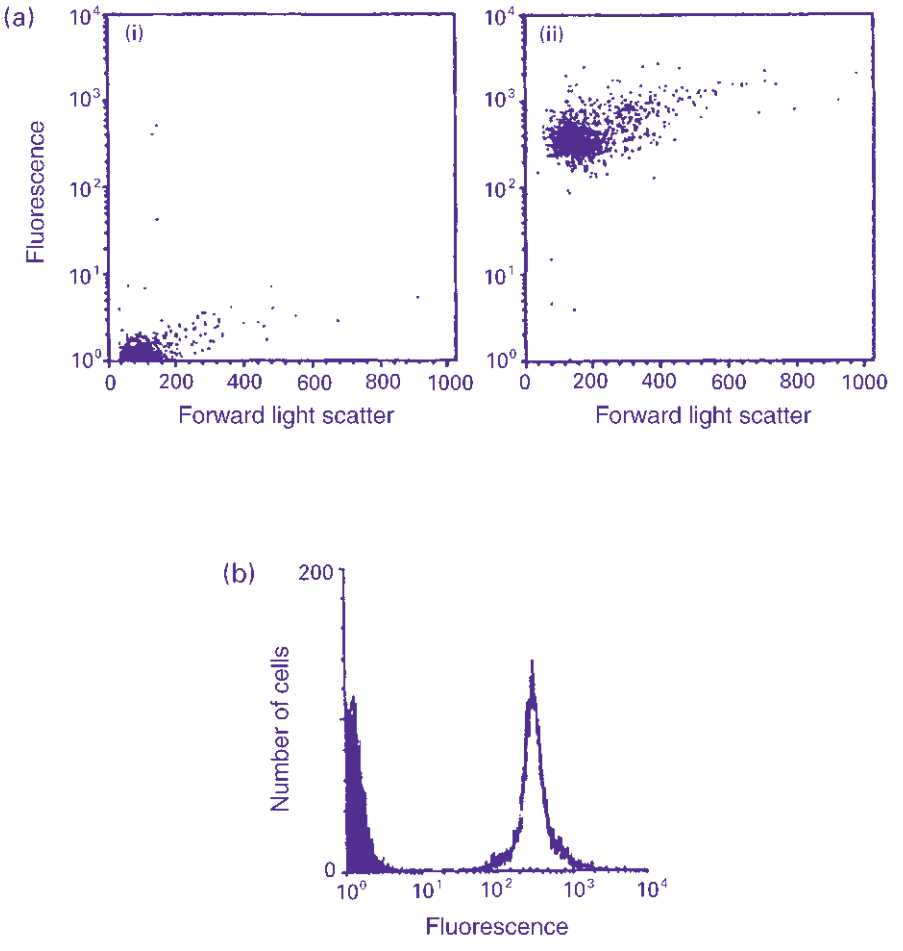


Fig. 7.28. Examples of flow cytometry displays. (a) Dot plots of erythrocytes (red blood cells) stained with a fluorescently labelled mouse monoclonal anti-haemoglobin antibody (ii) or a fluorescently labelled irrelevant mouse monoclonal antibody (i). Each dot represents an erythrocyte. The intensity of fluorescence is on the *y*-axis; forward light scatter (i.e. size) is on the *x*-axis. The erythrocytes are not fluorescently stained with the irrelevant antibody (i), but virtually all the cells are stained with the anti-haemoglobin antibody (ii). (b) The same data displayed as a profile histogram with number of cells on the *y*-axis and intensity of fluorescence on the *x*-axis.

either by blocking these sites with non-immune serum that contains high levels of immunoglobulin, or by the use of antibody $F(ab')_2$ fragments.

7.8.6 Immunoelectron microscopy

Subcellular detail that is not discernible by conventional microscopy can be resolved using electron microscopy, since electrons have a shorter wavelength

than white or ultraviolet light. Antibodies labelled with electron-dense reagents such as gold or ferritin (an iron-containing protein) or with HRP, which can yield an electron-dense product with an appropriate substrate, are used in immunoelectron microscopy to immunostain ultrathin sections of tissue or cells.

7.9 AFFINITY AND AVIDITY

Determining the affinity of an antibody can be important for predicting and/or explaining its immunochemical characteristics. Affinity is defined as the equilibrium constant when 'a monovalent antibody reacts with (binds) a monovalent antigen, i.e. an antigenic determinant'. As antibodies are usually di- or multivalent, and antigens usually have more than one antigenic determinant, this concept is relatively rarely encountered. The term avidity is normally used to describe the equilibrium constant applicable to whole antibody–antigen interactions and this includes the affinity component plus additive factors due to multiple valency of binding and other considerations. The terms affinity and avidity are sometimes replaced by intrinsic affinity and functional affinity, respectively, and these are certainly more descriptive.

7.9.1 Measurement of affinity and avidity

The affinity of monovalent antibody for its epitope can be expressed arithmetically as the equilibrium association constant using the following relationship:

$$K_a = \frac{[AgAb]}{[Ag]\,[Ab]}$$

where K_a is the equilibrium association constant, [Ag] is the concentration of antigen, [Ab] is the concentration of antibody, and [AgAb] is the concentration of the antigen–antibody complex.

Practically, this requires that all components are absolutely pure and relate to equilibrium in homogeneous solution. Such conditions rarely apply to most immunochemical techniques as applied to biochemical methodology. However, calculating the relative affinity of antibodies can be useful in predicting their use in immunochemical techniques (see also Section 16.2.1).

7.10 IMMUNOCHEMICAL USE OF SURFACE PLASMON RESONANCE

Surface plasmon resonance (Section 16.3.2) uses the optical properties at the surface of a thin gold-film-coated glass 'chip' to study binding phenomena. The physical principles of the method are as follows. A beam of polarised light will be internally reflected by the chip (mounted on a glass prism). The angle of reflection is changed if material binds to the surface of the chip and the alteration in the angle of reflection is proportional to the *mass* of substance bound to the chip surface. Instruments for the application of surface plasmon resonance technology

to immunochemistry are commercially available. The Biacore™ biosensor system is probably most appropriate for general immunochemical use, can be programmed for a variety of differing applications and is automated. In this system, the chip is coated with a dextran matrix to which antigen or antibody can be chemically coupled, and thus immobilised. Samples to be analysed for antigen (if antibody has been immobilised) or antibody (if antigen has been immobilised) are allowed to flow over the chip surface, and binding is continuously detected by measuring the alteration in the angle of reflection of light incident on the prism side of the chip. The measurements are directly proportional to the mass of substance binding and can be used to compare the antigen or antibody content of samples and also for calculation of kinetic parameters related to antigen–antibody interaction, for example association and dissociation affinity constants.

Surface plasmon resonance is a rapid procedure (measurements are made in a few minutes), does not require labelling of antibodies or antigens or the use of anti-immunoglobulin reagents and can be adapted for a wide range of purposes. It is not usually particularly sensitive, for example compared with immunoassays, but can be used to allow detection of very low affinity interactions.

7.11 SUGGESTIONS FOR FURTHER READING

DABBS, D. J. (2002). *Diagnostic Immunohistochemistry*. Churchill Livingstone, New York. (Good coverage of the clinical applications of this technique.)

HAYAT, M. A. (2002). *Microscopy, Immunohistochemistry and Antigen Retrieval Methods: For Light and Electron Microscopy.* Plenum Press, London. (An authoritative text on all aspects of the application of immunological techniques to microscopic and histochemical analytical methods.)

HERMANSON, G. T. (1996). *Bioconjugate Techniques.* Academic Press Inc., London and New York. (A very comprehensive, detailed account of just about every procedure for congugating labels to biological molecules. Includes a large number of methods used to produce congugates employed in immunochemical techniques.)

JOHNSTONE, A. P. and THORPE, R. (1996). *Immunochemistry in Practice*, 3rd edn. Blackwell Science, Oxford. (Contains detailed protocols for many immunochemical techniques plus a resumé of their underlying scientific basis.)

JOHNSTONE, A. P. and TURNER, M. W. (eds.) (1997). *Immunochemistry – A Practical Approach.* IRL Press, Oxford. (Chapters devoted to many immunochemical procedures.)

LACHMANN, P. J., PETERS, D. K. AND ROSENS, F. S. (eds.) (1992). *Clinical Aspects Of Immunology*, 5th edn. Blackwell Science, Oxford. (A large three-volume work describing most aspects of clinical immunology and much theoretical immunology.)

ROITT, I. M. (2001). *Essential Immunology*, 10th edn. Blackwell Science, Oxford. (An excellent general textbook on immunology.)

Chapter 8

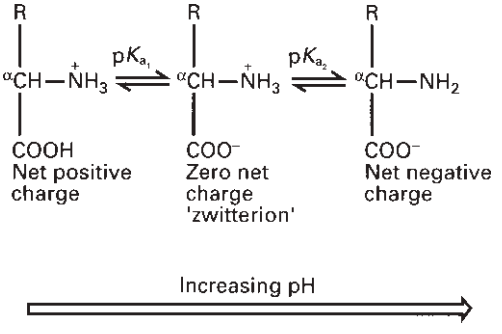
Protein structure, purification, characterisation and function analysis

8.1 IONIC PROPERTIES OF AMINO ACIDS AND PROTEINS

Twenty amino acids varying in size, shape, charge and chemical reactivity are found in proteins and each has at least one codon in the genetic code (Section 5.3.5). Nineteen of the amino acids are α-amino acids (i.e. the amino and carboxyl groups are attached to the carbon atom that is adjacent to the carboxyl group) with the general formula $RCH(NH_2)COOH$, where R is an aliphatic, aromatic or heterocyclic group. The only exception to this general formula is proline, which is an imino acid in which the $-NH_2$ group is incorporated into a five-membered ring. With the exception of the simplest amino acid glycine (R = H), all the amino acids found in proteins contain one asymmetric carbon atom and hence are optically active and have been found to have the L configuration.

For convenience, each amino acid found in proteins is designated by either a three-letter abbreviation, generally based on the first three letters of their name, or a one-letter symbol, some of which are the first letter of the name. Details are given in Table 8.1.

Since they possess both an amino group and a carboxyl group, amino acids are ionised at all pH values, i.e. a neutral species represented by the general formula does not exist in solution irrespective of the pH. This can be seen as follows:



Thus at low pH values an amino acid exists as a cation and at high pH values as an anion. At a particular intermediate pH the amino acid carries no net charge, although it is still ionised, and is called a zwitterion. It has been shown that, in the

Table 8.1 Abbreviations for amino acids

Amino acid	Three-letter symbol	One-letter symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Asparagine or aspartic acid	Asx	B
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glutamine or glutamic acid	Glx	Z
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

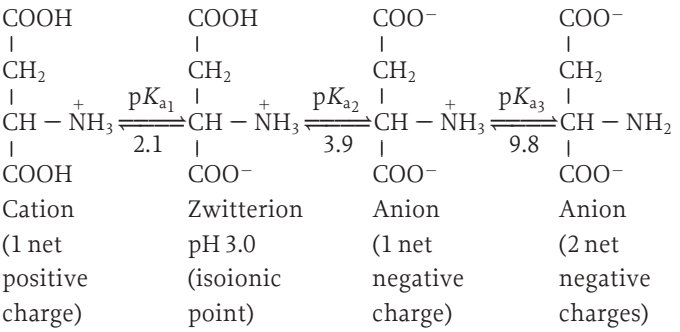
crystalline state and in solution in water, amino acids exist predominantly as this zwitterionic form. This confers upon them physical properties characteristic of ionic compounds, i.e. high melting point and boiling point, water solubility and low solubility in organic solvents such as ether and chloroform. The pH at which the zwitterion predominates in aqueous solution is referred to as the isoionic point, because it is the pH at which the number of negative charges on the molecule produced by ionisation of the carboxyl group is equal to the number of positive charges acquired by proton acceptance by the amino group. In the case of amino acids this is equal to the isoelectric point (pI), since the molecule carries no net charge and is therefore electrophoretically immobile. The numerical value of this pH for a given amino acid is related to its acid strength (pK_a values) by the equation:

$$\mathrm{pI} = \frac{pK_{a_1} + pK_{a_2}}{2} \tag{8.1}$$

where pK_{a_1} and pK_{a_2} are equal to the negative logarithm of the acid dissociation constants, K_{a_1} and K_{a_2} (Section 1.4.2).

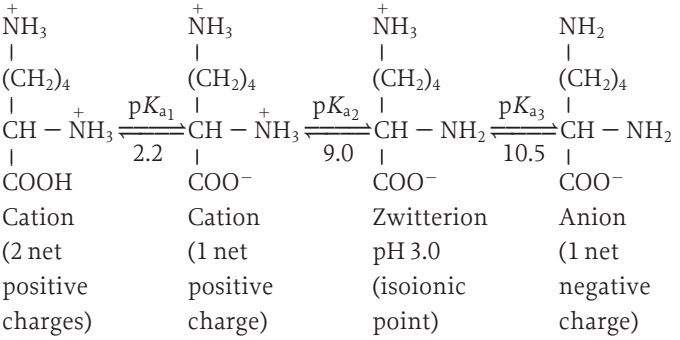
In the case of glycine, pK_{a_1} and pK_{a_2} are 2, 3 and 9.6, respectively, so that the isoionic point is 6.0. At pH values below this, the cation and zwitterion will coexist in equilibrium in a ratio determined by the Henderson–Hasselbalch equation (Section 1.4.2), whereas at higher pH values the zwitterion and anion will coexist in equilibrium.

For acidic amino acids such as aspartic acid, the ionisation pattern is different owing to the presence of a second carboxyl group:



In this case, the zwitterion will predominate in aqueous solution at a pH determined by pK_{a_1} and pK_{a_2}, and the isoelectric point is the mean of pK_{a_1} and pK_{a_2}.

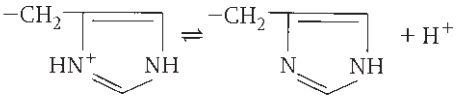
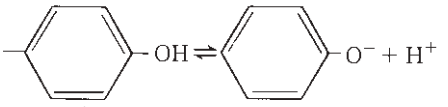
In the case of lysine, which is a basic amino acid, the ionisation pattern is different again and its isoionic point is the mean of pK_{a_2} and pK_{a_3}:



As an alternative to possessing a second amino or carboxyl group, an amino acid side chain may contain in the R of the general formula a quite different chemical group that is also capable of ionising at a characteristic pH. Such groups include a phenolic group (tyrosine), guanidino group (arginine), imidazolyl group (histidine) and sulphydryl group (cysteine) (Table 8.2). It is clear that the state of ionisation of the main groups of amino acids (acidic, basic, neutral) will be grossly different at a particular pH. Moreover, even within a given group there will be minor differences due to the precise nature of the R group. These differences are exploited in the electrophoretic and ion-exchange chromatographic separation of mixtures of amino acids such as those present in a protein hydrolysate (Section 8.4.2).

Proteins are formed by the condensation of the α-amino group of one amino acid with the α-carboxyl of the adjacent amino acid (Section 8.2). With the exception of the two terminal amino acids, therefore, the α-amino and carboxyl groups are all involved in peptide bonds and are no longer ionisable in the protein. Amino, carboxyl, imidazolyl, guanidino, phenolic and sulphydryl groups in the side chains are, however, free to ionise and of course there may be many of these.

Table 8.2 Ionisable groups found in proteins

Amino acid group	pH-dependent ionisation	Approx. pK_a
N-terminal α-amino	$-NH_3 \rightleftharpoons NH_2 + H^+$	8.0
C-terminal α-carboxyl	$-COOH \rightleftharpoons COO^- + H^+$	3.0
Asp-β-carboxyl	$-CH_2COOH \rightleftharpoons CH_2COO^- + H^+$	3.9
Glu-γ-carboxyl	$-(CH_2)_2COOH \rightleftharpoons (CH_2)_2COO^- + H^+$	4.1
His-imidazolyl		6.0
Cys-sulphydryl	$-CH_2SH \rightleftharpoons -CH_2S^- + H^+$	8.4
Tyr-phenolic		10.1
Lys-ε-amino	$-(CH_2)_4\overset{+}{N}H_3 \rightleftharpoons -(CH_2)_4NH_2 + H^+$	10.3
Arg-guanidino	$-NH-C(=\overset{+}{N}H_2)-NH_2 \rightleftharpoons -NH-C(=NH)-NH_2 + H^+$	12.5

Proteins fold in such a manner that the majority of these ionisable groups are on the outside of the molecule, where they can interact with the surrounding aqueous medium. Some of these groups are located within the structure and may be involved in electrostatic attractions that help to stabilise the three-dimensional structure of the protein molecule. The relative numbers of positive and negative groups in a protein molecule influence aspects of its physical behaviour, such as solubility and electrophoretic mobility.

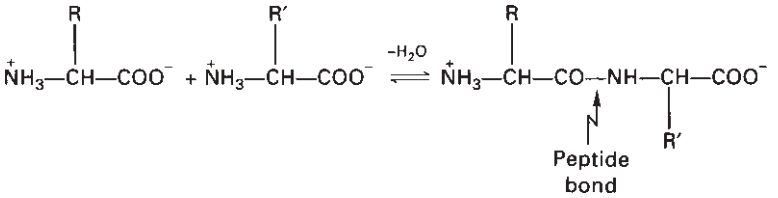
The isoionic point of a protein and its isoelectric point, unlike that of an amino acid, are generally not identical. This is because, by definition, the isoionic point is the pH at which the protein molecule possesses an equal number of positive and negative groups formed by the association of basic groups with protons and dissociation of acidic groups, respectively. In contrast, the isoelectric point is the pH at which the protein is electrophoretically immobile. In order to determine electrophoretic mobility experimentally, the protein must be dissolved in a buffered medium containing anions and cations, of low relative molecular mass, that are capable of binding to the multi-ionised protein. Hence the observed balance of charges at the isoelectric point could be due in part to there being more bound mobile anions (or cations) than bound cations (anions) at this pH. This could mask an imbalance of charges on the actual protein.

In practice, protein molecules are always studied in buffered solutions, so it is the isoelectric point that is important. It is the pH at which, for example, the protein has minimum solubility, since it is the point at which there is the greatest

opportunity for attraction between oppositely charged groups of neighbouring molecules and consequent aggregation and easy precipitation.

8.2 PROTEIN STRUCTURE

Proteins are formed by condensing the α-amino group of one amino acid or the imino group of proline with the α-carboxyl group of another, with the concomitant loss of a molecule of water and the formation of a peptide bond.



The progressive condensation of many molecules of amino acids gives rise to an unbranched polypeptide chain. By convention, the N-terminal amino acid is taken as the beginning of the chain and the C-terminal amino acid the end of the chain (proteins are biosynthesised in this direction). Polypeptide chains contain between 20 and 2000 amino acids residues and hence have a relative molecular mass ranging between about 2000 and 200 000. Many proteins have a relative molecular mass in the range 20 000 to 100 000. The distinction between a large peptide and a small protein is not clear. Generally, chains of amino acids containing fewer than 50 residues are referred to as peptides, and those with more than 50 are referred to as proteins. Most proteins contain many hundreds of amino acids (ribonuclease is an extremely small protein with only 103 amino acid residues) and many biologically active peptides contain 20 or fewer amino acids, for example oxytocin (9 amino acid residues), vasopressin (9), enkephalins (5), gastrin (17), somatostatin (14) and lutenising hormone (10).

The primary structure of a protein defines the sequence of the amino acid residues and is dictated by the base sequence of the corresponding gene(s). Indirectly, the primary structure also defines the amino acid composition (which of the possible 20 amino acids are actually present) and content (the relative proportions of the amino acids present).

The peptide bonds linking the individual amino acid residues in a protein are both rigid and planar, with no opportunity for rotation about the carbon–nitrogen bond, as it has considerable double bond character due to the delocalisation of the lone pair of electrons on the nitrogen atom; this, coupled with the tetrahedral geometry around each α-carbon atom, profoundly influences the three-dimensional arrangement which the polypeptide chain adopts.

Secondary structure defines the localised folding of a polypeptide chain due to hydrogen bonding. It includes structures such as the α-helix and β-pleated sheet. Certain of the 20 amino acids found in proteins, including proline, isoleucine, tryptophan and asparagine, disrupt α-helical structures. Some proteins have up to 70% secondary structure but others have none.

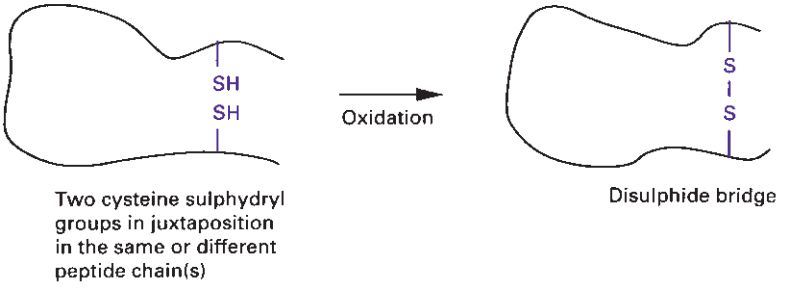


Fig. 8.1. The formation of a disulphide bridge.

Tertiary structure defines the overall folding of a polypeptide chain. It is stabilised by electrostatic attractions between oppositely charged ionic groups ($-\overset{+}{N}H_3$, COO^-), by weak van der Waals forces, by hydrogen bonding, hydrophobic interactions and, in some proteins, by disulphide (-S — S-) bridges formed by the oxidation of spatially adjacent sulphydryl groups (-SH) of cysteine residues (Fig. 8.1). The three-dimensional folding of polypeptide chains is such that the interior consists predominantly of non-polar, hydrophobic amino acid residues such as valine, leucine and phenylalanine. The polar, ionised, hydrophilic residues are found on the outside of the molecule, where they are compatible with the aqueous environment. However, some proteins also have hydrophobic residues on their outside and the presence of these residues is important in the processes of ammonium sulphate fractionation (Section 8.3.4) and hydrophobic interaction chromatography (Sections 8.3.4 and 11.4.3).

Quaternary structure is restricted to oligomeric proteins, which consist of the association of two or more polypeptide chains held together by electrostatic attractions, hydrogen bonding, van der Waals forces and occasionally disulphide bridges. Thus disulphide bridges may exist within a given polypeptide chain (intra-chain) or linking different chains (inter-chain). An individual polypeptide chain in an oligomeric protein is referred to as a subunit. The subunits in a protein may be identical or different: for example, haemoglobin consists of two α- and two β-chains, and lactate dehydrogenase of four (virtually) identical chains.

Traditionally, proteins are classified into two groups – globular and fibrous. The former are approximately spherical in shape, are generally water soluble and may contain a mixture of α-helix, β-pleated sheet and random structures. Globular proteins include enzymes, transport proteins and immunoglobulins. Fibrous proteins are structural proteins, generally insoluble in water, consisting of long cable-like structures built entirely of either helical or sheet arrangements. Examples include hair keratin, silk fibroin and collagen. The native state of a protein is its biologically active form.

The process of protein denaturation results in the loss of biological activity, decreased aqueous solubility and increased susceptibility to proteolytic degradation. It can be brought about by heat and by treatment with reagents such as acids

and alkalis, detergents, organic solvents and heavy-metal cations such as mercury and lead. It is associated with the loss of organised (tertiary) three-dimensional structure and exposure to the aqueous environment of numerous hydrophobic groups previously located within the folded structure.

In enzymes, the specific three-dimensional folding of the polypeptide chain(s) results in the juxtaposition of certain amino acid residues that constitute the active site or catalytic site. Oligomeric enzymes may possess several such sites. Many enzymes also possess one or more regulatory site(s). X-ray crystallography studies have revealed that the active site is often located in a cleft that is lined with hydrophobic amino acid residues but which contains some polar residues. The binding of the substrate at the catalytic site and the subsequent conversion of substrate to product involves different amino acid residues.

Some oligomeric enzymes exist in multiple forms called isoenzymes or isozymes (Section 15.1). Their existence relies on the presence of two genes that give similar but not identical subunits. One of the best-known examples of isoenzymes is lactate dehydrogenase, which reversibly interconverts pyruvate and lactate. It is a tetramer and exists in five forms (LDH1 to 5) corresponding to the five permutations of arranging the two types of subunits (H and M), which differ only in a single amino acid substitution, into a tetramer:

H_4	LDH1
H_3M	LDH2
H_2M_2	LDH3
HM_3	LDH4
M_4	LDH5

Each isoenzyme promotes the same reaction but has different kinetic constants (K_m, V_{max}), thermal stability and electrophoretic mobility. The tissue distribution of isoenzymes within an organism is frequently different, for example, in humans LDH1 is the dominant isoenzyme in heart muscle but LDH5 is the most abundant form in liver and muscle. These differences are exploited in diagnostic enzymology to identify specific organ damage, for example following myocardial infarction, and thereby aiding clinical diagnosis and prognosis.

8.2.1 Post-translational modifications

Proteins are synthesised at the ribosome and as the growing polypeptide chain emerges from the ribosome it folds up into its native three-dimensional structure. However, this is often not the final active form of the protein. Many proteins undergo modifications once they leave the ribosome, where one or more amino acid side chains are modified by the addition of a further chemical group; this is referred to as post-translational modification. Such changes include extensive modifications of the protein structure, for example the addition of chains of carbohydrates to form glycoproteins (see Section 8.4.4), where in some cases the final protein consists of as much as over 40% carbohydrate. Less dramatic, but equally important modifications include the addition of a hydroxyl group to proline to

produce hydroxyproline (found in the structure of collagen), or the phosphorylation of one or more amino acids (tyrosine, serine and threonine residues are all capable of being phosphorylated). Many cases are known, for example, where the addition of a single phosphate group (by enzymes known as kinases) can activate a protein molecule, and the subsequent removal of the phosphate group (by a phosphatase) can inactivate the molecule; protein phosphorylation reactions are a central part of intracellular signalling (Sections 16.5 and 16.6). Another example can be found in the post-translational modification of proline residues in the transcription factor HIF (the α subunit of the hypoxia-inducible factor), which is a key oxygen-sensing mechanism in cells. Many proteins therefore are not in their final active, biological form until post-translational modifications have taken place. Over 200 different post-translational modifications have been reported for proteins from microbial, plant and animal sources. Mass spectrometry is used to determine such modifications (see Section 9.5.6).

8.3 PROTEIN PURIFICATION

8.3.1 Introduction

At first sight, the purification of *one* protein from a cell and tissue homogenate that will typically contain 10 000–20 000 different proteins, seems a daunting task. However, in practice, on average, only four different fractionation steps are needed to purify a given protein. Indeed, in exceptional circumstances proteins have been purified in a single chromatographic step. Since the reason for purifying a protein is normally to provide material for structural or functional studies, the final degree of purity required depends on the purposes for which the protein will be used, i.e. you may not need a protein sample that is 100% pure for your studies. Indeed, to define what is meant by a 'a pure protein' is not easy. Theoretically, a protein is pure when a sample contains only a single protein species, although in practice it is more or less impossible to achieve 100% purity. Fortunately, many studies on proteins can be carried out on samples that contain as much as 5–10% or more contamination with other proteins. This is an important point, since each purification step necessarily involves loss of some of the protein you are trying to purify. An extra (and unnecessary) purification step that increases the purity of your sample from, say, 90% to 98% may mean that you now have a more pure protein, but insufficient protein for your studies. Better to have studied the sample that was 90% pure and have enough to work on!

For example, a 90% pure protein is sufficient for amino acid sequence determination studies as long as the sequence is analysed quantitatively to ensure that the deduced sequence does not arise from a contaminant protein. Similarly, immunisation of a rodent to provide spleen cells for monoclonal antibody production (Section 7.2.3) can be carried out with a sample that is considerably less than 50% pure. As long as your protein of interest raises an immune response it matters not at all that antibodies are also produced against the contaminating proteins. For

kinetic studies on an enzyme, a relatively impure sample can be used provided it does not contain any competing activities. On the other hand, if you are raising a monospecific polyclonal antibody in an animal (see Section 7.2.3), it is necessary to have a highly purified protein as antigen, otherwise immunogenic contaminating proteins will give rise to additional antibodies. Equally, proteins that are to have a therapeutic use must be extremely pure to satisfy regulatory (safety) requirements. Clearly, therefore, the degree of purity required depends on the purpose for which the protein is needed.

8.3.2 The determination of protein concentration

The need to determine protein concentration in solution is a routine requirement during protein purification. The only truly accurate method for determining protein concentration is to acid hydrolyse a portion of the sample and then carry out amino acid analysis on the hydrolysate (see Section 8.4.2). However, this is relatively time-consuming, particularly if multiple samples are to be analysed. Fortunately, in practice, one rarely needs decimal place accuracy and other, quicker methods that give a reasonably accurate assessment of protein concentrations of a solution are acceptable. Most of these (see below) are colorimetric methods, where a portion of the protein solution is reacted with a reagent that produces a coloured product. The amount of this coloured product is then measured spectrophotometrically and the amount of colour related to the amount of protein present by appropriate calibration. However, none of these methods is absolute, since, as will be seen below, the development of colour is often at least partly dependent on the amino acid composition of the protein(s). The presence of prosthetic groups (e.g. carbohydrate) also influences colorimetric assays. Many workers prepare a standard calibration curve using bovine serum albumin (BSA), chosen because of its low cost, high purity and ready availability. However, it should be understood that, since the amino acid composition of BSA will differ from the composition of the sample being tested, any concentration values deduced from the calibration graph can only be approximate.

Ultraviolet absorption

The aromatic amino acid residues tyrosine and tryptophan in a protein exhibit an absorption maximum at a wavelength of 280 nm. Since the proportions of these aromatic amino acids in proteins vary, so too do extinction coefficients for individual proteins. However, for most proteins the extinction coefficient lies in the range 0.4–1.5; so for a complex mixture of proteins it is a fair approximation to say that a solution with an absorbance at 280 nm (A_{280}) of 1.0, using a 1 cm pathlength, has a protein concentration of approximately 1 mg cm^{-3}. The method is relatively sensitive, being able to measure protein concentrations as low as 10 μg cm^{-3}, and, unlike colorimetric methods, is non-destructive, i.e. having made the measurement, the sample in the cuvette can be recovered and used further. This is particularly useful when one is working with small amounts of protein and cannot afford to waste any. However, the method is subject to

interference by the presence of other compounds that absorb at 280 nm. Nucleic acids fall into this category having an absorbance as much as 10 times that of protein at this wavelength. Hence the presence of only a small percentage of nucleic acid can greatly influence the absorbance at this wavelength. However, if the absorbances (A) at 280 and 260 nm wavelengths are measured it is possible to apply a correction factor:

$$\text{Protein (mg cm}^{-3}) = 1.55\, A_{280} - 0.76\, A_{260}$$

The great advantage of this protein assay is that it is non-destructive and can be measured continuously, for example in chromatographic column effluents.

Even greater sensitivity can be obtained by measuring the absorbance of ultraviolet light by peptide bonds. The peptide bond absorbs strongly in the far ultraviolet, with a maximum at about 190 nm. However, because of the difficulties caused by the absorption by oxygen and the low output of conventional spectrophotometers at this wavelength, measurements are usually made at 205 or 210 nm. Most proteins have an extinction coefficient for a 1 μg cm^{-3} solution of about 30 at 205 nm and about 20 at 210 nm. Clearly therefore measuring at these wavelengths is 20 to 30 times more sensitive than measuring at 280 nm, and protein concentration can be measured to less than 1 μg cm^{-3}. However, one disadvantage of working at these lower wavelengths is that a number of buffers and other buffer components commonly used in protein studies also absorb strongly at this wavelength, so it is not always practical to work at this lower wavelength.

Nowadays all purpose-built column chromatography systems (e.g. fast protein liquid chromatography and high-performance liquid chromatography (HPLC)) have in-line variable wavelength ultraviolet light detectors that monitor protein elution from columns.

Lowry (Folin–Ciocalteau) method

In the past this has been the most commonly used method for determining protein concentration, although it is tending to be replaced by the more sensitive methods described below. The Lowry method is reasonably sensitive, detecting down to 10 μg cm^{-3} of protein, and the sensitivity is moderately constant from one protein to another. When the Folin reagent (a mixture of sodium tungstate, molybdate and phosphate), together with a copper sulphate solution, is mixed with a protein solution, a blue-purple colour is produced which can be quantified by its absorbance at 660 nm. As with most colorimetric assays, care must be taken that other compounds that interfere with the assay are not present. For the Lowry method this includes Tris, zwitterionic buffers such as Pipes and Hepes, and EDTA. The method is based on both the Biuret reaction, where the peptide bonds of proteins react with Cu^{2+} under alkaline conditions producing Cu^{+}, which reacts with the Folin reagent, and the Folin–Ciocalteau reaction, which is poorly understood but essentially involves the reduction of phosphomolybdotungstate to heteropolymolybdenum blue by the copper-catalysed oxidation of aromatic amino acids. The resultant strong blue colour is therefore partly dependent on the tyrosine and tryptophan content of the protein sample.

The bicinchoninic acid method

This method is similar to the Lowry method in that it also depends on the conversion of Cu^{2+} to Cu^{+} under alkaline conditions. The Cu^{+} is then detected by reaction with bicinchoninic acid (BCA) to give an intense purple colour with an absorbance maximum at 562 nm. The method is more sensitive than the Lowry method, being able to detect down to 0.5 μg protein cm^{-3}, but perhaps more importantly it is generally more tolerant of the presence of compounds that interfere with the Lowry assay, hence the increasing popularity of the method.

The Bradford method

This method relies on the binding of the dye Coomassie Brilliant Blue to protein. At low pH the free dye has absorption maxima at 470 and 650 nm, but when bound to protein has an absorption maximum at 595 nm. The practical advantages of the method are that the reagent is simple to prepare and that the colour develops rapidly and is stable. Although it is sensitive down to 20 μg protein cm^{-3}, it is only a relative method, as the amount of dye binding appears to vary with the content of the basic amino acids arginine and lysine in the protein. This makes the choice of a standard difficult. In addition, many proteins will not dissolve properly in the acidic reaction medium.

Example 1 PROTEIN ASSAY

Question

A series of dilutions of bovine serum albumin (BSA) was prepared and 0.1 cm^3 of each solution subjected to a Bradford assay. The increase in absorbance at 595 nm relative to an appropriate blank was determined in each case, and the results are shown in the table.

Concentration of BSA (mg cm^{-3})	A_{595}
1.5	1.40
1.0	0.97
0.8	0.79
0.6	0.59
0.4	0.37
0.2	0.17

A sample (0.1 cm^3) of a protein extract from *E. coli* gave an A_{595} of 0.84 in the same assay. What was the concentration of protein in the *E. coli* extract?

Answer

If a graph of BSA concentration against A_{595} is plotted it is seen to be linear. From the graph, at an A_{595} of 0.84 it can be seen that the protein concentration of the *E. coli* extracted is 0.85 mg cm^{-3}.

Kjeldahl analysis

This is a general chemical method for determining the nitrogen content of any compound. It is not normally used for the analysis of purified proteins or for

monitoring column fractions but is frequently used for analysing complex solid samples and microbiological samples for protein content. The sample is digested by boiling with concentrated sulphuric acid in the presence of sodium sulphate (to raise the boiling point) and a copper and/or selenium catalyst. The digestion converts all the organic nitrogen to ammonia, which is trapped as ammonium sulphate. Completion of the digestion stage is generally recognised by the formation of a clear solution. The ammonia is released by the addition of excess sodium hydroxide and removed by steam distillation in a Markham still. It is collected in boric acid and titrated with standard hydrochloric acid using methyl red–methylene blue as indicator. It is possible to carry out the analysis automatically in an autokjeldahl apparatus. Alternatively, a selective ammonium ion electrode (Section 1.5.2) may be used to directly determine the content of ammonium ion in the digest. Although Kjeldahl analysis is a precise and reproducible method for the determination of nitrogen, the determination of the protein content of the original sample is complicated by the variation of the nitrogen content of individual proteins and by the presence of nitrogen in contaminants such as DNA. In practice, the nitrogen content of proteins is generally assumed to be 16% by weight.

8.3.3 Cell disruption and production of initial crude extract

The initial step of any purification procedure must, of course, be to disrupt the starting tissue to release proteins from within the cell. The means of disrupting the tissue will depend on the cell type (see Cell disruption, below), but thought must first be given to the composition of the buffer used to extract the proteins.

Extraction buffer

Normally extraction buffers are at an ionic strength (0.1–0.2 M) and pH (7.0–8.0) that is considered to be compatible with that found inside the cell. Tris or phosphate buffers are most commonly used. However, in addition a range of other reagents may be included in the buffer for specific purposes. These include:

- *An anti-oxidant:* Within the cell the protein is in a highly reducing environment, but when released into the buffer it is exposed to a more oxidising environment. Since most proteins contain a number of free sulphydryl groups (from the amino acid cysteine) these can undergo oxidation to give inter- and intramolecular disulphide bridges. To prevent this, reducing agents such as dithiothreitol, β-mercaptoethanol, cysteine or reduced glutathione are often included in the buffer.
- *Enzyme inhibitors:* Once the cell is disrupted the organisational integrity of the cell is lost, and proteolytic enzymes that were carefully packaged and controlled within the intact cells are released, for example from lysosomes. Such enzymes will of course start to degrade proteins in the extract, including the protein of interest. To slow down unwanted proteolysis, all extraction and purification steps are carried out at 4 °C, and in addition a range of protease

inhibitors is included in the buffer. Each inhibitor is specific for a particular type of protease, for example serine proteases, thiol proteases, aspartic proteases and metalloproteases. Common examples of inhibitors include: di-isopropylphosphofluoridate (DFP), phenylmethyl sulphonylfluoride (PMSF) and tosylphenylalanyl-chloromethylketone (TPCK) (all serine protease inhibitors); iodoacetate and cystatin (thiol protease inhibitors); pepstatin (aspartic protease inhibitor); EDTA and 1,10-phenanthroline (metalloprotease inhibitors); and amastatin and bestatin (exopeptidase inhibitors).

- *Enzyme substrate and cofactors:* Low levels of substrate are often included in extraction buffers when an enzyme is purified, since binding of substrate to the enzyme active site can stabilise the enzyme during purification processes. Where relevant, cofactors that otherwise might be lost during purification are also included to maintain enzyme activity so that activity can be detected when column fractions, etc. are screened.
- *EDTA:* This can be present to remove divalent metal ions that can react with thiol groups in proteins giving *mercaptids*.

$$R-SH + Me^{2+} \rightarrow R-S-Me^{+} + H^{+}$$

- *Polyvinylpyrrolidone (PVP):* This is often added to extraction buffers for plant tissue. Plant tissues contain considerable amounts of phenolic compounds (both monomeric, such as *p*-hydroxybenzoic acid, and polymeric, such as tannins) that can bind to enzymes and other proteins by non-covalent forces, including hydrophobic, ionic and hydrogen bonds, causing protein precipitation. These phenolic compounds are also easily oxidised, predominantly by endogenous phenol oxidases, to form quinones, which are highly reactive and can combine with reactive groups in proteins causing cross-linking, and further aggregation and precipitation. Insoluble PVP (which mimics the polypeptide backbone) is therefore added to adsorb the phenolic compounds which can then be removed by centrifugation. Thiol compounds (reducing agents) are also added to minimise the activity of phenol oxidases, and thus prevent the formation of quinones.
- *Sodium azide:* For buffers that are going to be stored for long periods of time, antibacterial and/or antifungal agents are sometimes added at low concentrations. Sodium azide is frequently used as a bacteriostatic agent.

Membrane proteins

Membrane-bound proteins (normally glycoproteins) require special conditions for extraction as they are not released by simple cell disruption procedures alone. Two classes of membrane proteins are identified. Extrinsic (or peripheral) membrane proteins are bound only to the surface of the cell, normally via electrostatic and hydrogen bonds. These proteins are predominantly hydrophilic in nature and are relatively easily extracted either by raising the ionic concentration of the extraction buffer (e.g. to 1 M NaCl) or by changes of pH (e.g. to pH 3–5 or pH 9–12). Once extracted, they can be purified by conventional chromatographic procedures. Intrinsic membrane proteins are those that are embedded in the membrane

(integrated membrane proteins). These invariably have significant regions of hydrophobic amino acids (those regions of the protein that are embedded in the membrane, and associated with lipids) and have low solubility in aqueous buffer systems. Hence, once extracted into an aqueous polar environment, appropriate conditions must be used to retain their solubility. Intrinsic proteins are usually extracted with buffer containing detergents. The choice of detergent is mainly one of trial and error but can include ionic detergents such as sodium dodecyl sulphate (SDS), sodium deoxycholate, cetyl trimethylammonium bromide (CTAB) and CHAPS, and non-ionic detergents such as Triton X-100 and Nonidet P-40.

Once extracted, intrinsic membrane proteins can be purified using conventional chromatographic techniques such as gel filtration, ion-exchange chromatography or affinity chromatography (using lectins). However, in each case it is necessary to include detergent in all buffers to maintain protein solubility. The level of detergent used is normally 10- to 100-fold less than that used to extract the protein, in order to minimise any interference of the detergent with the chromatographic process.

Cell disruption

Unless one is isolating proteins from extracellular fluids such as blood, protein purification procedures necessarily start with the disruption of cells or tissue to release the protein content of the cells into an appropriate buffer. This initial extract is therefore the starting point for protein purification. Clearly one chooses, where possible, a starting material that has a high level of the protein of interest. Depending on the protein being isolated one might therefore start with a microbial culture, plant tissue, or mammalian tissue. The last of these has generally been the tissue of choice where possible, owing to the relatively large amounts of starting material available. However, the ability to clone and overexpress genes for proteins from any source, in both bacteria and yeast, means that nowadays more and more protein purification protocols are starting with a microbial lysate. The different methods available for disrupting cells are described below. Which method one uses depends on the nature of the cell wall/membrane being disrupted.

Mammalian cells Mammalian cells are of the order of 10 μm in diameter and enclosed by a plasma membrane, weakly supported by a cytoskeleton. These cells therefore lack any great rigidity and are easy to disrupt by shear forces.

Plant cells Plant cells are of the order of 100 μm in diameter and have a fairly rigid cell wall, comprising carbohydrate complexes and lignin or wax that surround the plasma membrane. Although the plasma membrane is protected by this outer layer, the large size of the cell still makes it susceptible to shear forces.

Bacteria Bacteria have cell diameters of the order of 1 to 4 μm and generally have extremely rigid cell walls. Bacteria can be classified as either Gram positive or Gram negative depending on whether or not they are stained by the Gram stain (crystal violet and iodine). In Gram-positive bacteria (Fig. 8.2) the plasma

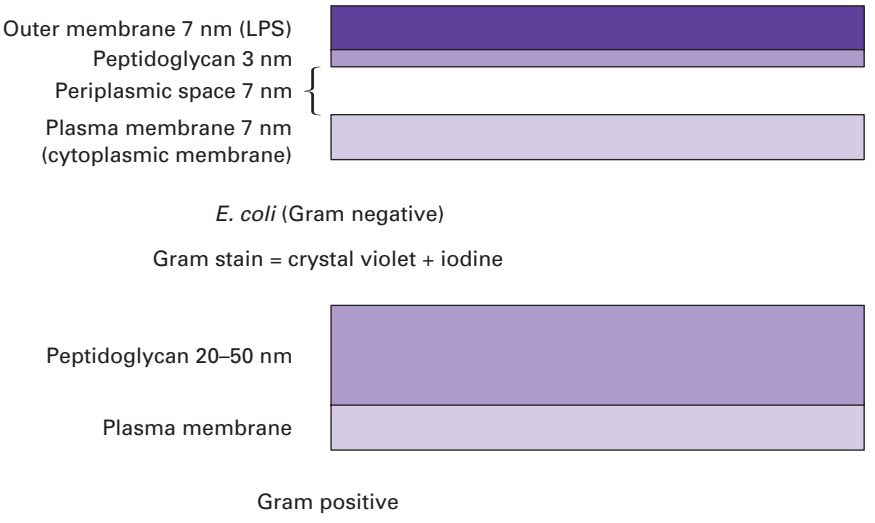


Fig. 8.2. The structure of the cell wall of Gram-positive and of Gram-negative bacteria. LPS, lipopolysaccharide.

membrane is surrounded by a thick shell of peptidoglycan (20–50 nm), which stains with the Gram stain. In Gram-negative bacteria (e.g. *Escherichia coli*) the plasma membrane is surrounded by a thin (2–3 nm) layer of peptidoglycan but this is compensated for by having a second outer membrane of lipopolysaccharide. The negatively charged lipopolysaccharide polymers interact laterally, being linked by divalent cations such as Mg^{2+}. A number of Gram-negative bacteria secrete proteins into the periplasmic space.

Fungi and yeast Filamentous fungi and yeasts have a rigid cell wall that is composed mainly of polysaccharide (80–90%). In lower fungi and yeast the polysaccharides are mannan and glucan. In filamentous fungi it is chitin cross-linked with glucans. Yeasts also have a small percentage of glycoprotein in the cell wall, and there is a periplasmic space between the cell wall and cell membrane. If the cell wall is removed the cell content, surrounded by a membrane, is referred to as a spheroplast.

Cell disruption methods

Blenders These are commercially available, although a typical domestic kitchen blender will suffice. This method is ideal for disrupting mammalian or plant tissue by shear force. Tissue is cut into small pieces and blended, in the presence of buffer, for about 1 min to disrupt the tissue, and then centrifuged to remove debris. This method is inappropriate for bacteria and yeast, but a blender can be used for these microorganisms if small glass beads are introduced to produce a bead mill. Cells are trapped between colliding beads and physically disrupted by shear forces.

Grinding with abrasives Grinding in a pestle and mortar, in the presence of sand or alumina and a small amount of buffer, is a useful method for disrupting bacterial or plant cells; cell walls are physically ripped off by the abrasive. However, the method is appropriate for handling only relatively small samples. The Dynomill is a large-scale mechanical version of this approach. The Dynomill comprises a chamber containing glass beads and a number of rotating impeller discs. Cells are ruptured when caught between colliding beads. A 600 cm^3 laboratory scale model can process 5 kg of bacteria per hour.

Presses The use of a press such as a French Press, or the Manton–Gaulin Press, which is a larger-scale version, is an excellent means for disrupting microbial cells. A cell suspension ($\sim$ 50 cm^3) is forced by a piston-type pump, under high pressure (10 000 PSI = lbf $in.^{-2} \approx$ 1450 kPa) through a small orifice. Breakage occurs due to shear forces as the cells are forced through the small orifice, and also by the rapid drop in pressure as the cells emerge from the orifice, which allows the previously compressed cells to expand rapidly and effectively burst. Multiple passes are usually needed to lyse all the cells, but under carefully controlled conditions it can be possible to selectively release proteins from the periplasmic space. The X-Press and Hughes Press are variations on this method; the cells are forced through the orifice as a frozen paste, often mixed with an abrasive. Both the ice crystal and abrasive aid in disrupting the cell walls.

Enzymatic methods The enzyme lysozyme, isolated from hen egg whites, cleaves peptidoglycan. The peptidoglycan cell wall can therefore be removed from Gram-positive bacteria (see Fig. 8.2) by treatment with lysozyme, and if carried out in a suitable buffer, once the cell wall has been digested the cell membrane will rupture owing to the osmotic effect of the suspending buffer.

Gram-negative bacteria can similarly be disrupted by lysozyme but treatment with EDTA (to remove Ca^{2+}, thus destabilising the outer lipopolysaccharide layer) and the inclusion of a non-ionic detergent to solubilise the cell membrane are also needed. This effectively permeabilises the outer membrane, allowing access of the lysozyme to the peptidoglycan layer. If carried out in an isotonic medium so that the cell membrane is not ruptured, it is possible to selectively release proteins from the periplasmic space.

Yeast can be similarly disrupted using enzymes to degrade the cell wall and either osmotic shock or mild physical force to disrupt the cell membrane. Enzyme digestion alone allows the selective release of proteins from the periplasmic space. The two most commonly used enzyme preparations for yeast are zymolyase or lyticase, both of which have β-1,3-glucanase activity as their major activity, together with a proteolytic activity specific for the yeast cell wall. Chitinase is commonly used to disrupt filamentous fungi. Enzymic methods tend to be used for laboratory-scale work, since for large-scale work their use is limited by cost.

Sonication This method is ideal for a suspension of cultured cells or microbial cells. A sonicator probe is lowered into the suspension of cells and high frequency

sound waves (>20 kHz) generated for 30–60 s. These sound waves cause disruption of cells by shear force and cavitation. Cavitation refers to areas where there is alternate compression and rarefaction, which rapidly interchange. The gas bubbles in the buffer are initially under pressure but, as they decompress, shock waves are released and disrupt the cells. This method is suitable for relatively small volumes (50–100 cm^3). Since considerable heat is generated by this method, samples must be kept on ice during treatment.

8.3.4 Fractionation methods

Monitoring protein purification

As will be seen below, the purification of a protein invariably involves the application of one or more column chromatographic steps, each of which generates a relatively large number of test tubes (fractions) containing buffer and protein eluted from the column. It is necessary to determine how much protein is present in each tube so that an elution profile (a plot of protein concentration versus tube number) can be produced. Appropriate methods for detecting and quantifying protein in solution are described in Section 8.3.2. A method is also required for determining which tubes contain the protein of interest so that their contents can be pooled and the pooled sample progressed to the next purification step. If one is purifying an enzyme, this is relatively easy as each tube simply has to be assayed for the presence of enzyme activity (Section 15.2.2).

For proteins that have no easily measured biological activity, other approaches have to be used. If an antibody to the protein of interest is available then samples from each tube can be dried onto nitrocellulose and the antibody used to detect the protein-containing fractions using the dot blot method (Section 5.9.2). Alternatively, an immunoassay such as ELISA or radioimmunoassay (Section 7.7) can be used to detect the protein. If an antibody is not available, then portions from each fraction can be run on a sodium dodecyl sulphate–polyacrylamide gel and the protein-containing fraction identified from the appearance of the protein band of interest on the gel (Section 10.3.1).

An alternative approach that can be used for cloned genes that are expressed in cells is to express the protein as a fusion protein, i.e. one that is linked via a short peptide sequence to a second protein. This can have advantages for protein purification (see Section 8.3.5). However, it can also prove extremely useful for monitoring the purification of a protein that has no easily measurable activity. If the second protein is an enzyme that can be easily assayed (e.g. using a simple colorimetric assay), such as β-galactosidase, then the presence of the protein of interest can be detected by the presence of the linked β-galactosidase activity.

A successful fractionation step is recognised by an increase in the specific activity of the sample, where the specific activity of the enzyme relates its total activity to the total amount of protein present in the preparation:

$$\text{specific activity} = \frac{\text{total units of enzyme in fraction}}{\text{total amount of protein in fraction}}$$

The measurement of units of an enzyme relies on an appreciation of certain basic kinetic concepts and upon the availability of a suitable analytical procedure. These are discussed in Section 15.2.2.

The amount of enzyme present in a particular fraction is expressed conventionally not in terms of units of mass or moles but in terms of units based upon the rate of the reaction that the enzyme promotes. The international unit (IU) of an enzyme is defined as the amount of enzyme that will convert 1 μmole of substrate to product in 1 minute under defined conditions (generally 25 or 30 °C at the optimum pH). The SI unit of enzyme activity is defined as the amount of enzyme that will convert 1 mole of substrate to product in 1 second. It has units of katal (kat) such that 1 kat $= 6 \times 10^7$ IU and 1 IU $= 1.7 \times 10^{-8}$ kat. For some enzymes, especially those where the substrate is a macromolecule of unknown relative molecular mass (e.g. amylase, pepsin, RNase, DNase), it is not possible to define either of these units. In such cases arbitrary units are used generally that are based upon some observable change in a chemical or physical property of the substrate.

For a purification step to be successful, therefore, the specific activity of the protein must be greater after the purification step than it was before. This increase is best represented as the fold purification:

$$\text{fold purification} = \frac{\text{specific activity of fraction}}{\text{original specific activity}}$$

A significant increase in specific activity is clearly necessary for a successful purification step. However, another important factor is the yield of the step. It is no use having an increased specific activity if you lose 95% of the protein you are trying to purify. Yield is defined as follows:

$$\text{yield} = \frac{\text{units of enzyme in fraction}}{\text{units of enzyme in original preparation}}$$

A yield of 70% or more in any purification step would normally be considered as acceptable. Table 8.3 shows how yield and specific activity vary during a purification schedule.

Preliminary purification steps

The initial extract, produced by the disruption of cells and tissue, and referred to at this stage as a homogenate, will invariably contain insoluble matter. For example, for mammalian tissue there will be incompletely homogenised connective and/or vascular tissue, and small fragments of non-homogenised tissue. This is most easily removed by filtering through a double layer of cheesecloth or by low speed (5000 ***g***) centrifugation. Any fat floating on the surface can be removed by coarse filtration through glass wool or cheesecloth. However, the solution will still be cloudy with organelles and membrane fragments that are too small to be conveniently removed by filtration or low speed centrifugation. These may not be much of a problem as they will often be lost in the preliminary stages of protein purification, for example during salt fractionation. However, if necessary they can be removed first by

Table 8.3 **Example of a protein purification schedule**

Fraction	Volume (cm^3)	Protein concentration (mg U cm^{-3})	Total protein (mg)	Activity[a] (mg U cm^{-3})	Total activity (U)	Specific activity (U mg^{-1})	Purification factor[b]	Overall yield[c] (%)
Homogenate	8500	40	340 000	1.8	15 300	0.045	1	100
45%–70% $(NH_4)_2SO_4$	530	194	103 000	23.3	12 350	0.12	2.7	81
CM-cellulose	420	19.5	8 190	25	10 500	1.28	28.4	69
Affinity chromotography	48	2.2	105.6	198	9500	88.4	1964	62
DEAE-Sepharose	12	2.3	27.6	633	7600	275	6110	50

[a] The unit of enzyme activity (U) is defined as that amount which produces 1 μmole of product per minute under standard assay conditions.
[b] Defined as: purification factor = (specific activity of fraction/specific activity of homogenate).
[c] Defined as: overall yield = (total activity of fraction/total activity of homogenate).
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Example 2 ENZYME FRACTIONATION

Question

A tissue homogenate was prepared from pig heart tissue as the first step in the preparation of the enzyme aspartate aminotransferase (AAT). Cell debris was removed by filtration and nucleic acids removed by treatment with polyethyleneimine, leaving a total extract (solution A) of 2 dm^3. A sample of this extract (50 mm^3) was added to 3 cm^3 of buffer in a 1 cm pathlength cuvette and the absorbance at 280 nm shown to be 1.7.

(i) Determine the approximate protein concentration in the extract, and hence the total protein content of the extract.

(ii) One unit of AAT enzyme activity is defined as the amount of enzyme in 3 cm^3 of substrate solution that causes an absorbance change at 260 nm of 0.1 min^{-1}. To determine enzyme activity, 100 mm^3 of extract was added to 3 cm^3 of substrate solution and an absorbance change of 0.08 min^{-1} was recorded. Determine the number of units of AAT actively present per cm^3 of extract A, and hence the total number of enzyme units in the extract.

(iii) The initial extract (solution A) was then subjected to ammonium sulphate fractionation. The fraction precipitating betweeen 50% and 70% saturation was collected and redissolved in 120 cm^3 of buffer (solution B). Solution B (5 mm^3 (0.005 cm^3)) was added to 3 cm^3 of buffer and the absorbance at 280 nm determined to be 0.89 using a 1 cm pathlength cuvette. Determine the protein concentration, and hence total protein content, of solution B.

(iv) Solution B 20 mm^3 was used to assay for AAT activities and an absorbance change of 0.21 per min at 260 nm was recorded. Determine the number of AAT units cm^{-3} in solution B and hence the total number of enzyme units in solution B.

(v) From your answers to (i) to (iv), determine the specific activity of AAT in both solutions A and B.

(vi) From your answers to question (v), determine the fold purification achieved by the ammonium sulphate fractionation step.

(vii) Finally, determine the yield of AAT following the ammonium sulphate fractionation step.

Answer

(i) Assuming the approximation that a 1 mg protein cm^{-3} solution has an absorbance of 1.0 at 280 nm using a 1 cm pathlength cell, then we can deduce that the protein concentration *in the cuvette* is approximately 1.7 mg cm^{-3}. Since 50 μl (0.05 cm^3) of the solution A was added to 3.0 cm^3 then the solution A sample had been diluted by a factor of 3.05/0.05 = 61.

Therefore the protein concentration of solution A is 61×1.7 mg cm^{-3} $= \sim 104$ mg cm^{-3}. Since there is 2 dm^3 (2000 cm^3) of solution A, the *total* amount of protein in solution A is $2000 \times 104 = 208\,000$ mg or 208 g.

(ii) Since one enzyme unit causes an absorbance change of 0.1 per minute, there was 0.08/0.1 = 0.8 enzyme units in the cuvette. These 0.8 enzyme units came from the 100 mm^3 of solution A that was added to the cuvette.

Therefore in 100 mm^3 of solution A there is 0.8 enzyme unit.

Therefore in 1 cm^3 of solution A there are 8.0 enzyme units.

Since we have 2000 cm^3 of solution A there is a total of $2000 \times 8.0 = 16\,000$ enzyme units in solution A.

(iii) Using the same approach as in Example 2(i), the protein concentration of solution B is $3.005/0.005 \times 0.89 = 601 \times 0.89 = 535$ mg cm^{-3}.

Therefore the total protein present in solution B $= 120 \times 535 = 64\,200$ mg.

i(iv) Using the same approach as in Example 2(ii), there are $0.21/0.1 = 2.1$ units of enzyme activity in the cuvette. These units came from the 20 mm^3 that was added to the cell.

Therefore, 20 mm^3 (0.020 cm^3) of solution B contains 2.1 enzyme units. Thus, 1 cm^3 of solution B contains $1.0/0.02 \times 2.1 = 105$ units. Therefore, solution B has 105 units cm^{-3}.

Since there are 120 cm^3 of solution B, total units in solution B $= 120 \times 105 = 12\,600$.

(v) For solution A, specific activity $= 16\,000/208\,000 = 0.077$ units mg^{-1}.
For solution B, specific activity $= 12\,600/64\,200 = 0.197$ units mg^{-1}.

(vi) Fold purification $= 0.197/0.077 = 2.6$ (approx.).

(vii) Yield $= (12\,600/16\,000) \times 100\% = 79\%$.

precipitation using materials such as Celite (a diatomaceous earth that provides a large surface area to trap the particles), Cell Debris Remover (CDR) a cellulose-based absorber, or any number of flocculants such as starch, gums, tannins or polyamines, the resultant precipitate being removed by centrifugation or filtration.

It is tempting to assume that the cell extract contains only protein, but of course a range of other molecules is present such as DNA, RNA, carbohydrate and lipid as well as any number of small molecular weight metabolites. Small molecules tend to be removed later on during dialysis steps or steps that involve fractionation based on size (e.g. gel filtration) and therefore are of little concern. However, specific attention has to be paid at this stage to macromolecules such as nucleic acids and polysaccharides. This is particularly true for bacterial extracts, which are particularly viscous owing to the presence of chromosomal DNA. Indeed microbial extracts can be extremely difficult to centrifuge to produce a supernatant extract. Some workers include DNase I in the extraction buffer to reduce viscosity, the small DNA fragments generated being removed at later dialysis/gel filtration steps. Likewise RNA can be removed by treatment with RNase. DNA and RNA can also be removed by precipitation with protamine sulphate. Protamine sulphate is a mixture of small, highly basic (i.e. positively charged) proteins, whose natural role is to bind to DNA in the sperm head. (Protamines are usually extracted from fish organs, which are obtained as a waste product at canning factories.) These positively charged proteins bind to negatively charged phosphate groups on nucleic acids, thus masking the charged groups on the nucleic acids and rendering them insoluble. The addition of a solution of protamine sulphate to the extract therefore precipitates most of the DNA and RNA, which can subsequently be removed by centrifugation. An alternative is to use polyethyleneimine, a synthetic long chain cationic (i.e. positively charged) polymer (molecular mass 24 kDa). This also binds to the phosphate groups in nucleic acids, and is very

effective, precipitating DNA and RNA almost instantly. For bacterial extracts, carbohydrate capsular gum can also be a problem as this can interfere with protein precipitation methods. This is best removed by totally precipitating the protein with ammonium sulphate (see below) leaving the gum in solution. The protein can then be recovered by centrifugation and redissolved in buffer. However, if lysozyme (plus detergent) is used to lyse the cells (see Section 8.3.3) capsular gum will not be a problem as it is digested by the lysozyme.

The clarified extract is now ready for protein fractionation steps to be carried out. The concentration of the protein in this initial extract is normally quite low, and in fact the major contaminant at this stage is water! The initial purification step is frequently based on solubility methods. These methods have a high capacity, can therefore be easily applied to large volumes of initial extracts and also have the advantage of concentrating the protein sample. Essentially, proteins that differ considerably in their physical characteristics from the protein of interest are removed at this stage, leaving a more concentrated solution of proteins that have more closely similar physical characteristics. The next stages, therefore, involve higher resolution techniques that can separate proteins with similar characteristics. Invariably these high resolution techniques are chromatographic. Which technique to use, and in which order, is more often than not a matter of trial and error. The final research paper that describes in four pages a three-step, four-day protein purification procedure invariably belies the months of hard work that went into developing the final 'simple' purification protocol!

All purification techniques are based on exploiting those properties by which proteins differ from one another. These different properties, and the techniques that exploit these differences, are as follows.

Stability Denaturation fractionation exploits differences in the heat sensitivity of proteins. The three-dimensional (tertiary) structure of proteins is maintained by a number of forces, mainly hydrophobic interactions, hydrogen bonds and sometimes disulphide bridges. When we say that a protein is denatured we mean that these bonds have by some means been disrupted and that the protein chain has unfolded to give the insoluble, 'denatured' protein. One of the easiest ways to denature proteins in solution is to heat them. However, different proteins will denature at different temperatures, depending on their different thermal stabilities; this, in turn, is a measure of the number of bonds holding the tertiary structure together. If the protein of interest is particularly heat stable, then heating the extract to a temperature at which the protein is stable yet other proteins denature can be a very useful preliminary step. The temperature at which the protein being purified is denatured is first determined by a small-scale experiment. Once this temperature is known, it is possible to remove more thermolabile contaminating proteins by heating the mixture to a temperature 5–10 deg.C below this critical temperature for a period of 15–30 min. The denatured, unwanted protein is then removed by centrifugation. The presence of the substrate, product or a competitive inhibitor of an enzyme often stabilises it and allows an even higher heat denaturation temperature to be employed. In a similar way, proteins differ in the ease with which they

are denatured by extremes of pH (<3 and >10). The sensitivity of the protein under investigation to extreme pH is determined by a small-scale trial. The whole protein extract is then adjusted to a pH not less than 1 pH unit within that at which the test protein is preciptiated. More sensitive proteins will precipitate and are removed by centrifugation.

Solubility Proteins differ in the balance of charged, polar and hydrophobic amino acids that they display on their surfaces. Charged and polar groups on the surface are solvated by water molecules, thus making the protein molecule soluble, whereas hydrophobic residues are masked by water molecules that are necessarily found adjacent to these regions. Since solubility is a consequence of solvation of charged and polar groups on the surfaces of the protein, it follows that, under a particular set of conditions, proteins will differ in their solubilities. In particular, one exploits the fact that proteins precipitate differentially from solution on the addition of species such as neutral salts or organic solvents. It should be stressed here that these methods precipitate native (i.e. active) protein that has become insoluble by aggregation; we have not denatured the protein.

Salt fractionation is frequently carried out using ammonium sulphate. As increasing salt is added to a protein solution, so the salt ions are solvated by water molecules in the solution. As the salt concentration increases, freely available water molecules that can solvate the ions become scarce. At this stage those water molecules that have been forced into contact with hydrophobic groups on the surface of the protein are the next most freely available water molecules (rather than those involved in solvating polar groups on the protein surface, which are bound by electrostatic interactions and are far less easily given up) and these are therefore removed to solvate the salt molecules, thus leaving the hydrophobic patches exposed. As the ammonium sulphate concentration increases, the hydrophobic surfaces on the protein are progressively exposed. Thus revealed, these hydrophobic patches cause proteins to aggregate by hydrophobic interaction, resulting in precipitation. The first proteins to aggregate are therefore those with most hydrophobic residues on the surface, followed by those with less hydrophobic residues. Clearly the aggregates formed are made of mixtures of more than one protein. Individual identical molecules do not seek out each other, but simply bind to another adjacent molecule with an exposed hydrophobic patch. However, many proteins are precipitated from solution over a narrow range of salt concentrations, making this a suitably simple procedure for enriching the proteins of interest.

Organic solvent fractionation is based on differences in the solubility of proteins in aqueous solutions containing water-miscible organic solvents such as ethanol, acetone and butanol. The addition of organic solvent effectively ‘dilutes out’ the water present (reduces the dielectric constant) and at the same time water molecules are used up in hydrating the organic solvent molecules. Water of solvation is therefore removed from the charged and polar groups on the surface of proteins, thus exposing their charged groups. Aggregation of proteins therefore occurs by charge (ionic) interactions between molecules. Proteins consequently

precipitate in decreasing order of the number of charged groups on their surface as the organic solvent concentration is increased.

Organic polymers can also be used for the fractional precipitation of proteins. This method resembles organic solvent fractionation in its mechanism of action but requires lower concentrations to cause protein precipitation and is less likely to cause protein denaturation. The most commonly used polymer is polyethylene glycol (PEG), with a relative molecular mass in the range 6000–20 000.

The fractionation of a protein mixture using ammonium sulphate is given here as a practical example of fractional precipitation. As explained above, as increasing amounts of ammonium sulphate are dissolved in a protein solution, certain proteins start to aggregate and precipitate out of solution. Increasing the salt strength results in further, different proteins precipitating out. By carrying out a controlled pilot experiment where the percentage of ammonium sulphate is increased stepwise say from 10% to 20% to 30% etc., the resultant precipitate at each step being recovered by centrifugation, redissolved in buffer and analysed for the protein of interests, it is possible to determine a fractionation procedure that will give a significantly purified sample. In the example shown in Table 8.3, the original homogenate was made 45% in ammonium sulphate and the precipitate recovered and discarded. The supernatant was then made 70% in ammonium sulphate, the precipitate collected, redissolved in buffer, and kept, with the supernatant being discarded. This produced a purification factor of 2.7. As can be seen, a significant amount of protein has been removed at this step (237 000 mg of protein) while 81% of the total enzyme present was recovered, i.e. the yield was good. This step has clearly produced an enrichment of the protein of interest from a large volume of extract and at the same time has concentrated the sample.

Isoelectric precipitation fractionation is based upon the observations that proteins have their minimum solubility at their isoelectric point. At this pH there are equal numbers of positive and negative charges on the protein molecule; intermolecular repulsions are therefore minimised and protein molecules can approach each other. This therefore allows opposite charges on different molecules to interact, resulting in the formation of insoluble aggregates. The principle can be exploited either to remove unwanted protein, by adjusting the pH of the protein extract so as to cause the precipitation of these proteins but not that of the test protein, or to remove the test protein, by adjusting the pH of the extract to its pI. In practice, the former alternative is preferable, since some denaturation of the precipitation protein inevitably occurs.

Finally, an unusual solubility phenomenon can be utilised in some cases for protein purification from *E. coli*. Early workers who were overexpressing heterologous proteins in *E. coli* at high levels were alarmed to discover that, although their protein was expressed in high yield (up to 40% of the total cell protein), the protein aggregated to form insoluble particles that became known as inclusion bodies. Initially this was seen as a major impediment to the production of proteins in *E. coli*, the inclusion bodies effectively being a mixture of monomeric and polymeric denatured proteins formed by partial or incorrect folding, probably due to the reducing environment of the *E. coli* cytoplasm. However, it was soon realised

that this phenomenon could be used to advantage in protein purification. The inclusion bodies can be separated from a large proportion of the bacterial cytoplasmic protein by centrifugation, giving an effective purification step. The recovered inclusion bodies must then be solubilised and denatured and subsequently allowed to refold slowly to their active, native configuration. This is normally achieved by heating in 6 M guanidinium hydrochloride (to denature the protein) in the presence of a reducing agent (to disrupt any disulphide bridges). The denatured protein is then either diluted in buffer or dialysed against buffer, at which time the protein slowly refolds. Although the refolding method is not always 100% successful, this approach can often produce protein that is 50% or more pure.

Having carried out an initial fractionation step such as that described above, one would then move towards using higher resolution chromatographic methods. Chromatographic techniques for purifying proteins are summarised in Table 8.4, and some of the more commonly used methods are outlined below. The precise practical details of each technique are discussed in Chapter 11.

Charge Proteins differ from one another in the proportions of the charged amino acids (aspartic and glutamic acids, lysine, arginine and histidine) that they contain. Hence proteins will differ in net charge at a particular pH. This difference is exploited in ion-exchange chromatography (Section 11.6), where the protein of interest is bound onto a solid support material bearing charged groups of the opposite sign (ion-exchange resin). Proteins with the same charge as the resin pass through the column to waste, after which bound proteins, containing the protein of interest, are selectively released from the column by gradually increasing the strength of salt ions in the buffer passing through the column or by gradually changing the pH of the eluting buffer. These ions compete with the protein for binding to the resin, the more weakly charged protein being eluted at the lower salt strength and the more strongly charged protein being eluted at higher salt strengths.

Another feature of the different charged groups found in proteins is the fact that most proteins will differ in their isoelectric points (Section 8.1), i.e. they will differ in the pH value at which they have zero overall charge. This difference in pI can be exploited using chromatofocusing (Section 11.6.3).

Size Size differences between proteins can be exploited in molecular exclusion (also known as gel filtration) chromatography. The gel filtration medium consists of a range of beads with slighly differing amounts of cross-linking and therefore slightly different pore sizes. The separation process depends on the different abilities of the various proteins to enter some, all or none of the beads, which in turn relates to the size of this protein (Section 11.7). The method has limited resolving power, but can be used to obtain a separation between large and small protein molecules and therefore be useful when the protein of interest is either particularly large or particularly small. This method can also be used to determine the relative molecular mass of a protein (Section 11.7.2) and for concentrating or desalting a protein solution (Section 11.7.2).

Table 8.4 Summary of chromatographic techniques commonly used in protein purification

Technique	Property exploited	Capacity	Resolution	Practical points	Further details
Hydrophobic interaction	Hydrophobicity	High	Medium	Can cope with high ionic strength samples, e.g. ammonium sulphate precipitates. Fractions are of varying pH and/or ionic strength. Medium yield. Commonly used in early stages of purification protocol. Unpredictable	Section 11.4.3
Ion exchange	Charge	High	Medium	Sample ionic strength must be low. Fractions are of varying pH and/or ionic strength. Medium yield. Commonly used in early stages of purification protocol	Section 11.6
Affinity	Biological function	Medium (cost limited)	High	Limited by availability of immobilised ligand. Elution may denature protein. Yield medium–low. Commonly used towards end of purification protocol	Section 11.8
Dye affinity	Structure and hydrophobicity		High	Necessary to carry out initial screening of a wide range of dye–ligand supports	Section 11.8.6
Chromatofocusing	Charge and pI	High–medium	High–medium	Sample ionic strength must be low. Fractions contaminated with ampholytes	Section 11.6.3
Covalent	Thiol groups	Medium–low	High	Specific for thiol-containing proteins. Limited by high cost and long (3 h) regeneration time	Section 11.8.7
Metal chelate	Imidazole, thiol, tryptophan groups	Medium–low	High	Relatively few examples in literature. Expensive	Section 11.8.5
Exclusion	Molecular size	Medium	Low	Commonly used as a final stage of purification. Can give information about protein molecular weight. Good for desalting protein samples	Section 11.7

Affinity Certain proteins bind strongly to specific small molecules. One can take advantage of this by developing an affinity chromatography system where the small molecule (ligand) is bound to an insoluble support. When a crude mixture of proteins containing the protein of interest is passed through the column, the ligand binds the protein to the matrix whilst all other proteins pass through the column. The bound protein can then be eluted from the column by changing the pH, increasing salt strength or passing through a high concentration of unbound free ligand. For example, the protein concanavalin A (con A) binds strongly to glucose. An affinity column using glucose as the ligand can therefore be used to bind con A to the matrix, and the con A can be recovered by passing a high concentration of glucose through the column. Lectins (Section 11.8.3) are particularly useful ligands for purifying glycoproteins by affinity chromatography. Affinity chromatography is covered in detail in Section 11.8.

Hydrophobicity Proteins differ in the amount of hydrophobic amino acids that are present on their surface. This difference can be exploited in salt fractionation (see above) but can also be used in a higher resolution method using hydrophobic interaction chromatography (HIC) (Section 11.4.3). A typical column material would be phenyl-Sepharose, where phenyl groups are bonded to the insoluble support Sepharose. The protein mixture is loaded on the column in high salt (to ensure hydrophobic patches are exposed) where hydrophobic interaction will occur between the phenyl groups on the resin and hydrophobic regions on the proteins. Proteins are then eluted by applying a decreasing salt gradient to the column and should emerge from the column in order of increasing hydrophobicity. However, some highly hydrophobic proteins may not even be eluted in the total absence of salt. In this case it is necessary to add a small amount of water-miscible organic solvent such as propanol or ethylene glycol to the column buffer solution. This will compete with the proteins for binding to the hydrophobic matrix and will elute any remaining proteins.

8.3.5 Engineering proteins for purification

With the ability to clone and overexpress genes for proteins using genetic engineering methodology has also come the ability to aid considerably the purification process by manipulation of the gene of interest prior to expression. These manipulations are carried out either to ensure secretion of the proteins from the cell or to aid protein purification.

Ensuring secretion from the cell

For cloned genes that are being expressed in microbial or eukaryotic cells, there are a number of advantages in manipulating the gene to ensure that the protein product is secreted from the cell:

- *To facilitate purification:* Clearly if the protein is secreted into the growth medium, there will be far fewer contaminating proteins present than if the

cells had to be ruptured to release the protein, when all the other intracellular proteins would also be present.

- *Prevention of intracellular degradation of the cloned protein:* Many cloned proteins are recognised as 'foreign' by the cell in which they are produced and are therefore degraded by intracellular proteases. Secretion of the protein into the culture medium should minimise this degradation.
- *Reduction of the intracellular concentration of toxic proteins:* Some cloned proteins are toxic to the cell in which they are produced and there is therefore a limit to the amount of protein the cell will produce before it dies. Protein secretion should prevent cell death and result in continued production of protein.
- *To allow post-translational modification of proteins:* Most post-translational modifications of proteins occur as part of the secretory pathway, and these modifications, for example glycosylation (see Section 8.4.4), are a necessary process in producing the final protein structure. Since prokaryotic cells do not glycosylate their proteins, this explains why many proteins have to be expressed in eukaryotic cells (e.g. yeast) rather than in bacteria. The entry of a protein into a secretory pathway and its ultimate destination is determined by a short amino acid sequence (signal sequence) that is usually at the N terminus of the protein. For proteins going to the membrane or outside the cell the route is via the endoplasmic reticulum and Golgi apparatus, the signal sequence being cleaved-off by a protease prior to secretion. For example, human γ-interferon has been secreted from the yeast *Pichia pastoris* using the protein's native signal sequence. Also there are a number of well-characterised yeast signal sequences (e.g. the α-factor signal sequence) that can be used to ensure secretion of proteins cloned into yeast.

Fusion proteins to aid protein purification

This approach requires an additional gene to be joined to the gene of the protein of interest such that the protein is produced as a fusion protein (i.e. linked to this second protein, or tag). As will be seen below, the purpose of this tag is to provide a means whereby the fusion protein can be selectively removed from the cell extract. The fusion protein can then be cleaved to release the protein of interest from the tag protein. Clearly the amino acid sequence of the peptide linkage between tag and protein has to be carefully designed to allow chemical or enzymatic cleavage of this sequence. The following are just a few examples of different types of fusion proteins that have been used to aid protein purification.

Flag™ This is a short hydrophilic amino acid sequence that is attached to the N-terminal end of the protein, and is designed for purification by immunoaffinity chromatography.

Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-Protein

A monoclonal antibody against this Flag sequence is available on an immobilised support for use in affinity chromatography. The cell extract, which includes the Flag-labelled protein, is passed through the column where the antibody binds to

the Flag-labelled protein, allowing all other proteins to pass through. This is carried out in the presence of Ca^{2+}, since the binding of the Flag sequence to the monoclonal antibody is Ca^{2+} dependent. Once all unbound protein has been eluted from the column, the Flag-linked protein is released by passing EDTA through the column, which chelates the Ca^{2+}. Finally the Flag sequence is removed by the enzyme enterokinase, which recognises the following amino acid sequence and cleaves C-terminal to the lysine residue

N-Asp-Asp-Asp-Lys-C

Using this approach, granulocyte–macrophage colony-stimulating factor (GMCSF) was cloned in and secreted from yeast, and purified in a single step. GMCSF was produced in the cell as signal peptide–Flag–gene. The signal sequence used was the signal sequence for the outer membrane protein OmpA. The Flag–gene protein was thus secreted into the periplasm, the fusion protein purified, and finally the Flag sequence removed, as described above.

Glutathione affinity agarose In this method the protein of interest is expressed as a fusion protein with the enzyme glutathione *S*-transferase. The cell extract is passed through a column of glutathione-linked agarose beads, where the enzyme binds to the glutathione. Once all unbound protein has been washed through the column, the fusion protein is eluted by passing reduced glutathione through the column. Finally, cleavage of the fusion protein is achieved using human thrombin, which recognises a specific amino acid sequence in the linker region.

Protein A As described in Section 7.3.4, protein A binds to the Fc region of the immunoglobulin G (IgG) molecule. The protein of interest is cloned fused to the protein A gene, and the fusion protein purified by affinity chromatography on a column of IgG–Sepharose. The bound fusion protein is then eluted using either high salt or low pH, to disrupt the binding between the IgG molecule and the protein A–protein fusion product. Protein A is then finally removed by treatment with 70% (v/v) formic acid for 2 days, which cleaves an acid-labile Asp-Pro bond in the linker region.

Poly(arginine) This method requires the addition of a series of arginine residues to the C terminus of the protein to be purified. This makes the protein highly basic (positively charged at neutral pH). The cell extract can therefore be fractionated using cation-exchange chromatography. Bound proteins are sequentially released from the column by applying a salt gradient, with the poly(Arg)-containing protein, because of its high overall positive charge, being the last to be eluted. The poly(Arg) tail is then removed by incubation with the enzyme carboxypeptidase B. Carboxypeptidase B is an exoprotease that sequentially removes arginine or lysine residues from the C terminus of proteins. The arginine residues are therefore sequentially removed from the C terminus, the removal of amino acid residues stopping when the 'normal' (i.e. non-arginine) C-terminal amino acid residue of the protein is reached.

8.4 PROTEIN STRUCTURE DETERMINATION

8.4.1 Relative molecular mass

There are three methods available for determining protein relative molecular mass, M_r, frequently referred to as molecular weight. The first two described here are quick and easy methods that will give a value to ± 5–10%. For many purposes one simply needs a rough estimate of size and these methods are sufficient. The third method, mass spectrometry, requires expensive specialist instruments and can give accuracy to ± 0.001%. This kind of accuracy is invaluable in detecting postsynthetic modification of proteins.

SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

This form of electrophoresis, described in Section 10.3.1, separates proteins on the basis of their shape (size), which in turn relates to their relative molecular masses. A series of proteins of known molecular mass (molecular weight markers) are run on a gel on a track adjacent to the protein of unknown molecular mass. The distance each marker protein moves through the gel is measured and a calibration curve of log M_r versus distance moved is plotted. The distance migrated by the protein of unknown M_r is also measured, and from the graph its log M_r and hence M_r is calculated. The method is suitable for proteins covering a large M_r range (10 000–300 000). The method is easy to perform and requires very little material. If silver staining (Section 10.3.7) is used, as little as 1 ng of protein is required. In practice SDS–PAGE is the most commonly used method for determining protein M_r values.

Molecular exclusion (gel filtration) chromatography

The elution volume of a protein from a molecular exclusion chromatography column having an appropriate fractionation range is determined largely by the size of the protein such that there is a logarithmic relationship between protein relative molecular mass and elution volume (Section 11.7.3). By calibrating the column with a range of proteins of known M_r, the M_r of a test protein can be calculated. The method is carried out on HPLC columns ($\sim 1 \times 30$ cm) packed with porous silica beads. Flow rates are about 1 $cm^3\ min^{-1}$, giving a run time of about 12 min, producing sharp, well-resolved peaks. A linear calibration line is obtained by plotting a graph of log M_r versus K_d for the calibrating proteins. K_d is calculated from the following equation:

$$K_d = \frac{(V_e - V_o)}{(V_t - V_o)}$$

where V_o is the volume in which molecules that are wholly excluded from the column material emerge (the excluded volume), V_t is the volume in which small molecules that can enter all the pores emerge (the included volume) and V_e is the volume in which the marker protein elutes. This method gives values that are accurate to ± 10%.

Mass spectrometry

Using either electrospray ionisation (ESI) (Section 9.2.4) or matrix-assisted laser desorption ionisation (MALDI) (Section 9.3.7) intact molecular ions can be produced for proteins and hence their masses accurately measured by mass spectrometry. ESI produces molecular ions from molecules with molecular masses up to and in excess of 100 kDa, whereas MALDI produces ions from intact proteins up to and in excess of 200 kDa. In either case, only low picomole quantities of protein are needed. For example, $\alpha\beta_2$ crystallin gave a molecular mass value (20 200 $\pm$ 0.9), in excellent agreement with the deduced mass of 20 201. However, in addition about 10% of the analysed material produced an ion of mass 20 072.2. This showed that some of the purified protein molecules had lost their N-terminal amino acid (lysine). The deduced mass with the loss of N-terminal lysine was 20 072.8. Clearly mass spectrometry has the ability to provide highly accurate molecular mass measurements for proteins and peptides, which in turn can be used to deduce small changes made to the basic protein structure.

8.4.2 Amino acid analysis

The determination of which of the 20 possible amino acids are present in a particular protein, and in what relative amounts, is achieved by hydrolysing the protein to yield its component amino acids and identifying and quantifying them chromatographically. Hydrolysis is achieved by heating the protein with 6 M hydrochloride acid for 14 h at 110 °C *in vacuo*. Unfortunately, the hydrolysis procedure destroys or chemically modifies the asparagine, glutamine and tryptophan residues. Asparagine and glutamine are converted to their corresponding acids (Asp and Glu) and are quantified with them. Tryptophan is completely destroyed and is best determined spectrophotometrically on the unhydrolysed protein.

The amino acids in the protein hydrolysate may be separated chromatographically and quantified by postcolumn derivatisation with an appropriate reagent. In postcolumn derivatisation methods, the effluent stream from the chromatography column is mixed, in-line, with a reagent that reacts with the amino groups of amino acids to produce a coloured or fluorescent product. The effluent then continues to pass through an appropriate detector (colorimeter or fluorimeter) and the amount of colour/fluorescence recorded, on a chart recorder, where each amino acid is recorded as a separate peak, the area under the peak being proportional to the amount of that amino acid. The apparatus dedicated to the analysis of amino acids in mixtures by this technique is referred to as an amino acid analyser. In the original procedure, separation was achieved by ion-exchange chromatography on a sulphated polystyrene column and ninhydrin was used as the colour reagent and was sensitive down to about 50–100 pmol of the amino acid. Later, *o*-phthalaldehyde (Fig. 8.3) and fluorescamine, both of which give fluorescent products, became the reagents of choice, since they enabled as little as 10 pmol of an amino acid to be detected by fluorimetry.

In recent years, precolumn derivatisation of amino acids, followed by separation by reversed-phase HPLC has become attractive and has generally superseded the

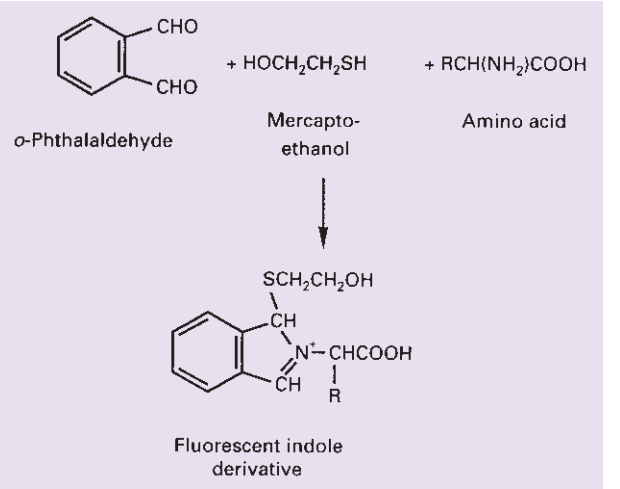

Fig. 8.3. The reaction of an amino acid with *o*-phthalaldehyde for pre- or postcolumn derivatisation.

original ion-exchange method for the quantification of amino acids in a protein hydrolysate. In this approach the amino acid hydrolysate is first treated with a molecule that (i) reacts with amino groups in amino acids, (ii) is hydrophobic, thus allowing separation of derivatised amino acids by reversed-phase HPLC and (iii) is easily detected by its ultraviolet absorbance of fluorescence. Reagents routinely used for precolumn derivatisation include *o*-phthalaldehyde (see Fig. 8.3) and 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC), which both produce fluorescent derivatives, and phenylisothiocyanate, which produces a phenylthiocarbamyl derivative that is detected by its absorbance at 254 nm. Analysis times can be as little as 20 min, and sensitivity is down to 1 pmole or less of amino acid.

8.4.3 Primary structure determination

For many years the amino acid sequence of a protein was determined from studies made on the purified protein alone. This in turn meant that sequence data available were limited to those proteins that could be purified in sufficiently large amounts. Knowledge of the complete primary structure of the protein was (and still is) a prerequisite for the determination of the three-dimensional structure of the protein, and hence an understanding of how that protein functions. However, nowadays the protein biochemist is normally satisfied with data from just a relatively short length of sequence either from the N terminus of the protein or from an internal sequence, obtained by sequencing peptides produced by cleavage of the native protein. The sequence data will then most likely be used for one of three purposes:

- To search sequence databases to see whether the protein of interest has already been isolated, and hence can therefore be identified. For this type of search

extremely short lengths of sequence (three to five residues), known as sequence tags, need to be used. An example of this type of data search is given in Section 9.5.2.

- To search for sequence homology using computerised databases in order to identify the function of the protein. For example, the search may show significant sequence identity with the amino acid sequence of some known protein tyrosine kinases, strongly suggesting that the protein is also a tyrosine kinase.
- The sequence will be used to design an oligonucleotide probe for selecting appropriate clones from complementary DNA libraries. In this way the DNA coding for the protein can be isolated and the DNA sequence, and hence the protein sequence, determined. Obtaining a protein sequence in this way is far less laborious and time-consuming than having to determine the total protein sequence by analysis of the protein.

A further use of protein sequence data is in quality control in the biopharmaceutical industry. Many pharmaceutical companies produce products that are proteins, for example peptide hormones, antibodies, therapeutic enzymes, etc., and synthetic peptides also require analysis to confirm their identities. Sequence analysis, especially to determine sites and nature of postsynthetic modifications such as glycosylation, is necessary to confirm the structural integrity of these products.

Edman degradation

In 1950, Per Edman published a chemical method for the stepwise removal of amino acid residues from the N terminus of a peptide or protein. This series of reactions has come to be known as the Edman degradation, and the method remains, 50 years after its introduction, the most effective chemical means for removing amino acid residues in a stepwise fashion from a polypeptide chain. The reactions comprise three stages (see Fig. 8.4):

- *The coupling reaction:* In this step phenylisothiocyanate (PITC) reacts with the amino group to give the phenylthiocarbamyl (PTC) derivative of the peptide. The reaction is carried out in an inert atmosphere (argon) to avoid oxidation of the sulphur atom in PITC. Following the reaction, the PTC derivative is washed thoroughly with an organic solvent (e.g. benzene) to extract excess PITC and side products, and then dried under vacuum.
- *The cleavage reaction:* In this step, the dried PTC derivative is treated with an anhydrous acid (e.g. heptafluorobutyric acid). This results in the cleavage of the PTC-polypeptide at the peptide bond nearest to the PTC substituent thus releasing the original N-terminal amino acid residue as the 2-anilino-5-thiazolinone derivative, leaving the original polypeptide chain less its N-terminal amino acid residue. Following the cleavage reaction, the anhydrous acid is removed under vacuum and the thiazolinone derivative extracted from the remaining peptide with an organic solvent and recovered by evaporation of the organic solvent.

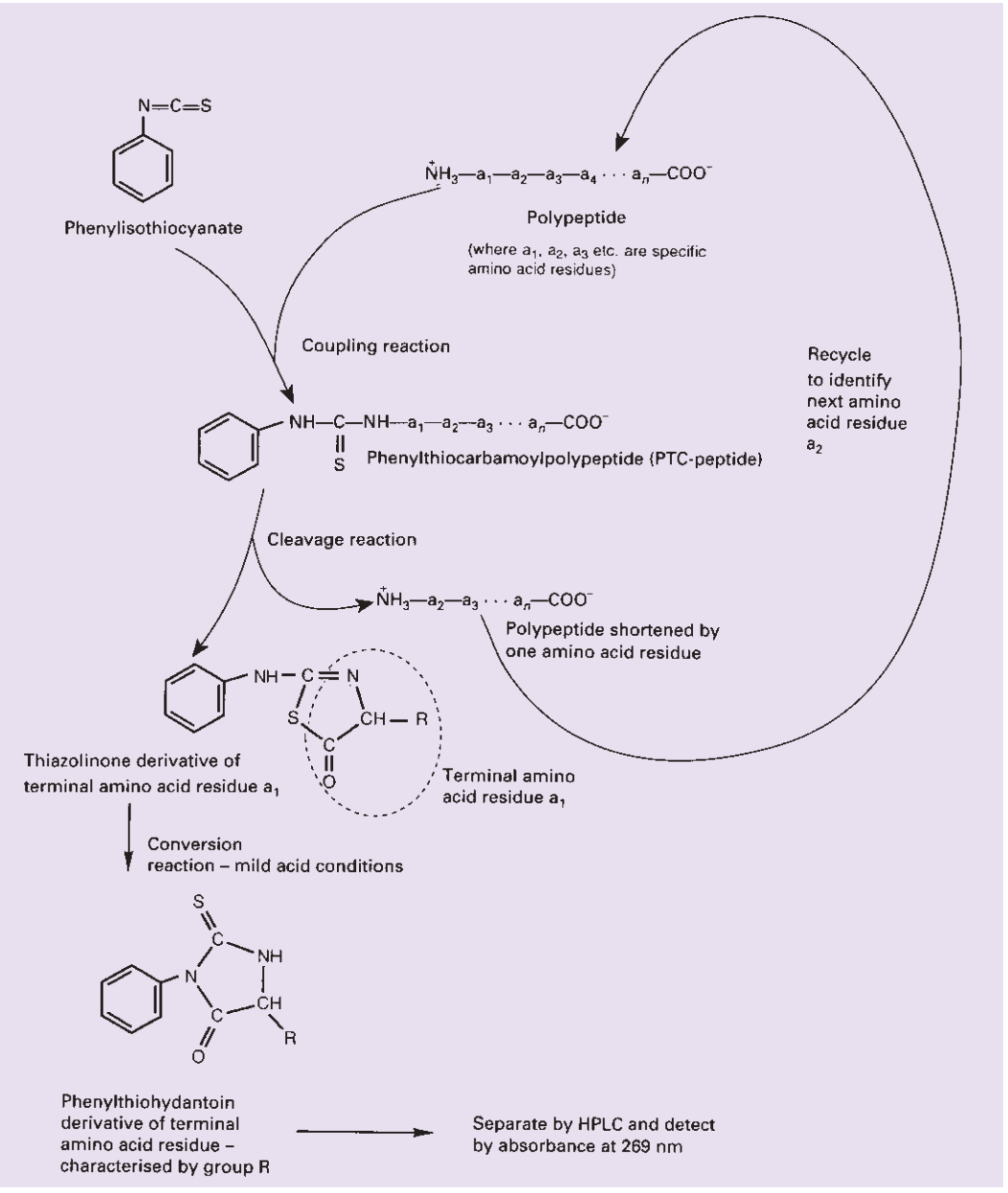

Fig. 8.4. Edman degradation for sequencing of amino acid residues (a, to a_n) in a polypeptide. Each cycle releases the N-terminal amino acid residue as a phenylthiohydantoin derivative, which is then identified chromatographically against reference compounds.

- *The conversion reaction:* Since the thiazolinone is a derivative of the N-terminal amino acid, it could, in principle, be used for identification of that amino acid. However, in practice this is not done, since thiazolinones are relatively unstable. A more stable derivative is obtained by heating the thiazolinone in 1 M HCl at 80 °C for 10 min to convert it to the more stable, isomeric 3-phenyl-2-thiohydantoin (PTH) derivative. This PTH-amino acid is therefore the end-product of one cycle of the Edman degradation and can be easily identified by reversed-phase (RP) HPLC. Thus, if PTH-alanine is identified, we know that the first amino acid residue in the protein was alanine. The remaining polypeptide chain may now be subjected to further cycles of Edman degradation. At the end of each cycle, a PTH-amino acid is recovered and identified. In this way the amino acid sequence of a polypeptide chain may be determined. Theoretically one should be able to apply a series of Edman degradation reactions to a protein and obtain a complete sequence. In practice this is not possible, since the Edman degradation gives only a 96–98% repetitive yield at each step. Thus, after a number of cycles, the yield of PTH-amino acid drops and the background level of PTH-amino acids increases. However, sequences of 20–40 amino acid residues are not uncommon and are frequently sufficient for the purposes outlined above.

The Edman degradation is invariably carried out in an automated analyser, where all steps, including injection onto the reversed-phase HPLC column and identification of the PTH derivative, are carried out automatically. Analysers can therefore be loaded and run overnight and data obtained first thing the following morning. Protein samples in solution are dried onto a small glass fibre disc that is inserted into the reaction chamber of the machine. This ensures that the protein is spread over a very large surface area (i.e. over every glass thread that goes to make up the glass fibre disc). Therefore, even though the protein is denatured by the rigorous chemical treatments of the Edman degradation, the insoluble protein is precipitated as an extremely thin film, thus allowing high reactivity at each step of the Edman cycle. Even proteins that have been blotted onto membranes from gels can be sequenced in the machine. Following blotting (e.g. from a two-dimensional gel), the protein spot of interest can be cut out and placed in the reaction cartridge. In this case it is necessary to use a particulary resistant form of membrane, poly(vinylidene difluoride) (PVDF), since normal nitrocellular membranes are unstable to many of the reagents involved in the Edman degradation. Nowadays sequence data can be obtained from as little as 10–100 ng of protein, the sensitivity of this method being limited only by the sensitivity of detection of the PTH derivatives by ultraviolet absorbance during HPLC.

Protein cleavage and peptide production

Clearly the Edman method determines the amino acid sequence from the N terminus of the protein and requires a free amino group at the N terminus for reaction with PITC. However, it is estimated that 50–70% of all proteins have their N-terminal amino group blocked (e.g. by a formyl, acetyl or acyl group). For such

Table 8.5 Specific cleavage of polypeptide

Reagent	Specificity
Enzymic cleavage	
Chymotrypsin	C-terminal side of hydrophobic amino acid residues, e.g. Phe, Try, Tyr, Leu
Endoproteinase Arg-C	C-terminal side of arginine
Endoproteinase Asp-N	Peptide bonds N-terminal to aspartate or cysteine residues
Trypsin	C-terminal side of arginine and lysine residues but Arg-Pro and Lys-Pro poorly cleaved
Endoproteinase Glu-C	C-terminal side of glutamate residues and some aspartate residues
Endoproteinase Lys-C	C-terminal side of lysine
Thermolysin	N-terminal side of hydrophobic amino acid residues excluding Trp
Chemical cleavage	
BNPS skatole *N*-Bromosuccinimide *o*-Iodosobenzoate	C-terminal side of tryptophan residues
Cyanogen bromide	C-terminal side of methionine residues
Hydroxylamine	Asparagine–glycine bonds
2-Nitro-5-thiocyanobenzoate	N-terminal side of cysteine residues

proteins determining an N-terminal amino acid sequence is not possible, so they have to be cleaved to produce peptides, one or more of which can be purified and sequenced to give details of a region from within the protein. (For the reasons described above for requiring protein sequence data, it is usually immaterial whether the sequence comes from the N terminus or from within the protein.) Peptides can be produced by either chemical or enzymatic cleavage of the native protein (see Table 8.5). Chemical methods include the use of cyanogen bromide, which cleaves at methionine residues, and *N*-bromosuccinimide, which cleaves at tryptophan residues. Methione is a relatively rare amino acid in proteins, and tryptophan even rarer, so these methods tend to produce large peptides. Enzymatic methods include the use of trypsin, which cleaves C-terminal to arginine and lysine residues, endoproteinase Arg-C, which cleaves C-terminal to arginine only, and endoproteinase Glu-C, which cleaves C-terminal to glutamate and some aspartate residues. Clearly it is useful to have the amino acid composition of the protein (Section 8.4.2) to help to decide which method to use; obviously it is better to cleave at an amino acid that is present at relatively low amounts, thus producing a small number of large peptides rather than a more complex mixture of smaller peptides. The peptide hydrolysate thus produced is then fractionated using RP-HPLC. It may seem a little odd to be separating peptides that invariably contain a large proportion of charged and polar groups using a method based on hydrophobicity. However, the standard conditions used to separate peptides mask the polar groups of peptides and give the peptides an overall hydrophobic

characteristic. Peptides are frequently dissolved in 1% (v/v) trifluoroacetic acid prior to RP-HPLC. Under these acid conditions, carboxyl groups (-COO^-) in the peptide (from the C terminus and the side-chains of any Asp or Glu residues present) are protonated (-COOH) thus masking the charged nature of this group. Any positively charged groups (form the N-terminal amino group and the side-chains of Lys, His and Arg residues) can pair with the trifluoroacetyl group (CF_3COO^-), masking the positive charge and indeed now giving these groups hydrophobic character due to the hydrophobic trifluoroacetyl group. The overall appearance of the peptide under these conditions is therefore of a non-charged, hydrophobic molecule, with of course the side-chains of any hydrophobic residues present in the peptide (Leu, Tyr, Phe, etc.) also contributing to the peptide's hydrophobicity. All peptides then bind to a RP-HPLC column and can be sequentially eluted, in order of increasing hydrophobicity, by the application of a linear gradient of acetonitrile (methyl cyanide), which competes for the hydrophobic interaction between the peptide and the column material.

Mass spectrometry

Because of the absolute requirement to produce ions in the gas phase for the analysis of any sample by mass spectrometry (MS), for many years MS analysis was applicable only to small, non-polar molecules (< 500 M_r). However, recent developments in ionisation technology such as the introduction of fast atom bombardment (FAB), ESI and MALDI methods (see Chapter 9), now means that the analysis of large, charged molecules such as proteins and peptides are routinely achieved. Indeed, the analysis of proteins and peptides by MS is now becoming routine, particularly with the introduction of smaller (and cheaper) bench-top mass spectrometers. Although the Edman degradation still has applications in protein structure analysis, mass spectrometry is now used more routinely to determine amino acid sequence data. Also, of course, proteins with blocked N-terminal residues can be sequenced directly by MS. When peptides are fragmented it is fortunate that the break occurs predominantly at the peptide bond (although it must be noted that other fragmentations, such as internal cleavages, secondary fragmentations, etc. do occur, thus complicating the mass spectrum). This means that the peptide fragments produced each differ sequentially by the mass of one amino acid residue. The amino acid sequence can thus be readily deduced. In particular, if side-chain modifications occur, these can also be observed due to the corresponding increase in mass difference. The use of mass spectrometry to obtain sequence data from proteins and peptides is described more fully in Section 9.3. Tandem mass spectrometry (MS/MS or MS^2) is also increasingly being used to obtain sequence data. A digest of the protein (e.g. with trypsin) is separated by MS. The ion corresponding to one peptide is selected in the first analyser and collided with argon gas in a collision cell to generate fragment ions. The fragment ions thus generated are then separated, according to mass, in a second analyser, identified, and the sequence determined as described in Section 9.3.

A further method, ladder sequencing (Section 9.5.5), has been developed, and combines the Edman chemistry with MS. Edman sequencing is carried out using a

mixture of PITC and phenylisocyanate (PIC) (at about 5% of the concentration of PITC). N-terminal amino groups that react with PIC are effectively blocked as they are not cleaved at the acid cleavage step. Consequently, at each cycle, approximately 5% of the protein molecules are blocked. Thus, after 20 to 30 cycles of Edman degradation, a nested set of peptides is produced, each differing by the loss of one amino acid. Analysis of the mass of each of these polypeptides using ESI or MALDI allows the determination of the molecular mass of each polypeptide and the difference in mass between each molecule identifies the lost amino acid residue.

Detection of disulphide linkages

For proteins that contain more than one cysteine residue it is important to determine whether, and if so how many, cysteine residues are joined by disulphide bridges. The most commonly used method involves the use of MS (Section 9.5.6). The native protein (i.e. with disulphide bridges intact) is cleaved with a proteolytic enzyme (e.g. trypsin) to produce a number of small peptides. The same experiment is also carried out on proteins treated with dithiothreitol (DTT) which reduces (cleaves) the disulphide bridges. MALDI spectra of the tryptic digest before and after reduction with DTT allows identification of disulphide-linked peptides. Linked peptides from the native protein will disappear from the spectrum of the reduced protein and reappear as *two* peptides of lower mass. Knowledge of the exact mass of each of the two peptides, and knowledge of the cleavage site of the enzyme used, will allow easy identification of the two peptides from the known protein sequence. Thus, if the mass of two disulphide-linked peptides is M, and this is reduced to two separate chains of masses A and B, respectively, then $A + B = M + 2$. The extra two mass units derive from the fact that reduction of the disulphide bond results in an increase of mass of + 1 for both cysteine residues.

$$\text{-S} - \text{S-} \xrightarrow{2\text{H}} - \text{SH} + \text{HS-}$$

Hydrophobicity profile

Having determined the amino acid sequence of a protein, analysis of the distribution of hydrophobic groups along the linear sequence can be used in a predictive manner. This requires the products of a hydrophobicity profile for the protein, which graphs the average hydrophobicity per residue against the sequence number. Averaging is achieved by evaluating, using a predictive algorithm, the mean hydrophobicity within a moving window that is stepped along the sequence from each residue to the next. In this way, a graph comprising a series of curves is produced and reveals areas of minima and maxima in hydrophobicity along the linear polypeptide chain. For membrane proteins, such profiles allow the identification of potential membrane-spanning segments. For example, an analysis of a thylakoid membrane protein revealed seven general regions of the protein sequence that contained spans of 20–28 amino acid residues, each of which contained predominantly hydrophobic residues flanked on either side by

hydrophilic residues. These regions represent the seven membrane-spanning helical regions of the protein.

For membrane proteins defining aqueous channels, hydrophilic residues are also present in the transmembrane section. Pores comprise amphipathic α-helices, the polar sides of which line the channel, whereas the hydrophobic sides interact with the membrane lipids. More advanced algorithms are used to detect these sequences, since such helices would not necessarily be revealed by simple hydrophobicity analysis.

8.4.4 Glycoproteins

Glycoproteins result from the covalent attachment of carbohydrate chains (glycans), both linear and branched in structure, to various sites on the polypeptide backbone of a protein. These post-translational modifications are carried out by cytoplasmic enzymes within the endoplasmic reticulum and Golgi apparatus. The amount of polysaccharide attached to a given glycoprotein can vary enormously, from as little as a few per cent to more than 60% by weight. Glycoproteins tend to be found in the serum and in cell membranes. The precise role played by the carbohydrate moiety of glycoproteins includes stabilisation of the protein structure, protection of the protein from degradation by proteases, control of protein half-life in blood, the physical maintenance of tissue structure and integrity, a role in cellular adhesion and cell–cell interaction, and as an important determinant in receptor–ligand binding.

The major types of protein glycoconjugates are:

- N-linked;
- O-linked;
- glycosylphosphatidylinositol (GPI)-linked.

N-linked glycans are always linked to an asparagine residue side-chain (Fig. 8.5) at a consensus sequence Asn-X-Ser/Thr where X is any amino acid except proline. O-linked glycosylation occurs where carbohydrate is attached to the hydroxyl group of a serine or threonine residue (Fig. 8.5). However, there is no consensus sequence similar to that found for N-linked oligosaccharides. GPI membrane anchors are a more recently discovered modification of proteins. They are complex glycophospholipids that are covalently attached to a variety of externally expressed plasma membrane proteins. The role of this anchor is to provide a stable association of protein with the membrane lipid bilayer, and will not be discussed further here.

There is considerable interest in the determination of the structure of O- and N-linked oligosaccharides, since glycosylation can affect both the half-life and function of a protein. This is particularly important of course when producing therapeutic glycoproteins by recombinant methods as it is necessary to ensure that the correct carbohydrate structure is produced. It should be noted that prokaryotic cells do not produce glycoproteins, so cloned genes for glycoproteins need to be expressed in eukaryotic cells. The glycosylation of proteins is a complex

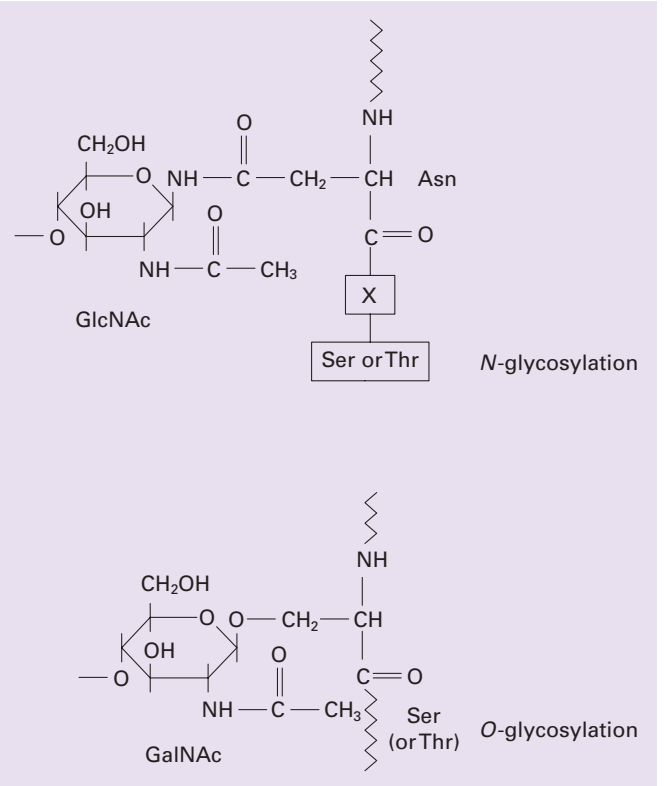

Fig. 8.5. The two types of oligosaccharide linkages found in glycoproteins.

subject. From one glycoprotein to another there are variations in the sites of glycosylation (e.g. only about 30% of consensus sequences for N-linked attachments are occupied by polysaccharide; the nature of the secondary structure at this position also seems to play a role in deciding whether glycosylation takes place), variations in the type of amino acid–carbohydrate bond, variations in the composition of the sugar chains, and variations in the particular carbohydrate sequences and linkages in each chain. There are eight monosaccharide units commonly found in mammalian glycoproteins, although other less common units are also known to occur. These eight are *N*-acetyl neuraminic acid (NeuNAc), *N*-glycolyl neuraminic acid (NeuGc), D-galactose (Gal), *N*-acetyl-D-glucosamine (GlcNac), *N*-acetyl-D-galactosamine (GalNAc), D-mannose (Man), L-fucose (Fuc) and D-xylose (Xyl). To further complicate the issue, within any population of molecules in a purified glycoprotein there can be considerable heterogeneity in the carbohydrate structure (glycoforms). This can include some molecules showing increased branching of sugar side-chains, reduced chain length and further addition of single carbohydrate units to the same polypeptide chain. The complete determination of the glycosylation status of a molecule clearly requires considerable effort. However, the steps involved are fairly straightforward and the following therefore provides a generalised (and idealised) description of the overall procedures used.

The first question to be asked about a purified protein is 'Is it a glycoprotein?' Glycoprotein bands in gels (e.g. on SDS-polyacrylamide gels) can be stained with cationic dyes such as Alcian Blue, which bind to negatively charged glycosaminoglycan side-chains, or by the periodic acid–Schiff reagent (PAS), where carbohydrate is initially oxidised by periodic acid then subsequently stained with Schiff's reagent. However, although they are both carbohydrate specific (i.e. non-glycosylated proteins are not stained) both methods suffer from low sensitivity. A more sensitive, and informative, approach is to use the specific carbohydrate-binding proteins known as lectins. Blots from SDS–PAGE, dot blots of the glycoprotein sample, or the glycoprotein sample adsorbed onto the walls of a microtitre plates can be challenged with enzyme-linked lectins. Lectins that bind to the glycoprotein can be identified by the associated enzymic activity. By repeating the experiment with a range of different lectins, one can not only confirm the presence of a glycoprotein but also identify which sugar residues are, or are not, present. Having confirmed the presence of glycoprotein the following procedures would normally be carried out.

- *Identification of the type and amount of each monosaccharide:* Release of monosaccharides is achieved by hydrolysis in methanolic HCl at 80 °C for 18 h. The released monosaccharide can be separated and quantified by gas chromatography.
- *Protease digestion to release glycopeptide:* A protease is chosen that cleaves the glycoprotein into peptides and glycopeptides of ideally 5–15 amino acid residues. Glycopeptides are then fractionated by HPLC and purified glycopeptides subjected to N-terminal sequence analysis to allow identification of the site of glycosylation.
- *Oligosaccharide profiling:* Oligosaccharide chains are released from the polypeptide backbone either chemically, for example by hydrazinolysis to release N-linked oligosaccharide, or enzymatically using peptide-*N*-glucosidase F (PNGase F), which cleaves sugars at the asparagine link, or using endo-α-*N*-acetylgalactosaminidase (*O*-glycanase), which cleaves O-linked glycans. These released oligosaccharides can then be separated either by HPLC or by high performance anion exchange chromatography (HPAEC).
- *Structure analysis of each purified oligosaccharide:* This requires the determination of the composition, sequence and nature of the linkages in each purified oligosaccharide. A detailed description is beyond the scope of this book, but would involve a mixture of complementary approaches including analysis by FAB–MS, gas chromatography–MS, lectin analysis following partial release of sugars and nuclear magnetic resonance (NMR) analysis.

8.4.5 Tertiary structure

The most commonly used method for determining protein three-dimensional structure is X-ray crystallography. A detailed description of the theory and methodology is beyond the scope of this book, requiring a detailed mathematical understanding of the process and computer analysis of the extensive data that are generated. The

following is therefore a brief and idealised description of the overall process, and ignores the multitude of pitfalls and problems inherent in determining three-dimensional structures.

- Clearly the first step must be to produce a crystal of the protein (a crystal should be thought of as a three-dimensional lattice of molecules). Protein crystallisation is attempted using as homogeneous a preparation as possible, such preparations having a greater chance of yielding crystals than material that contains impurities. Because of our inadequate understanding of the physical processes involved in crystallisation, methods for growing protein crystals are generally empirical, but basically all involve varying the physical parameters that affect solubility of the protein – for example pH, ionic strength, temperature, presence of precipitating agents – to produce a state of supersaturation. The process involves extensive trial and error to find a procedure that results in crystals for a particular protein. Initially this involves a systematic screen of methods to identify those conditions that indicate crystallinity, followed by subsequent experiments that involve fine-tuning of these conditions. Basically, nucleation sites of crystal growth are formed by chance collisions of molecules forming molecular aggregates, and the probability that these aggregates will occur will be greater in a saturated solution. Clearly, to produce saturated solutions, tens of milligrams of proteins are required. This used to represent a considerable challenge for other than the most abundant proteins, but nowadays genetic engineering methodology allows the overproduction of most proteins from cloned genes almost on demand. The following are some of the methods that have proved successful.

  (a) *Dialysis.* A state of supersaturation is achieved by dialysis of the protein solution against a solution containing a precipitant, or by a gradual change in pH or ionic strength. Because of frequent limitations on the amount of protein available, this approach often uses small volumes ($< 50\ mm^3$) for which a number of microdialysis techniques exist.

  (b) *Vapour diffusion.* This process relies on controlled equilibration through the vapour phase to produce supersaturation in the sample. For example, in the hanging-drop method, a microdroplet ($2–20\ mm^3$) of protein is deposited on a glass coverslip; then the coverslip is inverted and placed over a sealed reservoir containing a precipitant solution, with the droplet initially having a precipitant concentration lower than that in the reservoir. Vapour diffusion will then gradually increase the concentration of the protein solution. Because of the small volumes involved this method readily lends itself to screening large numbers of different conditions.

  When produced, crystals may not be of sufficient size for analysis. In this case larger crystals can be obtained by using a small crystal to seed a supersaturated protein solution, which will result in a larger crystal.

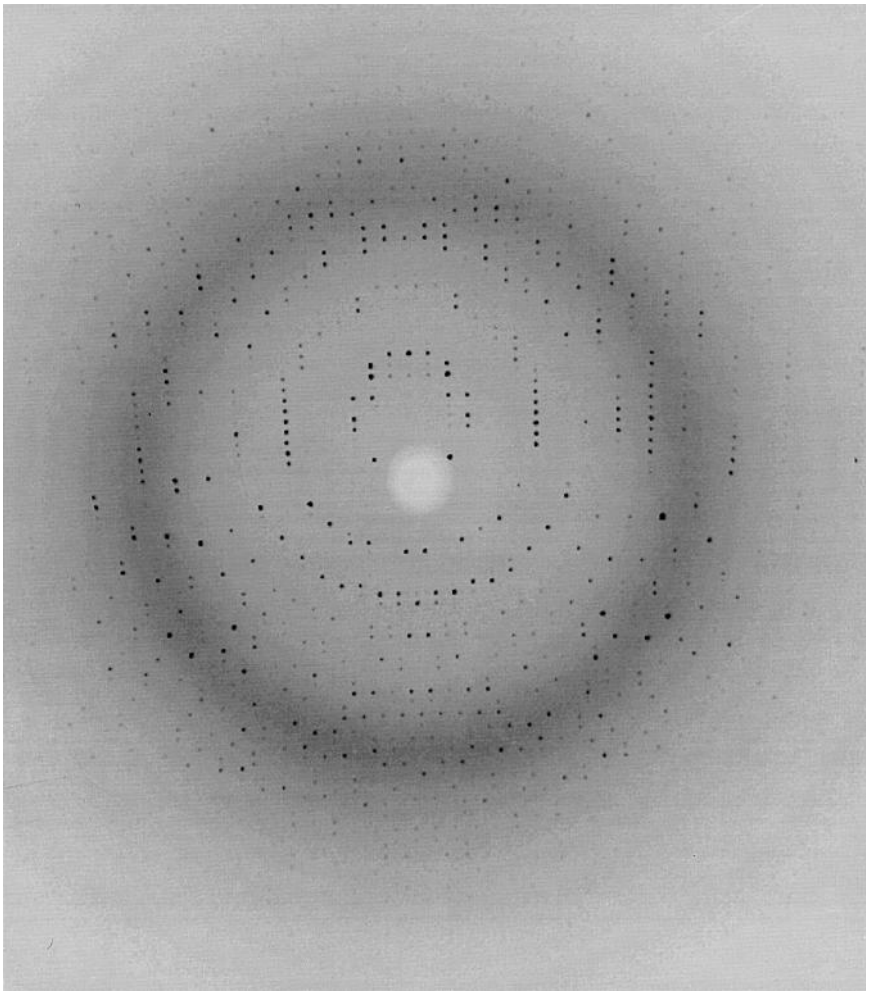

Fig. 8.6. X-ray diffraction frame of data from a crystal of herpes simplex virus type 1 thymidine kinase, complexed with substrate deoxythymidine, at 2 Å resolution. (Picture provided by John N. Champness, Matthew S. Bennett and Mark R. Sanderson of King's College London.)

- Once prepared, the crystal (which is extremely fragile) is mounted inside a quartz or glass capillary tube, with a drop of either mother liquor (the solution from which it was crystallised) or a stabilising solution drawn into one end of the capillary tube to prevent the crystal from drying out. The tube is then sealed and the crystal exposed to a beam of X-rays. Since the wavelength of X-rays is comparable to the planar separation of atoms in a crystal lattice, the crystal can be considered to act as a three-dimensional grating. The X-rays are therefore diffracted, interfering both in phase and out of phase to produce a diffraction pattern as shown in Fig. 8.6. Data collection technology necessary for recording the diffraction pattern is now highly sophisticated. Originally,

conventional diffractometers and photographic film were used to detect diffracted X-rays. This involved wet developing of the film and subsequent digital scanning of the negative. Data collection by this method took many weeks. By contrast, modern area-detectors can collect data in under 24 h.

- Unfortunately the diffraction pattern alone is insufficient to determine the crystal structure. Each diffraction maximum has both an amplitude and a phase associated with it, and both need to be determined. But the phases are not directly measurable in a diffraction experiment and must be estimated from further experiments. This is usually done by the method of isomorphous replacement (MIR). The MIR method requires at least two further crystals of the protein (derivatives), each being crystallised in the presence of a different heavy-metal ion (e.g. Hg^{2+}, Cu^{2+}, Mn^{2+}). Comparison of the diffraction patterns from the crystalline protein and the crystalline heavy-metal atom derivative allows phases to be estimated. A more recent approach to producing a heavy-metal derivative is to clone the protein of interest into a methionine auxotroph, and then grow this strain in the presence of selenomethionine (a selenium-containing analogue of methionine). Selenomethionine is therefore incorporated into the protein in the place of methionine, and the final purified and crystallised protein has the selenium heavy metal conveniently included in its structure.
- Diffraction data and phase information having been collected, these data are processed by computer to construct an electron density map. The known sequence of the protein is then fitted into the electron density map using computer graphics, to produce a three-dimensional model of the protein (Fig. 8.7.). In the past there had been concern that the three-dimensional structure determined from the rigid molecules found in a crystal may differ from the true, more flexible, structure found in free solution. These concerns have been effectively resolved by, for example, diffusing substrate into an enzyme crystal and showing that the substrate is converted into product by the crystalline enzyme (there is sufficient mother liquor within the crystal to maintain the substrate in solution). In a more recent development, it is now becoming possible to determine the solution structure of protein using NMR. At present the method is capable of determining the structure of a protein up to about 20 000 kDa but will no doubt be developed to study larger proteins. Although the time-consuming step of producing a crystal is obviated, the methodology and data analysis involved are at present no less time-consuming and complex than that for X-ray crystallography.

8.5 PROTEOMICS AND PROTEIN FUNCTION

In order to completely understand how a cell works, it is necessary to understand the function (role) of every single protein in that cell. The analysis of any specific disease (e.g. cancer) will also require us to understand what changes have taken place in the protein component of the cell, so that we can use this information to understand the molecular basis of the disease, and thus design appropriate drug

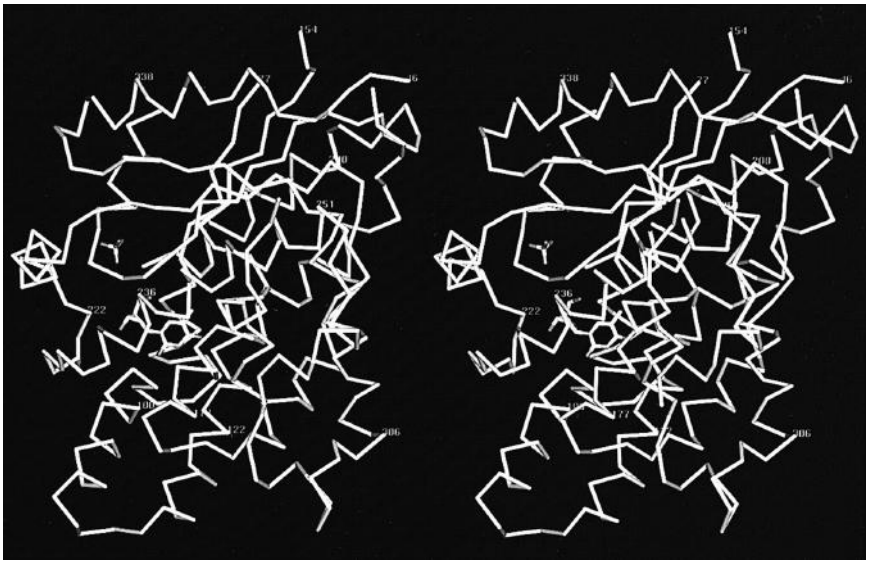

Fig. 8.7. (*Relaxed-eye stereo pair*): A C^{α}-trace of herpes simplex virus type 1 thymidine kinase from a crystallographic study of a complex of the enzyme with one of its substrates, deoxythymidine. The enzyme is an α–β protein, having a five-stranded parallel β-sheet surrounded by 14 α-helices. The active site, occupied by deoxythymidine, is a volume surrounded by four of the helices, the C-terminal edge of the β-sheet and a short 'flap' segment; a sulphate ion occupies the site of the β-phosphate of the absent co-substrate ATP. (Short missing regions of chain indicate where electron density calculated from the X-ray data could not be interpreted.) (Picture provided by John N. Champness, Matthew S. Bennett and Mark R. Sanderson of King's College London.)

therapies and develop diagnostic methods. (Just about every therapeutic drug that is currently in use has a protein as its target.) The completion of the Human Genome Project might suggest that it is not now necessary to study proteins directly, since the amino acid sequence of each protein can be deduced from the DNA sequence. This is not true for the following reasons:

- First, although the DNA in each cell type in the body is the same, different sets of genes are expressed in different tissues, and hence the protein component of a cell varies from cell type to cell type. For example, some proteins are found in nearly all cells (the so-called house-keeping genes) such as those involved in glycolysis, whereas specific cell types such as kidney, liver, brain, etc. contain specific proteins unique to that tissue and necessary for the functioning of that particular tissue/organ. It is therefore only by studying the protein component of a cell directly that we can identify which proteins are actually present.
- Secondly, it is now appreciated that a single DNA sequence (gene) can encode multiple proteins. This can occur in a number of ways:
  - (i) Alternative splicing of the mRNA transcript.
  - (ii) Variation in the translation 'stop' or 'start' sites.
  - (iii) Frameshifting, where a different set of triplet codons is translated, to give a totally different amino acid sequence.

(iv) Post-translational modifications. The genome sequence defines the amino acid sequence of a protein, but tells us nothing of any post-translational modifications (Sections 8.2.1 and 9.5.3) that can occur once the polypeptide chain is synthesised at the ribosome. Up to 10 different forms (variants) of a single polypeptide chain can be produced by phosphorylation, glycosylation, etc.

The consequence of the above is that the total protein content of the human body is an order of magnitude more complex than the genome. The human genome sequence suggests there may be 30 000–40 000 genes (and hence proteins) whereas estimates of the actual number of proteins in human cells suggests possibly as many as 200 000 or even more. The dogma that one gene codes for one protein has been truly demolished!

From the above, I hope it is easy to appreciate the need to directly analyse the protein component of the cell, and the need for an understanding of the function of each individual protein in the cell. In recent years, development of new techniques (discussed below) has enhanced our ability to study the protein component of the cell and has led to the introduction of the terms proteome and proteomics. The total DNA composition of a cell is referred to as the genome, and the study of the structure and function of this DNA is called genomics. By analogy, the proteome is defined as the total protein component of a cell, and the study of the structure and function of these proteins is called proteomics. The ultimate aim of proteomics is to catalogue the identity and amount of every protein in a cell, and determine the function of each protein.

Earlier sections of this chapter and Chapter 11 describe the traditional, but still very valid approach to studying proteins, where individual proteins are extracted from tissue and purified so that studies can be made of the structure and function of the purified proteins. The subject of proteomics has developed from a different approach, where modern techniques allow us to view and analyse much of the total protein content of the cell in a single step. The development of these newer techniques has gone hand-in-hand with the development of techniques for the analysis of proteins by mass spectrometry, which has revolutionised the subject of protein chemistry. The cornerstone of proteomics has been two-dimensional (2-D) PAGE (described in Section 10.3) and the applications of this technique in proteomics are described below. However, although 2-D PAGE remains central to proteomics, the study of proteomics has stimulated the development of further methods for studying proteins and these will also be described below.

8.5.1 2-D PAGE

2-D PAGE has found extensive use in detecting changes in gene expressions between two different biological states, for example comparing normal and diseased tissue. In this case, a 2-D gel pattern would be produced of an extract from a diseased tissue such as a liver tumour and compared with the 2-D gel patterns of an extract from normal liver tissue. The two gel patterns are then compared to see

whether there are any differences in the two patterns. If it is found that a protein is present (or is absent) only in the liver tumour sample, then by identifying this protein we are directed to the gene for this protein and can thus try to understand why this gene is expressed (or not) in the diseased state. In this way it is possible to obtain an understanding of the molecular basis of diseases. This approach can be taken to study *any* disease process where normal and diseased tissue can be compared, for example arthritis, kidney disease, or heart valve disease.

Under favourable circumstances up to 5000 protein spots can be identified on a large format 2-D gel. Thus with 2-D PAGE we now have the ability to follow changes in the expression of a significant proportion of the proteins in a cell or tissue type, rather than just one or two, which has been the situation in the past. The potential applications of proteome analysis are vast. Initially one must produce a 2-D map of the proteins expressed by an organism, tissue or cell under 'normal' conditions. This 2-D reference map and database can then be used to compare similar information from 'abnormal' or treated organisms, tissues or cells. For example, as well as comparing normal tissue with diseased tissue (as described above), we can:

- analyse the effects of drug treatment or toxins on cells;
- observe the changing protein component of the cell at different stages of tissue development;
- observe the response to extracellular stimuli such as hormones or cytokines;
- compare pathogenic and non-pathogenic bacterial strains;
- compare serum protein profiles from healthy individuals and Alzheimer or cancer patients to detect proteins, produced in the serum of patients, which can then be developed as diagnostic markers for diseases (e.g. by setting up an enzyme-linked immunosorbent assay (ELISA) to measure the specific protein).

As a typical example, a research group studying the toxic effect of drugs on the liver can compare the 2-D gel patterns from their 'damaged' livers with the normal liver 2-D reference map, thus identifying protein changes that occur as a result of drug treatment.

The sheer complexity and amount of data available from 2-D gel patterns is daunting, but fortunately there is a range of commercial 2-D gel analysis software, compatible with personal computer workstations, which can provide both qualitative and quantitative information from gel patterns, and can also compare patterns between two different 2-D gels (see below). This has allowed the construction of a range of databases of quantitative protein expression in a range of tissue and cell types. For example, an extensive series of 2-DE databases, known as SWISS-2D PAGE is maintained at Geneva University Hospital and is accessible via the World Wide Web at <http://au.expasy.org/ch2d/>. This facility therefore allows an individual laboratory to compare their own 2-D protein database with that in another laboratory.

The comparison of two gel patterns is made by using any one of a number of software packages designed for this purpose. One of the more interesting approaches to comparing gel patterns is the use of the Flicker program, which

is available on the Web at <http://open2dprot.sourceforge.net/Flicker>. This program superimposes the two 2-D patterns to be compared and then alternately, and rapidly, displays one pattern and then the other. Spots that appear on both gel patterns (the majority) will be seen as fixed spots, but a spot that appears on one gel and not the other will seen to be flashing (hence 'flicker'). When one has compared two 2-DE patterns and identified any proteins spot(s) of interest, it is then necessary to identify each specific protein. In the majority of cases this is done by peptide mass-fingerprinting. The spot of interest is cut out of the gel and incubated in a solution of the proteolytic enzyme trypsin, which cleaves the protein C-terminal to each arginine and lysine residue. In this way the protein is reduced to a set of peptides. This collection of peptides is then analysed by MALDI–MS (see Section 9.3.7) to give an accurate mass measurement for each of the peptides in the sample. This set of masses, derived from the tryptic digestion of the protein, is highly diagnostic for this protein, as no other protein would give the same set of peptide masses (fingerprint). Using Web-based programs, such as Mascot or Protein Prospector (Section 9.7.2) this experimentally derived peptide mass-fingerprint is compared with databases of tryptic peptide mass-fingerprints generated from sequences of known proteins (or predicted sequences deduced from nucleotide sequences). If a match is found with a fingerprint from the database then the protein will be identified.

However, sometimes results from peptide mass-fingerprinting can be ambiguous. In this case it is necessary to obtain some partial amino acid sequence data from one of the peptides. This is done by tandem mass spectrometry (MS/MS; Section 9.3.3), where one of the peptides separated for mass-fingerprinting is further fragmented in a second analyser, and from the fragmentation pattern sequence data can be deduced (mass spectrometry conveniently fragments peptides at the peptide bond, such that the difference in the mass of fragments produced can be related to the loss of specific amino acids; Section 9.5.5). This partial sequence data is then used to search the protein sequence databases for sequence identity. Universal databases are available that store information on all types of protein from all biological species. These databases can be divided into two categories: (i) databases that are a simple repository of sequence data, mostly deduced directly from DNA sequences, for example the Tr EMBL database; and (ii) annotated databases where information in addition to the sequence is extracted by the biologist (the annotator) from the literature, review article, etc., for example the SWISS-PROT database.

An example of how sequence data can be produced is shown in Fig. 8.8. A lysate of 2×10^6 rat basophil leukaemic (RBL) cells were separated by 2-D electrophoresis and spot 2 chosen for analysis. This spot was digested *in situ* using trypsin and the resultant peptides extracted. This sample was then analysed by tandem MS using a triple quadrupole instrument (ESI–MS^2). MS of the peptide mixture showed a number of molecular ions relating to peptides. One of these (*m/z* 890) was selected for further analysis, being further fragmented in a quadrupole mass spectrometer to give fragment ions ranging from *m/z* 595.8 to 1553.6 (Fig. 8.8). The ions at *m/z* 1002.0, 1116.8, 1280.0, 1466.2 and 1553.6 are likely to be part of a Y ion series

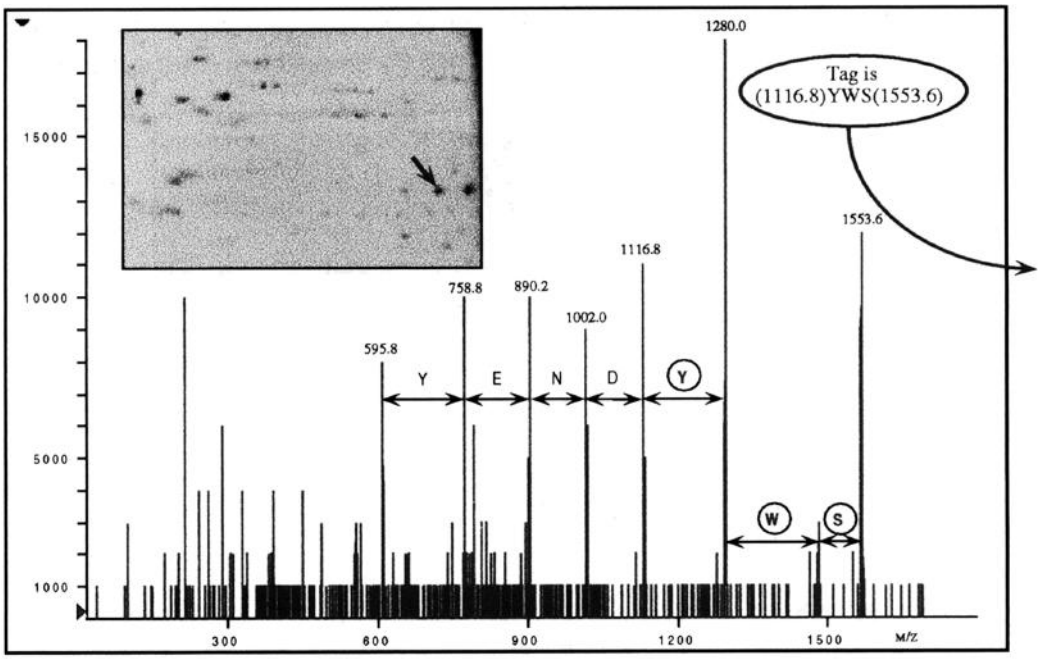

Fig. 8.8. Nano-ESI MS^2 spectrum of *m/z* 890 from RBL spot 2 showing construction of a sequence tag. The *y*-axis shows relative intensity. (Courtesy of Glaxo SmithKline, Stevenage, UK.)

(see Fig. 8.8) as they appear at higher *m/z* than the precursor at *m/z* 890. The gap between adjacent Y ions is related directly to an amino acid residue because the two flanking Y ions result from cleavage of two adjacent amide bonds. Therefore, with a knowledge of the relative molecular masses of each of the 20 naturally occurring amino acids, it is possible to determine the presence of a particular residue at any point within the peptide. The position of the assigned amino acid is deduced by virtue of the *m/z* ratio of the two ions. By reading several amino acids it was possible to assemble a sequence of amino acids, in this case (using the one-letter code) YWS. Database searching was then possible using the peptide 1778 Da, the position of the lower *m/z* Y ion (1116.8), the proposed amino acid sequence (YWS) and the higher Y ion at *m/z* 1553.6. This provides a sequence tag, which is written as (1116.8) YWS (1553.6).

A search of the SWISS-PROT database (Fig. 8.9), showed just two 'hits' from 40 000 entries, suggesting the protein is glyceraldehyde-3-phosphate dehydrogenase. The full sequence of this peptide is LISWYDNEYGYSNR and the MS/MS fragmentation data give a perfect match. Other peptides in the sample can also be analysed in the same manner, confirming the identity of the protein.

A further development of 2-D PAGE has been the introduction of difference gel electrophoresis (DIGE). This again allows the comparison of protein components of similar mixtures, but has the advantage that only one 2-D gel has to be run

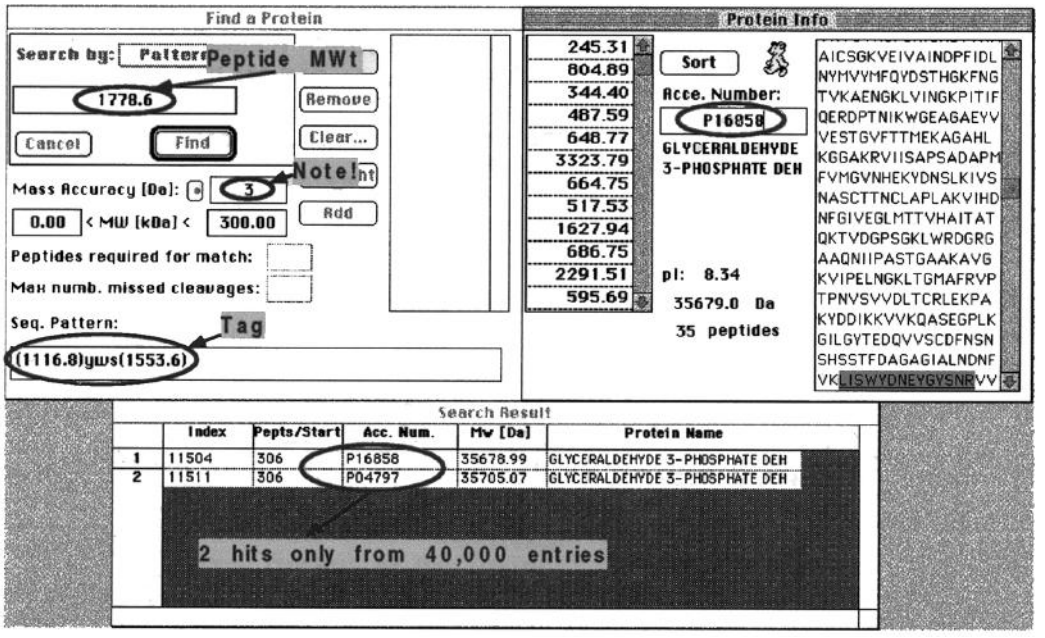

Fig. 8.9. The PeptideSearch™ input form and search result based on data obtained from nano-ESI MS2 of m/s 890 from RBL Spot 2. (Courtesy of Glaxo SmithKline, Stevenage, UK.)

rather than two. In this method the two samples to be compared are each treated with one of two different, yet structurally very similar, fluorescent dyes (cy3 and cy5). Each dye reacts with amino groups, so that each protein is fluorescently labelled by the dye binding to lysine residues and the N-terminal amino groups. The two protein solutions to be compared are then mixed and run on a *single* 2-D gel. Thus every protein in one sample superimposes with its differentially labelled identical counterpart in the other sample. Scanning of the gel at two different wavelengths that excite the two dye molecules reveals whether any individual spot is associated with only one dye molecule rather than two. Most spots will, of course, fluoresce at both wavelengths, but if a spot is associated with only one dye molecule then this tells us that that protein can have been present in only one of the extracts, and the wavelength at which it fluoresces tells you which extract it was originally in.

8.5.2 Isotope-coded affinity tags (ICAT)

Isotope-coded affinity tags (ICAT) uses mass spectrometry (rather than 2-D gels) to identify differences in the protein content of two complex mixtures. For example, the method can be used to identify protein differences between tumour and normal tissue, in the same way that 2-D PAGE can be used to address the same question (Section 8.5.1). This method uses two protein 'tags' that, whilst being in

every other respect identical, differ slightly in molecular mass; hence one is 'heavy' and one is 'light'. Both contain (a) a chemical group that reacts with the amino acid cysteine, and (b) a biotin group. In both molecules these groups are joined by a linker region, but in one case the linker contains eight hydrogen atoms, in the other, eight deuterium atoms; one molecule (tag) is thus heavier than the other by 8 Da (see Fig. 9.26). One cell extract (e.g. from cancer cells) is thus treated with one tag (which binds to cysteine residues in all the proteins in the extract) and the second tag is used to treat the second extract (e.g. from normal cells). Both extracts are then treated with trypsin to produce mixtures of peptides, those peptides that contain cysteine having been 'tagged'. The two extracts are then combined and an avidin column used to affinity-purify the labelled peptides by binding to the biotin moiety. When released from the column this mixture of labelled peptides will contain pairs of identical peptides (derived from identical proteins) from the two cell extracts, each pair differing by a mass of 8 Da.

Analysis of this peptide mixture by liquid chromatography–MS will then reveal a series of peptide mass signals, each one existing as a 'pair' of signals separated by eight mass units. These data will reveal the relative abundance of each peptide in the pair. Since most proteins present in the two samples originally being compared will be present at much the same levels, most peptide pairs will have equal signal strengths. However, for proteins that exist in greater or lesser amounts in one of the extracts, different signal strengths will be observed for each of the peptides in the pair, reflecting the relative abundance of this protein in the two samples. Further analysis of either of these pairs via tandem mass spectrometry will provide some sequence data that should allow the protein to be identified. ICAT is discussed in more details in Section 9.6.2.

8.5.3 Determining the function of a protein

Successfully applied, the methods described in the preceding section will have provided the amino acid sequence (or partial sequence) of a protein of interest. The next step is to identify the function and role of this protein. The first step is invariably to search the databases of existing protein sequences to find a protein or proteins that have sequence homology with the protein of interest (the homology method). This is done using programs such as BLAST and PSI-BLAST. If sequence homology is found with a protein of known function, either from the same or different species, then this invariably identifies the function of the protein. However, this approach does not always work. For example, when the genome of the yeast *Saccharomyces cerevisiae* was completely sequenced in 1996, 6000 genes were identified. Of these, approximately 2000 coded for proteins that were already known to exist in yeast (i.e. had been purified and studied in previous years), 2000 had homology with known sequences and hence their function could be deduced by the homology method but 2000 could not be matched to any known genes, i.e. they were 'new', previously undiscovered genes. In these cases, there are a number of other computational methods that can be used to help to identify the protein's function. These include:

	A	B	C	D	E
P1	1	1	1	0	0
P2	0	0	1	1	1
P3	1	0	1	1	0
P4	0	1	1	0	1
P5	1	1	0	0	1
P6	0	1	1	0	1
P7	1	0	0	1	0
P8	1	0	1	1	0

Fig. 8.10. Phylogenic profile method. Five genomes, A–E, are shown (e.g. *E. coli*, *S. cerevisiae*, etc.). The presence (1) or absence (0) of eight proteins (P1–P8) in each of these genomes is shown. It can be seen that proteins P3 and P8 have the same phylogenic profile and therefore may have a functional linkage. P4 and P6 are similarly linked.

- *Phylogenic profile method:* This method aims to identify any other protein(s) that has the same phylogenic profile (i.e. the same pattern of presence or absence) as the unknown protein, in all known genomes. If such proteins are found it is inferred that the unknown protein is involved in the same cellular process as these other protein(s) (i.e. they are said to have a functional link) and will give a strong clue as to the function of the unknown protein. This method is based on the premise that two proteins would not always both be inherited into a new species (or neither inherited) unless the two proteins have a functional link. At the time of writing there are over 100 published genome sequences that can be surveyed with this method. Fig. 8.10 shows a simple, hypothetical example, where just five genomes are analysed.
- *Method of correlated gene neighbours:* If two genes are found to be neighbours in several different genomes, a functional linkage may be inferred between the two proteins. The central assumption of this approach is based on the observation that functionally related genes in prokaryotes tend to be linked to form operons (e.g. the *lac* operon). Although operons are rare in eukaryotic species, it does appear that proteins involved in the same biological process/pathway within the cell have their genes situated in close proximity (e.g. within 500 bp) in the genome. Thus, if two genes are found to be in close

proximity across a number of genomes, it can be inferred that the protein products of these genes have a functional linkage. This method is most robust for microbial genomics but works to some extent in human cells where operon-like clusters are also observed. As an example, this method correctly identified a functional link between eight enzymes in the biosynthetic pathway for the amino acid arginine in *Mycobacterium tuberculosis*.

- *Analysis of fusion:* This method is based on the observation that two genes may exist separately in one organism, whereas the genes are fused into a single multifunctional gene in another organism. The existence of the protein product of the fused gene, in which the two functions of the protein clearly interact (being part of the same protein molecule), suggests that in the first organism the two separate proteins also interact. It has been suggested that gene fusion events occur to reduce the regulational load of multiple interacting gene products.
- *Protein–protein interactions:* A further clue to identifying protein function can come from identifying protein–protein interactions, and methods to identify these are described in the next section.

8.5.4 Protein–protein interactions

Given the complex network of pathways that exist in the cell (signalling pathways, biosynthetic pathways, etc.), it is clear that all proteins must interact with other molecules to fulfil their role. Indeed, it is now apparent that proteins do not exist in isolation in the cell; proteins involved in a common pathway appear to exist in a loose interaction, sometimes referred to as a biomodule. Therefore, if one can identify an interaction between our unknown protein and a well-characterised protein, it can be inferred that the former has a function somehow related to the latter. For example, if the unknown protein is shown to interact with one or more proteins involved in the biosynthetic pathways for arginine, then this strongly suggests that the unknown protein is also involved in this pathway. Using this approach networks of interacting proteins are being identified in individual organisms. This has led to the development of the Database of Interacting Proteins (DIP), which can be found at <http://dip.doe-mbi.ucla.edu>. Given the current fad for inventing new words ending in 'ome', some refer to these maps of protein interactions as the interactome.

One of the most widely used, and successful, methods for investigating protein–protein interaction is the yeast two-hybrid (Y2H) system, which exploits the modular architecture of transcription factors. A transcription factor gene (GAL4) is split into the coding regions for two domains, a DNA-binding domain and a *trans*-activation domain. Both these domains are expressed, each linked to a different protein (one being the unknown protein, the other a protein with which it may interact), in separate yeast cells, which are then mated to produce diploid cells (the two proteins being studied are often referred to as the bait and prey). If, in this diploid cell, the bait and prey proteins bind to each other, they will bring together the two domains of the transcription factor, which will then be active and

will bind to the promoter of a reporter gene (e.g. the *his* gene), inducing its expression. Identification of cells expressing the reporter gene product is evidence that the bait and prey proteins interact. In practice, following mating, diploids are selected on deficient medium (in this case, medium deficient in histidine), thus only yeast cells expressing interacting proteins survive (as they are capable of synthesising histidine). Once such a positive interaction is identified, the two interacting open reading frames (ORFs) are simply identified by sequencing a small part of the protein gene.

Using this approach, all 6000 ORFs from *S. cerevisiae* were individually cloned as both bait and prey. When the pool of 6000 prey clones was screened against each of the 6000 bait clones, 691 interactions were identified, only 88 of which were previously known. This therefore gave an indication of the function of over 600 proteins whose function was previously unknown. On a much larger scale, the same approach was used to identify protein–protein interactions in the fruit fly, *Drosophila melanogaster*. All 14 000 predicted *D. melanogaster* ORFs were amplified using the polymerase chain reaction (PCR) and each cloned into two-hybrid bait and prey vectors. A total of 45 417 two-hybrid positive colonies were obtained, from which 10 021 protein interactions involving 4500 proteins were obtained. The yeast 2-hybrid system is described in greater detail in Section 6.8.3.

8.5.5 Protein arrays

A newly developing area for studying protein–protein interactions is the use of protein arrays (chips). Although the basic principle for screening and identifying interacting molecules is much the same as for DNA arrays (Section 6.8.8), the production of protein arrays is more technically demanding owing mainly to the difficulty of binding proteins to a surface and ensuring that the protein is not denatured at any stage of the assay procedure.

In a protein array, proteins are immobilised as small spots (150–200 μm) onto a solid support (typically glass or a nitrocellulose membrane), using high precision contact printing (not unlike a dot-matrix printer) at a spot density of the order of 1500 spots cm^{-2}. A solution of the protein of unknown function is then incubated on the array surface for a period of time, then washed off, and the position(s) where the protein has bound, identified (see below). Since it is known which protein was immoblised in each position of the chip, each pair of interacting proteins can be identified.

Saccharomyces cerevisiae again provides a good example of the successful use of this technology where a protein array was used to identify yeast proteins that bind to the protein calmodulin (an important protein involved in calcium regulation). Five thousand eight hundred yeast ORFs were cloned into a yeast high copy expression vector, and each of the expressed proteins purified. Each protein was then spotted at high density onto nickel-coated glass microscope slides. Since each protein also contained a $(His)_6$-Tag (which binds to nickel; see Section 11.8.5) introduced at the C terminus, proteins were attached to the surface in an orientated manner, the C terminus being linked to the nickel-coated glass through the

$(His)_6$ sequence, while the rest of the molecule was therefore suitably orientated away from the surface of the array to be available for interaction with another protein. The array was then incubated in a solution of calmodulin that had been labelled with biotin. The calmodulin was then washed off and the positions where calmodulin had bound to the array were identified by incubating the array with a solution of fluorescently labelled avidin (the protein avidin binds strongly to the small molecular mass vitamin biotin: see Section 7.5.5). The use of ultraviolet light thus identified fluorescence where the screening molecules had bound. In total, 33 new proteins that bind calmodulin were discovered in this way.

Fig. 8.11 (see colour section) shows an interaction map of the yeast proteome. The authors constructed the map from published data on protein–protein interactions in yeast. The map contains 1584 proteins and 2358 interactions. Proteins are coloured according to their functional role, e.g. proteins involved in membrane fusion (blue), lipid metabolism (yellow), cell structure (green), etc. If one views the electronic version of this publication it is possible for the reader to zoom in and search for protein names and to read interactions more clearly.

Fig. 8.12 (see colour section) is a summary of Fig. 8.11 showing the number of interactions of proteins from each functional group with proteins of their own and other groups. The word function means the cellular role of the protein. Numbers in parentheses indicate, first, the number of interactions within a group and, secondly, the number of proteins within a group. Numbers on connecting lines indicate the numbers of interactions between proteins of the two connected groups. For example, in the upper left-hand corner, there are 77 interactions between the 21 proteins involved in membrane fusion and 141 proteins involved in vesicular transport. Looking at the bottom right of the diagram it can be seen that some proteins involved in RNA processing/modification not surprisingly also interact with proteins involved in RNA turnover, RNA splicing, RNA transcription and protein synthesis.

8.5.6 Systems biology

It can be seen from the section on proteomics that the study of proteins is moving away from methods that involve the purification and study of individual proteins. Nowadays proteins are more likely to be studied as a stained spot on a complex 2-D gel pattern, often present in as little as nanogram amounts, more often than not using analytical techniques such as mass spectrometry (see Chapter 9) and invariably requiring the interrogation of protein and genome sequence data on the Web (bioinformatics, Section 5.8). It is then necessary to determine which other proteins interact with the protein being studied. Proteomics is thus moving us away from studying proteins in isolation and encouraging us to consider the proteins in the cell as part of a dynamic interacting system. This has led to the development of the concept of systems biology, which can be defined as the study of living organisms in terms of their underlying network structure rather than just their individual molecular components. Since systems biology requires a study of all interacting components in the cell the new high throughput and quantitative

techniques of proteomics are central to systems biology. Needless to say, the analysis of complex biological systems will generate massive volumes of data, which can be handled only by the computational methods that make up the subject of bioinformatics (see Section 5.8). The study of the cell is thus no longer the remit of the biologist alone. Systems biology has introduced cross-disciplinary studies involving biologists, computer scientists, chemists, engineers and mathematicians who can understand the language of each other's disciplines and who can integrate their work with the data acquisition, storage and analysis tools of bioinformatics.

8.6 SUGGESTIONS FOR FURTHER READING

CUTLER, P. (2004). *Protein Purification Protocols*. Humana Press, Totowa, NJ. (Detailed theory and practical procedures for a range of protein purification techniques.)

LIEBLER, D. C. (2002). *Introduction to Proteomics*. Humana Press, Totowa, NJ. (A good introduction to all aspects of proteomics, in particular the analysis of two-dimensional gels by mass spectrometry.)

SCOPES, R. K. (1996). *Protein Purification*, 3rd edn. Springer-Verlag, Berlin. (Principles and methods for a range of protein purification techniques.)

WALKER, J. M. (2005). *Proteomics Protocols*. Humana Press, Totowa, NJ, in press. (Theory and techniques of a spectrum of methods applied to proteomics.)