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Permeability of Biological Membranes

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ISBN 978-3-319-28096-7 ISBN 978-3-319-28098-1 (eBook)
DOI 10.1007/978-3-319-28098-1

Library of Congress Control Number: 2016932313

Printed on acid-free paper

This Springer imprint is published by SpringerNature
The registered company is Springer International Publishing AG Switzerland.

Summary

The ultimate energy source for life on Earth is the solar energy of Sun. Cells convert the radiation energy to other types of energy. The major chemical energy produced by cells is ATP. In turn the energy needed for cellular work is provided by ATP, the energy currency of cells. Most of the chemical energy of ATP in cells is used for (i) mechanical work, (ii) biosynthetic processes, and (iii) transport by passing molecules and ions across the cell membranes to maintain concentration gradients.

By the analogy of "Space: the final frontier" (Star Track), the cell membrane is the living frontier of cells. However, the cell membrane is not quite the final frontier of cells due to their connection with the environment known as permeability. The cell membrane or cytoplasmic membrane and the intracellular membranes of organelles are biological membranes. The cytoplasmic membrane separates the cell from the outside environment and cells from each other. The intracellular membranes separate specialized subunits with specific function known as organelles. The cytoskeleton may form appendage-like organelles, such as cilia, lamellopodia and finger-like projections known as microvilli covered by cell membrane. The cell membrane is selectively permeable to organic molecules and ions. This book deals with biological membranes, focuses on the permeability of cells, on methods of permeabilization, with particular attention to the reversibility and applications of permeabilization.

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Chapter 1
Biological Membranes

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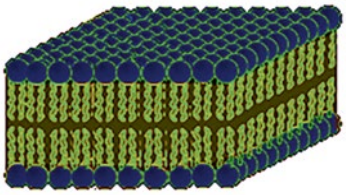
G. Banfalvi, *Permeability of Biological Membranes*,
DOI 10.1007/978-3-319-28098-1_1



Abstract Biological membranes (biomembranes) separate the interior of the cells from their external environment and cells from each other. Biomembranes are composed of lipid bilayers namely of two layers of amphipatic phospholipids with their hydrophobic tail regions turned inward and the polar head regions forming the intracellular (cytosolic) and extracellular (outer) faces of the bilayer. The selective permeability of biological membranes also referred to as semipermeability, partial permeability or differential permeability means that different molecules may diffuse, pass by passive and active or by other types of transport mediated by proteins. This chapter deals with basic properties of biological membranes describing different types of lipids that constitute the major components of membranes, characterizes cellular membranes of prokaryotic and eukaryotic cells, membranes of subcellular organelles and functions of biological membranes.

Keywords Amphipatic molecules • Artificial membranes • Bacterial membranes • Caveolae • Cell-cell interaction • Cell surface markers • Ceramide • Chemiosmosis • Cholesterol • Cholesterol esters • *Cis-trans* isomers • Cytoskeletal elements • Endomembrane systems • Energy transduction • Ether phospholipids • Extracellular matrix • Fatty acids • Fluid mosaic membrane • Focal adhesions • Functions of membranes • Galactocerebroside • Glycerolipids • Glycoshingolipids • Inner and outer membranes • Integrated proteins • Invadosomes • Lipid bilayer • Lipid rafts • Liposomes • Selective permeability • Membrane potential • Membrane receptors • Membrane transport • Micelles • Neutral fats • Organellar membranes • Plasmalemma • Plasmalogens • Plasma membrane • Phosphoinositides • Phospholipids • Porins • Postsynaptic densities • Sphingomyelins • Steroids • Supramembrane structures • Vesicular membranes

1.1 Definition of Biological Membranes

Biological membranes, in the form of cell membranes are not to be confused with layers forming tissues, that are also named membranes such as the mucous or basement membranes. By definition biological membrane, known as plasma membrane or plasmalemma, is the outer thin 60–100 Å film of protoplasm consisting of two lipid layers known as bilayer spanned by integrated proteins. All cells have an outer plasma membrane, but prokaryotes unlike eukaryotes lack membrane bond

organelles and are additionally covered from outside by the cell wall, with the notable exception of the *Mycoplasma* genus, which has no cell wall.

The biological cell membrane:

(i) separates the interior of the cells from the external environment and other cells,
(ii) is a highly selective permeability barrier with low permeability to charged (ATP, amino acids, glucose-6-phosphate) and larger polar substances (glucose, fructose) with medium permeability to neutral molecules and organic substances (urea, ethanol) and high permeability to water and respiratory gases (O_2, N_2, CO_2) (Darnell et al. 1986),
(iii) controls the transport in and out of the cells.

Biological membranes consist of lipids, proteins and carbohydrates. The phospholipid bilayer is the basic structure of all biological membranes. Other major lipids present in the membrane are glycolipids and cholesterol. The lipid bilayer forms the barrier, proteins mediate messages and distinct functions. Carbohydrate moieties are located on the outer surface of the membrane. Non-covalent self-assemblies of protein-lipid interactions form asymmetric fluid structures that are electronically polarized and maintain the negative charge (~ −70 mV) inside the cell with respect to the outside. Beside the electronic polarization, the cell membrane is responsible for keeping a well-defined, stable chemical composition and concentration inside the cell that differs significantly from the extracellular composition and concentration. The large concentration differences between the two sides of the membrane are built up and maintained by passive diffusion, and selective active transport mediated by ion channels and pumps built up by various proteins. The lipid bilayer containing an ion channel is shown in Fig. 1.1.

Most naturally occurring fatty acids contain 4–28 carbon acids (IUPAC Compendium 1997). There are two "essential" fatty acids for humans, the α-linoleic acid (an omega-3 fatty acid) and the linolenic acid (an omega-6 fatty acid) that humans must ingest since the body is unable to synthesize them (Burr et al. 1930; Goodhart and Shils 1980; Whitney and Rolfes 2008). Essential fatty acids of the diet are polyunsaturated. Saturated fatty acids are strait chains of hydrocarbons, have no double bonds and their numbering (1, 2, 3, etc.) starts from the carboxyl end (-COOH), while the Greek lettering starts at the carbon atom next to the carboxyl

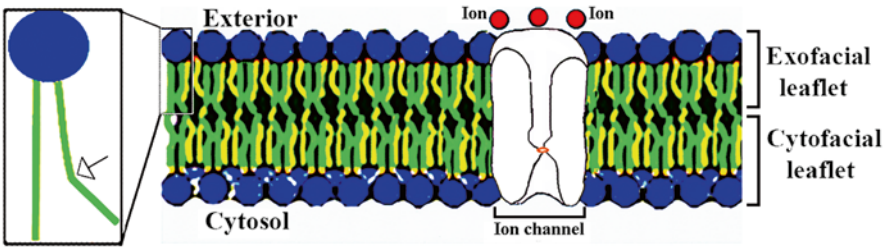


Fig. 1.1 Exofacial and cytofacial leaflets of a lipid bilayer containing a closed ion channel. *Boxed* image: phospholipid unit enlarged. *Cis* double bond in the fatty acid chain causing kink is indicated by the *white arrow*

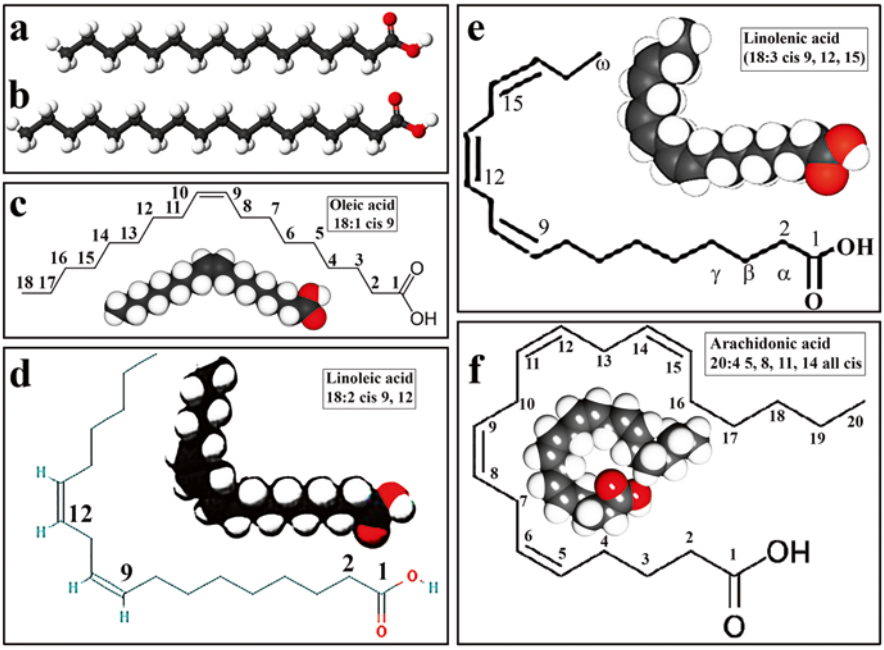


Fig. 1.2 Structures of fatty acids. Saturated fatty acids: (**a**) palmitic acid, (**b**) stearic acid. Unsaturated fatty acids: (**c**) oleic acid, (**d**) linoleic acid, (**e**) linolenic acid, (**f**) arachidonic acid

group (α, ß, γ, etc.). Due to the different lengths of fatty acids the last position is the so called "ω" carbon atom. See the numbering of fatty acids in Fig. 1.2. Unsaturated fatty acids contain at least one double bond in the fatty acid chain; polyunsaturated fatty acids contain more than one double bond.

1.2 Lipids

1.2.1 Fatty Acids

Biologically important fatty acids are either saturated straight, long chain fatty acids (e.g. palmitic, stearic acid) (Fig. 1.2a, b) or unsaturated long chain carboxylic acids, such as oleic, linoleic, linolenic and arachidonic acids, with 1, 2, 3 and 4 *cis*-double bounds, respectively (Fig. 1.2c–f and Table 1.1).

The double bond in the chain may generate either *trans* or *cis* geometry. In *trans* isomers the hydrogens of the double bond are on opposite side (Fig. 1.3a). *Cis* isomers contain the hydrogen atoms at the same side of the double bond (Fig. 1.3b). *Cis* isomers generate bent fatty acids. This arrangement prevents apolar interactions between fatty acid chains and lowers their melting point. Glycerolipids containing *cis* folded fatty acids are known as oils. In nature, unsaturated fatty acids generally have *cis* rather than *trans* configurations (Martin et al. 2007).

Table 1.1 Biologically important fatty acids

Carbon atoms	Number of double bonds	Position of double bond	Common name of fatty acid
14	0		Myristic acid
16	0		Palmitic acid
18	0		Stearic acid
18	1	cisΔ^{9}	Oleic acid
18	2	cis$\Delta^{9,12}$	Linoleic acid
18	3	cis$\Delta^{9,12,15}$	Linolenic acid
20	4	cis$\Delta^{5,8,11,14}$	Arachidonic acid

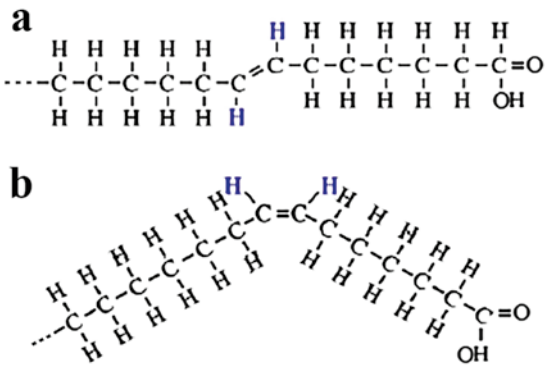


Fig. 1.3 Trans (**a**) and cis (**b**) geometry of fatty acids

Fatty acids are not to be confused with neutral lipids (fats and oils). Fats are important food molecules and play a significant role in nutrition. Fats store energy in the body; fat tissues are excellent insulators and cushion inner organs, transport lipid soluble vitamins (A, D, E, K) in the blood. The commercial importance of fats is not dealt with here.

1.2.2 Glycerolipids

Triglyceride (neutral fat) and cholesteryl ester are the forms in which fat is stored in fat cells (adipocytes). Neutral fats and oils as glycerolipids are not involved in membrane structures and are best known as fatty acid triesters of glycerols also named triglycerides (Fig. 1.4). In these glycerolipids the three hydroxy groups of glycerol are esterified with different fatty acids. In fats the saturated fatty acids will lie in close proximity to each other giving rise to apolar interactions and rendering triglycerides solid. Saturated fats are *trans*-fats present in lard and bacon grease, earlier named “animal” fat. As already noted, the presence of double bonds in unsaturated fatty acids will reduce the number of hydrogen atoms of carbon atoms at their double bonds. Most plants contain unsaturated fats known as oils. Full hydrogenation of plant oils in food processing in generating saturated fats making them

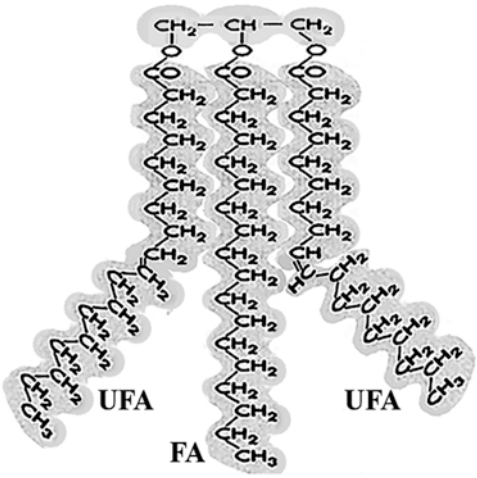


Fig. 1.4 Neutral fat. Triglyceride consisting of a glycerol esterified with one saturated fatty acid (*FA*) and two unsaturated fatty acids (*UFA*)

equivalent to the unhealthy saturated animal fats. This is normally avoided by partial hydrogenation of plant oils to maintain some of the double bonds and keeping them in *cis* configuration. The advantages of partial saturation are that hydrogenated double bonds of oils are less vulnerable to oxidation and rancidity, while the presence of double bonds softens the partially hydrogenated oils. The primary ingredient of margarine is vegetable oil that is highly processed to replace butter. It can be made with or without addition of solid fat. When butter having a pleasing flavor is not added, the vegetable oil is solidified by full or partial hydrogenation to remove the double bonds of the unsaturated fatty acids, mixed with water, milk powder, vitamins, citric acid, emulsifiers (e.g. lecithin), salt, preservatives. Block margarines contain more hydrogenated oils than the less hydrogenated soft tube margarines.

While the debate of saturated fat consumption and cardiovascular disease controversy remained an unsolved issue (Siri-Tarino et al. 2010), heart, medical, scientific authorities suggest that the consumption of saturated fats should be avoided to reduce the risk of contracting cardiovascular diseases by the lowering of LDL cholesterol level and increasing the HDL cholesterol level in the blood (Müller et al. 2003; Hu et al. 2001; Jeppesen et al. 2001).

1.2.3 Storage of Fats in Trans-Differentiated Adipose Tissues

Fats are stored in at least three, but probably four different types of adipocytes (fat cells) contained in: (i) white, (ii) brown, (iii) beige and (iv) pink adipose tissues. The transitions among these cell types represent transdifferentiation:

(i) White adipose tissue as an endocrine organ plays a central role in the understanding of metabolic abnormalities associated with the development of obesity (Vázquez-Vela et al. 2008). White adipocytes store lipids for release as free fatty acids during fasting periods (Giordano et al. 2014).

(ii) Brown fat functions to burn glucose and lipids to generate body heat and maintain thermal homeostasis by non-shivering thermogenesis rather than to produce ATP, especially in hibernating animals and in newborns. The brownish color of fat comes from the iron-containing mitochondria that are present in brown fat in much higher number than in white fat. The mitochondria of brown fat have a higher concentration of uncoupling protein (uncoupling protein 1) also known as thermogenin in their inner membrane and give off more heat and produce less ATP than white fat cells (Enerbäck 2009; Giordano et al. 2014).

(iii) Recent studies highlighted the importance of beige adipocytes by regarding them precursor cells obtained from white fat that is turning to beige. Brown fat in humans is composed primarily of beige adipocytes. Beige adipocytes have a high inducible thermogenic capacity and express distinct genes sensitive to irisin. Irisin is a myokine supposedly cleaved from its transmembrane precursor (FNDC5) to effect exercise-inducible human metabolism (Wu et al. 2012). Others doubt the magic effect and physiological role for irisin in humans and other species (Albrecht et al. 2015).

(iv) A fourth type of adipocytes, the pink adipocytes, have recently been described in mouse subcutaneous fat depots during pregnancy and lactation and could be involved in mammary duct development in the female breast. Pink adipocytes are likely to be derived from white adipocytes. Pink adipocytes produce and secrete milk (Barraclough and Rudland 1994; Smith-Kirwin et al. 1998; Giordano et al. 2014). The transdifferentiation pattern: from white → beige → brown → pink adipocytes demonstrates the extraordinary plasticity of fat cells, with predominantly white to brown adipocyte transdifferentiation taking place (Barbatelli et al. 2010). These two types of mammalian adipocytes, white and brown are well known, with their anatomy and physiology being different and acknowledged transdifferentiation properties in the adipose tissues (Cinti 2009). Nuclear transcription factors are responsible for the regulation of adipocyte differentiation and lipogenic genes during adipogenesis and induce also the transdifferentiation of myocytes into adipocytes (Yu et al. 2006).

To demonstrate that transdifferentiation is not a unique feature limited to adipocytes and myocytes the developmental conversions of podocytes is given as another example (Mundel et al. 1997; Pabst and Sterzl 1983). Based on our experiments with human podocytes (Banyai et al. 2014) it is hypothesized that the transdifferentiation of podocytes could involve several transitions (Banfalvi and Balla, unpublished):

(i) The conversions of epithelial cells of the proximal glomerular tubular parietal cells could turn to tubule committed parietal (Bowman's) cells at the exit of the Bowman's capsule.

(ii) Tubule committed progenitor parietal cells differentiate to adult parietal epithelial progenitor cells.

(iii) Parietal progenitor cells are converted to podocyte committed progenitor cells.

(iv) Transition takes place between podocyte committed progenitor cells and peripolar transitory podocytes.

(v) Undifferentiated U-podocytes develop into differentiated D-podocytes. Experimental evidence is provided that U ↔ D conversion is a bidirectional process.
(vi) Finally, D-podocytes are likely to be converted to macrophage-like cells.

Although, several steps of these transitions have been confirmed, the existing processes exemplify the maximization of economy through maturation, reversibility and aging of cells. A further conclusion that can be drawn from transdifferentiations is that they are related to the permeability of membranes and alterations in membrane structures take place in a so far unknown manner.

1.3 Major Lipids in Biological Membranes

1.3.1 Polyunsaturated Fatty Acids

Mammalian cell membranes are composed of higher proportion of polyunsaturated fatty acids than reptiles (Hulbert and Else 1999). Omega-3 fatty acids are polyunsaturated fatty acids (PUFAs) with a double bond (C=C) at the third carbon atom from the end of the carbon chain (Scorletti and Byrne 2013). Birds have similar higher proportion of polyunsaturated fatty acids in their cell membranes with the notable difference that their omega-3 fatty acid content is lower compared to the omega-6 fatty acids (Hulbert et al. 2002). As a consequence the cell membranes of birds are "leakier" i.e. more permeable to small ions such as H^+ and Na^+ and demand more energy to maintain the basic ionic potential. To maintain warm-blooded homeostasis mammals and birds compensate membrane leakage with higher metabolic rate. Polyunsaturation of fatty acids turned out to be a useful strategy in response to cold temperature. Fish that preferentially live in cold water developed a high content of mono- and polyunsaturated fatty acids to maintain the fluidity of cell membranes at lower temperatures (Hulbert 2003; Raynard and Cossins 1991).

1.3.2 Phospholipids

The major components of cell membranes are the phospholipid units. Phosphoglycerids are the most common class of phospholipids. Their structure is based on the three carbon alcohol glycerol: CH_2OH-CHOH-CH_2OH. The three carbon atoms in glycerol are denoted as Sn1, Sn2 and Sn3 positions. The glycerol in glycerophospholipids contains two fatty acids with even number carbon atoms between 14 and 20, attached to the first and second carbon atoms via esterification forming diglycerides, while the third hydroxy group of glycerol is esterified with a phosphoric acid giving rise to the basic membrane unit known as phosphatidic acid. In the diglyceride part of the phospholipid the saturated fatty acid is normally the

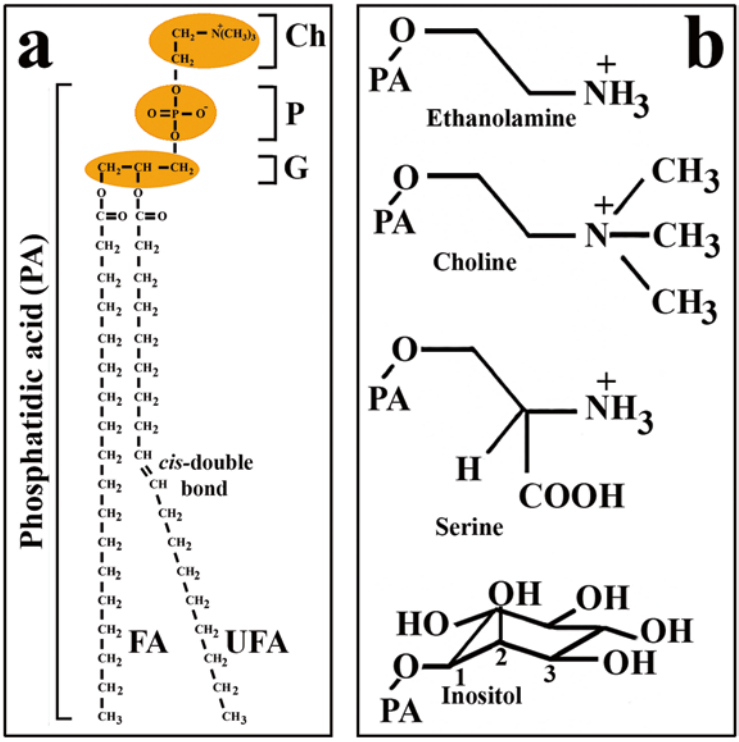


Fig. 1.5 Phosphoglycerides: (**a**) phosphatidylcholine, (**b**) small hydroxylated molecules that can be attached to phosphatidic acid. Abbreviations: *PA* phosphatidic acid, *FA* saturated fatty acid, *UFA* unsaturated fatty acid, *Ch* choline, *P* phosphate, *G* glycerol

C18 stearic acid, the unsaturated fatty acid the C18 *cis*-oleate. The phosphate moiety of phosphatidate can be further esterified with a small molecular weight alcohol, the hydroxy group of which is giving rise to specific phospholipids, such as phosphatidylcholine (Fig. 1.5a). Residues of phosphatidylethanolamine, phosphatidylcholine, phosphatidylserin and phosphatidylinositol are given in Fig. 1.5b.

Known phosphoglycerides are:

- Phosphatidic acid and its dissociated form known as phosphatidate
- Phosphatidylethanolamine (cephalin)
- Phosphatidylcholine (lecithin)
- Phosphatidylserine (PS)
- Phosphoinositides:
  - Phosphatidylinositol (PI)
  - Phosphatidylinositol phosphate (PIP)
  - Phosphatidylinositol bisphosphate (PIP_2) and
  - Phosphatidylinositol trisphosphate (PIP_3)

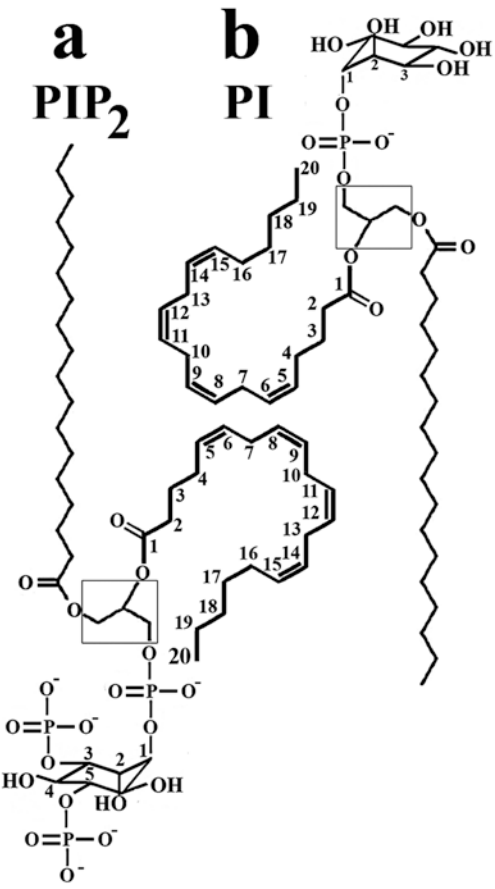


Fig. 1.6 Phosphoinositides. (**a**) Phosphatidylinositol bisphosphate (PIP_2). (**b**) Phosphatidylinositol (*PI*). *Boxed* area: the three carbon atoms of glycerol

In phosphoinositides the most often occurring unsaturated fatty acid is the arachidonic acid (C20) containing four *cis*-double bonds (Fig. 1.6).

Note
when two phosphates are coupled to each other with an anhydride bond the compound is a diphosphate. When the two phosphates are in different positions, the compound is a bisphosphate.

Phosphoglycerides are hydrolyzed by phospholipases. The specific cleavage sites of phospholipases are given in Fig. 1.7. Phospholipase A_1 catalyzes the hydrolysis of the ester bond in position 1 (Sn1) of glycerolipids. Phospholipase A_2 attacks the ester bond in position 2 (Sn2) of glycerolipids leaving behind a free fatty acid and a lysophospholipid, that can be reacylated by acyl-CoA catalyzed by an

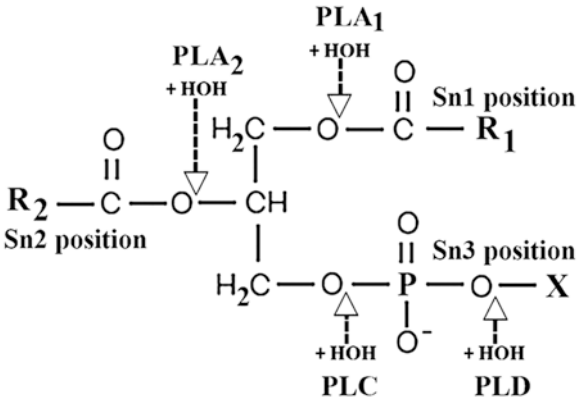


Fig. 1.7 Specific sites of the hydrolytic activity of phospholipases on phospholipid substrate. $R_1—C(=O)—$ and $R_2—C(=O)—$ are the esterified 1- and 2-acyl groups attacked and removed by phospholipase A_1 and phospholipase A_2, respectively. X is representing the nitrogen containing base. Abbreviations: *PLA*$_1$ phospholipase A_1, *PLA*$_2$ phospholipase A_2, *PLC* phospholipase C, *PLD* phospholipase D. The phosphoglyceride model also shows the positions of Sn1, Sn2 fatty acids and the Sn3 position of phosphate and nitrogen containing base on glycerol

acyltransferase. The remaining lysophospholipid such as lysolecitin can be degraded further to the glyceryl phosphoryl base by phospholipase B also known as lysophospholipase by hydrolyzing the remaining 1-acyl group. Finally, the glyceryl phosphoryl base can be split to glycerol 3-phosphate plus the X base. Phospholipase C catalyzes the hydrolytic cleavage in position 3 (Sn3), generating 1,2-diacylglycerol and liberating the phosphoryl base. Phospolipase D found mainly in plant cells catalyzes the hydrolysis of the nitrogenous base from phospholipids.

Phosphosphingolipids (Sphingomyelins) Phospholipids consist of a diglyceride, a phosphate group, and an alcoholic compound. Phosphosphingolipids (sphingomyelins) as phospholipids contain fatty acids, phosphate and choline that are attached to sphingosine, a complex amino alcohol, rather than to glycerol. Sphingolipids represent a different class of cell membrane lipids derived from sphingosine, an 18-carbon unit unsaturated hydrocarbon chain and amino alcohol (Fig. 1.8a). Ceramide is derived from sphingosine by amid formation between the amino group of sphingosine and the carboxyl group of a fatty acid (Fig. 1.8b). The hydroxy group at the end of the sphingosine moiety of the ceramide can be phosphorylated and further esterified with choline, ethanolamine giving rise to sphingomyelins. Alternatively, the hydroxy group can be subjected to O-linked glycosylation. O-glycosylation leads to cerebrosides with a single sugar residue such as galactocerebroside (Fig. 1.8c) or with oligosaccharides producing gangliosides (not shown).

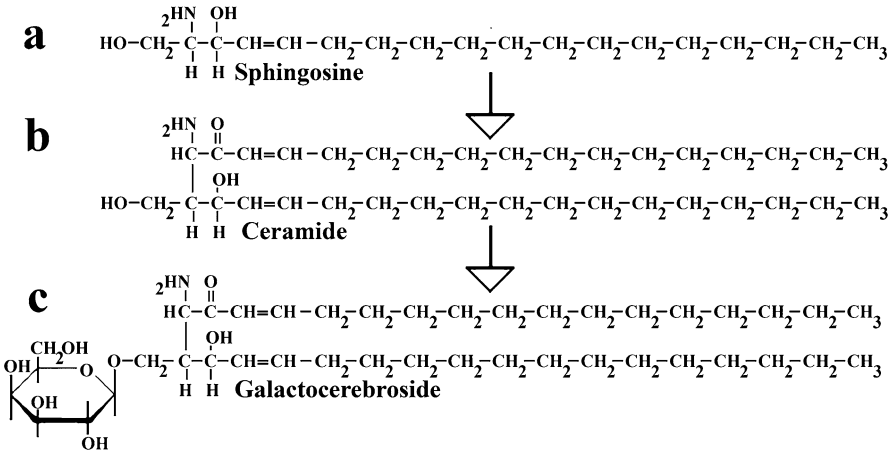


Fig. 1.8 Formation of glycolipid of plasma membranes from sphingosine (**a**), through ceramide (**b**), to membrane surface glycolipid galactocerebroside (**c**)

Glycosphingolipids Glycolipids are lipids with carbohydrate attached to them. They function as energy sources and also serve as markers for cellular recognition. The carbohydrates are located always on the outer monolayer of all eukaryotic cell membranes. They emerge from the phospholipid bilayer into the aqueous environment outside the cell where they act as recognition sites for specific chemicals and help to maintain the stability of the membrane and attach cells to each other to form tissues. The surface of a red blood cell may contain tens of millions of carbohydrate chains. Glycophorins containing one alpha helix, and carrying sugar molecules are rich in sialic acids (derivatives of neuraminic acid) and are characteristic to the membrane of red blood cells. The heavily glycosylated sialoglycoprotein coat gives the red blood cells a strong negative charge and protects them from adhering to each other and to the wall of blood vessels.

Glycosphingolipids contain sphingosine (Fig. 1.8a) esterified with fatty acid (ceramide) (Fig. 1.8b) in combination with mono-, or oligosaccharides. The simplest glycosphingolipid is galactocerebroside, the major lipid component of myelin (Fig. 1.8c).

Types of glycosylation:

(a) N-glycosylation. The initial biosynthesis of glycoproteins known as core-glycosylation in the endoplasmic reticulum takes place by N-glycosylation and generates glycans attached to the nitrogen of the asparagine and arginine moieties of nascent proteins. This type of glycosylation requires the special C20 lipid known as dolichol phosphate.
(b) O-glycosylation (or O-linked glycosylation) occurs mainly in the Golgi apparatus (Elmouelhi and Yarema 2008) through the attachment of glycans to the hydroxy residues of serine, threonine, tyrosine, hydroxyproline and hydroxylysine or to the hydroxy group on glycosphingolipids such as ceramide.

(c) In the less frequently occurring C-linked glycans the sugar is added to the carbon side chain of tryptophan.
(d) The glycolipid glycosylphosphatidylinositol (GPI) anchor can be attached to the C-terminal end of proteins *via* the ethanolamine and glycosidically bound to the inositol residue. GPI-linked proteins normally contain a signal peptide that directs these proteins to the rough endoplasmic reticulum.

Cell Surface Markers
Cell surface markers are antigenic determinants expressed on the surface of cells serving as markers of specific cell types to distinguish between self and nonself. Virtually all somatic cells carry such distinctive markers. Best known are lymphocytes: T cell and B cell surface markers identify their lineage and stage in the differentiation process. These lymphocytes differentiate into multiple cell subtypes, necessary for specific biological processes. During their differentiation, lymphocytes express different surface receptors, which are used to identify cellular subtypes, from progenitor cells to terminally differentiated T helper cells. Inappropriate differentiation may change the relative amounts of Th1 and Th2 cells and cause pathological conditions such as autoimmunity. Surface marker expression also helps to determine whether or not a drug or ligand will be recognized by the particular cell type of interest. Although, several biomarkers specific for cell types are known, including tumor markers indicating the presence of tumor cells, many more biomarkers remain to be discovered. The existence of cell surface markers underlines the importance of the the outer membrane.

Besides serving as energy carriers, glycolipids are markers for cellular recognition. Cell surface markers are specific antigenic determinants on the cell surface. Evidence has been provided that one of the functions of the glycolipid galactocerebroside is its involvement in opening Ca^{2+} channels in oligodendroglia cells (Dyer and Benjamins 1990). Galactocerebroside is a specific differentiation marker for myelin producing cells and plays an important role in myelin function and stability (Coetzee et al. 1996). It is the characteristic feature of axons of nerve cells in vertebrates that they are ensheathed with a multilamellar myelin membrane that is enriched in galactocerebroside.

Ether Phospholipids In ether lipids the hydroxy groups of the two carbon atoms of glycerol form ether bonds with two long chain alcohols at Sn1 and Sn2 positions, rather than ester bonds with fatty acids. The third hydroxy group of glycerol (Sn3) is esterified with phosphate. In plasmalogens the ether is at the first (Sn1) position, the esters are at second (Sn2) and third (Sn3) positions (Fig. 1.9).

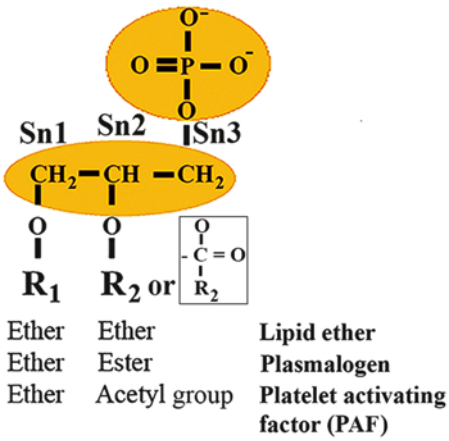


Fig. 1.9 Structure of ether phospholipids. R_1 is denoting a long chain fatty alcohol. R2 stands for a fatty acid or fatty alcohol chain. Corresponding to the stereospecific numbering on the Fischer projection, the first hydroxy group is by convention in Sn1, the middle one in Sn2 and the third one in Sn3 position

Ether-linked lipids are abundant and are typical to Archaea. The high chemical stability of ether bonds is contrasted by the hydrolysable ester bonds in glycerolipids that are characteristic to the lipid membranes of Bacteria and Eukarya. The chemical stability of ether lipids could explain the resistance and the survival of Archaea under extreme stress (heat, high salinity, etc.) placed on their cytoplasmic membranes.

Plasmalogens represent a special class of membrane glycerolipids with a fatty alcohol containing a vinyl (–CH = CH_2) bond at the Sn1 position and enriched in polyunsaturated fatty acids at the Sn2 position of the glycerol backbone. Although, plasmalogens make up to 20 % of the total phospholipid mass in humans, their physiological roles remained challenging, but are expected to play developmental roles in the cells of bone, brain, lens, lung, kidney and heart (Braverman and Moser 2012) and in their endurance and resistance to usage and stress.

In the platelet activating factor (PAF, acetyl-glyceryl-ether-phosphorylcholine) ether is at the first, and acetyl group at the second position (Fig. 1.9). PAF is produced by many cells, in those that are involved in host defense (platelets, endothelial cells) particularly in innate immune defense including polymorphonuclear leukocytes (PMNs or neutrophils) and monocytes (macrophages, dendritic cells). PAF as a phospholipid activator affects platelet aggregation and degranulation, inflammation, changes vascular permeability upon oxidative burst, influences arachidonic acid metabolism. The phospholipid structure of PAF confirms the notion that the composition of the cell membrane evolved and adapted in response to the environmental changes.

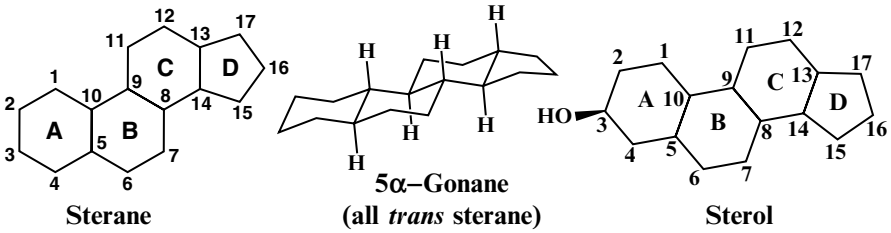


Fig. 1.10 Basic steroid structures: sterane, gonane and sterol

1.4 Steroids

The basic structure of steroids is sterane (cyclopentano-perhydrophenanthrene) representing a class of four cyclic compounds consisting of A, B, C and D rings with a side chain at carbon C17. The sterane structure shown in Fig. 1.10 may give rise to 64 stereoisomers. Probably the most important stereoisomer is the full-trans gonane (α-gonane) where all rings are in *trans* configuration. In steroids, including steroid hormones the rings are in full-*trans* configuration. Notable exceptions are the derivatives of bile acids where the A and B ring are *cis*-oriented, corresponding to ß-gonane, B–C and C–D rings have *trans*-configurations (not shown).

1.4.1 Sterols

Sterols are derivatives of steroids. These steroid alcohols are present in animals, plants, and fungi. Sterols of animals are zoosterols. The most frequently occurring and most important zoosterol is cholesterol; an indispensable component of the animal membrane structure, to be discussed in relation to the cellular membranes.

Phytosterols are produced by plants. The most important phytosterols include campesterol, sitosterol, and stigmasterol. Natural sources of phytosterols are vegetable oils and nuts. Cereals, vegetables, fruits and berries contain less phytosterols, nevertheless may contribute to a higher phytosterol intake. Due to their structure as cholesterol analogues they are blocking not only the intestinal absorption of cholesterol, but probably also other nutrients. This makes their use questionable as higher phytosterol intake particularly that of ß-sitosterol may inhibit the growth of breast and prostate cancer (Awad et al. 2000, 2001; Ju et al. 2004). Plant sterols (beta-sitosterol, campesterol, stigmasterol) reduce the gastric cancer risk (De Stefani et al. 2000), and improve lower urinary tract symptoms (Dreikorn 2002).

Ergosterol is a crystalline fungal sterol formerly isolated from the fungus grown on ergot (*Claviceps purpurea).* Ergosterol is present in the membranes of fungi and protozoa serving the same function as cholesterol in animal cells. Due to its importance in these cells it became a target to develop drugs against ergosterol to defend fungal and protozoan infections. Vitamin D_2 is produced from ergosterol upon the chemical reaction induced by ultraviolet light exposure.

1.4.2 Cholesterol, Cholesterol Esters

Cholesterol Cholesterol is a lipid molecule synthesized by animal cells. Major steps of cholesterol biosynthesis are:

Acetyl-CoA → hydroxymethylglutaryl-CoA → mevalonate → "biological isoprenes" (isopentenyl pyrophosphate, dimethylallyl pyrophosphate, geraniol pyrophosphate, farnesyl pyrophosphate) → squalene (Fig. 1.11) → lanosterol → cholesterol. Daily 1.5–2 g cholesterol is synthesized in the human body primarily by tissues producing steroid hormones (gonads, adrenal cortex).

Cholesterol as the principal steroid is the precursor of steroid hormones and vitamin D (Hanukoglu 1992). Cholesterol is present in the circulation as free cholesterol or as cholesterol ester. The degradation of cholesterol takes place in the liver and produces bile acids. Due to its low solubility high cholesterol level in blood may develop cholesterol deposits in blood vessels. Cholesterol deposits slow down the blood flow and their presence in the arteries of heart and brain is particularly dangerous by increasing the risk of heart attack and causing stroke. High cholesterol level (hypercholesterolemia) can be an inherited genetic trait, but more often can be the result of unhealthy diet that can be prevented and medicated by changing to healthy lifestyle and exercise. The rest of the book deals with cholesterol only as a membrane component.

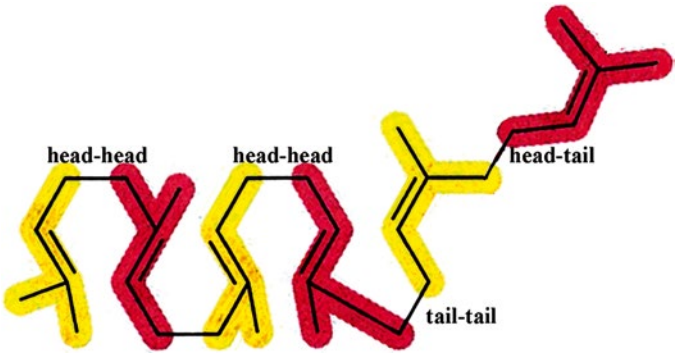


Fig. 1.11 Squalene (C30) formation by the connection of two farnezyl-pyrophosphates (C15)

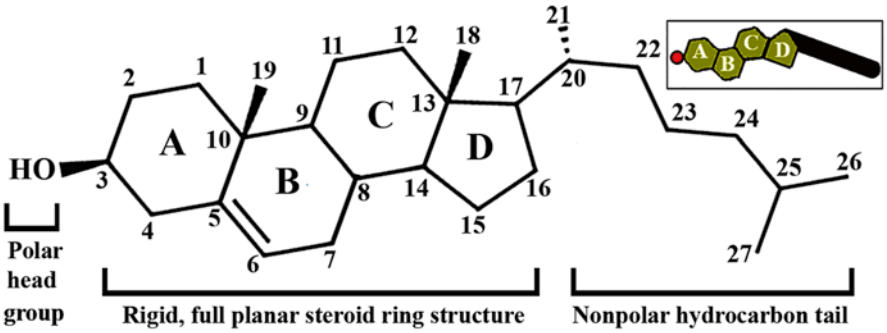


Fig. 1.12 Bond-line and shorthand formulas of cholesterol with numbered carbon atoms and capital letters of rings. *Upper right boxed* corner: shorthand structure of cholesterol. The shorthand structure of cholesterol will be used in membrane structures

Cholesterol is present in each human cell, contributing to roughly 50 % of the molecules of the cell membrane. Since it is smaller than other membrane components its mass in the membrane is only about 20 % (Alberts et al. 2002). The cholesterol content of organelles inside the cell is normally smaller. The proportion of cholesterol in mitochondria is exceptionally small, not more than three percent, and only 6 % in the endoplasmic reticulum (Alberts et al. 2002). As the double bilayer of the nuclear membrane is in close connection with the endoplasmic reticulum, the nuclear membrane also has lower cholesterol content. The membranes of organelles will be discussed individually.

The major function of cholesterol is to protect membrane integrity and cell viability by increasing or lowering its rigidity, to maintain fluidity and integrity of the animal cell membrane, to contribute to cellular movements, cell signaling through the membrane, to change the shape of the cell without necessitating a cell wall, unlike plant cells and bacteria. To understand the complex behavior of lipid membranes, it is necessary to elucidate the basic mechanical properties of cholesterol. Cholesterol is said to be a mobile molecule inside the continuous double-layer of phospholipid fluid membrane. Cholesterol has only one polar hydroxy group at position 3, while the four rigid steroid rings and the hydrocarbon tail are apolar (Fig. 1.12) providing the molecule only a week polar, and a strong, overwhelmingly apolar character and low solubility of the molecule. Under physiological conditions cholesterol increases the overall non-polar bonding with fatty acids inside the phospholipid bilayer, stiffening the surface of the membrane and making it less soluble and permeable to small soluble molecules that could pass in the absence of the apolar cholesterol. As dictated by the structure and amphipathic nature of cholesterol its hydrophobic core is buried within the hydrocarbon region of the bilayer. Cholesterol spans approximately one leaflet of the membrane, with its OH group protruding into the polar (head group) region of the bilayer (Kessel et al. 2001).

Cholesterol is not the only factor that influences the bending rigidity of membranes. Certain lipids including cholesterol, sphingomyelin and glycolipids segre-

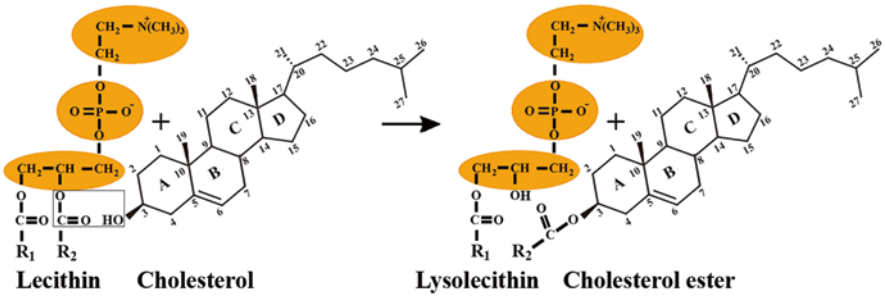


Fig. 1.13 Cholesterol ester derived by the reaction of lecithin (phosphathidylcholine) and cholesterol

gate into submicron-sized domains known as lipid rafts (Simons and Ikonen 1997; Brown and London 1998; Lipowsky and Dimova 2003). Double bonds of unsaturated fatty acids with hydrocarbon chains in *cis*-orientation increase the fluidity of the phospholipid bilayer, saturated straight hydrocarbon chains and cholesterol stiffen the lipid bilayer.

The stability of cell membranes is maintained by balancing the level of cholesterol, unsaturated and saturated fatty acids in the phospholipid bilayer. This equilibrium is directed by membrane-bound transcription factors known as sterol regulatory element-binding proteins (SREBPs) that activate genes encoding enzymes of cholesterol and fatty acid biosynthesis (Brown and Goldstein 1999). Cholesterol, along with sphingomyelin, form caveolae also referred to as plasma membrane rafts where signaling molecules are concentrated (Simons and Ikonen 1997; Anderson 1998). Lipid rafts enriched with transmembrane α-helices, surrounded by high amount of cholesterol and containing signaling molecules will be discussed with the caveolae.

Cholesterol Esters The synthesis of cholesterol esters is catalyzed by the enzyme lecithin-cholesterol acyltransferase (LCAT) through the transfer of Sn2 fatty acid of phosphatidylcholine (lecithin) to the C3 OH group of cholesterol where esterification takes place. The products are 1-lysophosphatidylcholine and cholesterol ester (Fig. 1.13).

1.5 Waxes

Waxes are low melting point solids formed by the esterification of long-chain carboxylic acids with long-chain alcohols. Best known examples of waxes are:

- Bee's wax: $C_{25}H_{31}$-COOH, secreted by worker honeybees
- Spermacetic wax (Spermacetil): $C_{15}H_{31}$-COOH, most often found in the head cavities of the sperm whale.

Waxes have not much to do with cellular membranes, and will not be further discussed.

1.6 Amphipathic Molecules Forming Synthetic Bilayers

Two-dimensional structures of oriented amphipathic molecules and proteins are spontaneously formed in water. These molecules possess both hydrophilic (polar, water-soluble) and lipophilic (apolar, fat-loving, water insoluble) portions in their structure and correspondingly are amphipathic. To these compounds belong soaps, detergents, fatty acids, bile acids, saponins, cholesterol, lipoproteins, phospholipids, glycolipids, etc. As the lipid membrane is present in each cell the study of artificial membranes and vesicles contributes to the understanding of membranes of real cells.

Due to the amphipathic nature phospholipids form not only bilayers in aqueous solutions, but also other structures such as micelles and liposomes (Fig. 1.14). The three major types of lipids in biological membranes are the most abundant phospholipids, cholesterol and glycolipids are amphipathic molecules. Due to their amphiphilic nature, phospholipid molecules tend to form liposomes, micelles and bilayers in aqueous environment. In aqueous solution amphipathic phospholipids spontaneously aggregate with their hydrophobic hydrocarbon tails turned inside and buried in the interior and their hydrophilic heads turned outward to the water.

Lipid Micelles The immersion of lipid molecules in aqueous environment at near physiological conditions (concentration, temperature), the spontaneous aggregation into a bilayer membrane or an encapsulated bag named vesicle takes place. In these micellar aggregates the head portions of phospholipids are in contact with water and the hydrophobic fatty acid tail regions turned inside the micelle (Fig. 1.14a). Similarly, the hydration of the head groups of other surfactant molecules forces

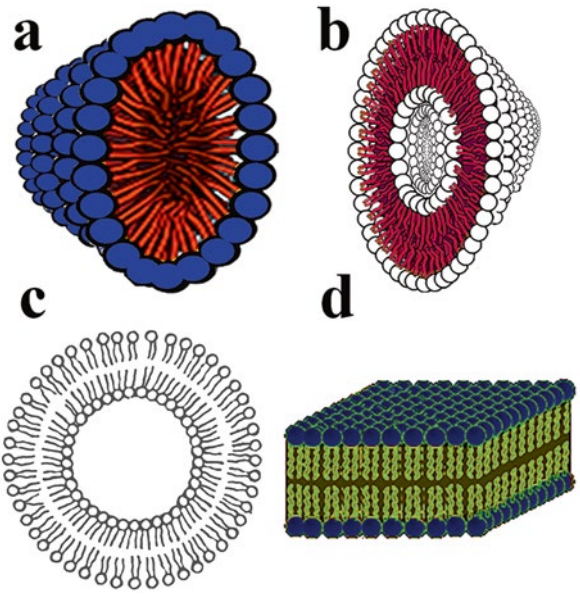


Fig. 1.14 Structures formed by phospholipids in aqueous solutions. (**a**) micelle, (**b**) liposome, (**c**) spherical liposome in cross-section, (**d**) phospholipid bilayer forming the essential backbone of cellular membrane

these monomers to turn their apolar portions inward forming a micelle. Individual surfactant molecules that are in the system but not being part of the micelle are "monomers". Lipid micelles represent a molecular assembly, where individual components are thermodynamically in equilibrium with monomers of the same species in the surrounding medium. In water, the hydrophilic "heads" of surfactant molecules are always in contact with the solvent, regardless of whether the surfactants exist as monomers or as part of a micelle. However, the lipophilic "tails" of surfactant molecules tend to avoid contact with water and are the basis for the energetic drive of micelle formation. In a micelle, the hydrophobic tails of several surfactant molecules assemble into an oil-like core, providing stability for the micelles by avoiding contact with water.

Liposomes (lipid vesicles) are formed when thin lipid films or lipid sandwiches are hydrated and stacks of liquid crystalline bilayers become fluid and swell. The hydrated lipid sheets detach during agitation and become self-closed to form large, multilamellar vesicles, most frequently liposomes (closed bilayers) (Fig. 1.14b, c).

Model Bilayers The packing of lipids within the bilayer affecting its mechanical properties, have been studied with artificial "model" bilayers produced in laboratories (Fig. 1.14d). Liposomes were made by model bilayers and have been used clinically to deliver drugs.

1.6.1 Synthetic Membranes, Artificial Cell Membranes

Artificial cell membranes are customized cell membranes that mimic live membranes. The composition of synthetic membranes can be quite different from the above mentioned synthetic bilayers. Among the commercially available synthetic membranes are organic polymers, but they can also be made of liquids and inorganic materials. Artificial membranes have been developed for laboratory purposes and widely used in the industry. They have the potential of containing targeted membrane proteins and other biological molecules that could be useful for bio sensing, detection of toxins and diseases (http://phys.org/news/2014-09-artificial-membranes-silicon-synthetic-solvents.html#jCp).

Synthetic membranes are utilized primarily by the separation industry, in membrane separation processes through filtration, microfiltration and ultrafiltration, depending on particle size. Filtration processes utilizing synthetic membranes are: water purification, reverse osmosis, removal of hydrogen from natural gas, removal of microorganisms from dairy products, beer clarification by microfiltration, dialysis, just to mention the most important ones. Reverse osmosis is a commercially available procedure since the 1970s (Loeb et al. 1976) for producing drinking water from seawater, by desalination using semi-permeable membranes and removing other substances from the water molecules. Dialysis is a filtration process to remove waste and excess water from the blood of patients, who have lost or damaged kidney function (Pendse et al. 2008).

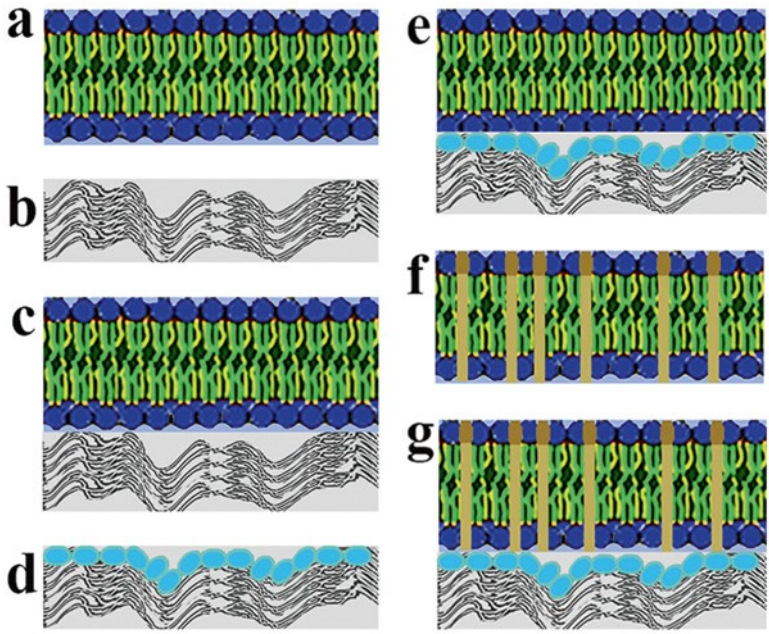


Fig. 1.15 Increasing complexity of artificial lipid membranes. (**a**) Simple phospholipid bilayer. (**b**) Polymer membrane (e.g. Chitosan). (**c**) Complex lipid-polymer membrane. (**d**) Protein coated polymer membrane. (**e**) Complex lipid-protein coated polymer membrane. (**f**) Channels in lipid membrane. (**g**) Complex channeled–lipid-polymer membrane

The first patented artificial cell membrane was developed in 2009 and was licensed to speed up drug discovery (http://phys.org/news/2014-01-artificial-cell-membranes-drug-discovery.html#jCp). Recently the formation of an artificial membrane composed of phospholipid bilayer composed of dipalmitoylphosphatidylcholine sitting on a polysaccharide mattress (Chitosan) keeping the membrane hydrated, has been synthesized with the aim to develop nano-device sensors (Retamal et al. 2014). The development of an artificial membrane of increasing complexity is seen in Fig. 1.15. Detailed description of artificially supported lipid membranes as model systems for the understanding of the phenomena occurring in the cell membrane was given by Váró and Szegletes (2012).

1.6.2 Artificial Cells

Engineered artificial or minimal cells may mimic certain functions of biological cells, but are not real cells that could reproduce themselves or would provide specific genetic information to make them individual. Although, synthetic DNA was implanted into a genetically emptied host cell and this “genome” was able to

duplicate itself (Gibson et al. 2010), this construction was not an artificial cell because the cell membranes including those of subcellular particles of the host remained intact and without them the cell would not have been able to maintain biological functions. Instead of creating so called artificial cells it is more reasonable to speak about bioactive polymeric systems that utilize biological or polymeric membranes to enclose biologically active polymers, such as natural polymers, synthetic polypeptides, pseudo enzymes, pseudo nucleic acids, and polymeric drugs and include immobilized bioactive materials, such as immobilized enzymes, antibodies, and other bioactive agents (Petersen 1985).

1.7 Lipid Membranes

1.7.1 Cytoplasm, Cytosol, Cytoskeleton

Cytoplasm Before discussing the membranes of the cell it is inescapable to review briefly the colorless cytoplasm consisting of the gel-like cytosol (~80 % water) and the organelles. Cytosol is the unstructured aqueous part of the cytoplasm that contains neither membranes, nor organelles, is not confined by the boundaries of a matrix, nor by insoluble cytoskeletal elements, which fill the inner part of the cell.

The density of the sol-phase cytoplasm solution is influenced by the presence of salts, proteins, including enzymes, amino acids, carbohydrates, lipids, nucleotides and other metabolites. In prokaryote organisms that lack nuclei all sub-structures are within the cell, in eukaryotes the nucleus has its own substructure known as nucleoplasm. Animal cells have larger cytoplasms than plant cells. The cytoplasm of plant cells may contain relatively large vacuoles. The thylakoid sacs in the stroma of the chloroplasts are the sites of photosynthesis where light-dependent reactions take place with photosynthetic components: cytochrome b6f complex, photosystem I, Photosystem II, NADP reductase, ATP synthase, plastoquinone embedded in the thylakoid membrane (McGraw 2007). The cytochrome b_6f complex (plastoquinol – plastocyanin reductase; EC1.10.99.1) transfers electrons between the two reaction complexes from Photosystem II to Photosystem I, introduces protons into the thylakoid space and generates an electrochemical gradient that is converted to the energy of ATP.

Most of the cellular activities take place in the cytoplasm *via* metabolic pathways. Metabolism includes all chemical reactions taking place in the cell. The inner part of the eukaryotic cytoplasm is also called endoplasm and the outer part cortical or ectoplasmic region. As a result of the active metabolism cytoplasmic components interact with each other and contribute to the intracellular movement of organelles and particles. The nature of these movements is incompletely understood. Part of the cytoplasmic streaming is tightly connected and dependent on the permeability of the cytoplasm. The shape of the cell, the size of subcellular compartments, the distinctly non-homogeneous and spatial distribution of molecules within the

cytoplasm influence molecular interactions and cellular behavior (Cowan et al. 2012). The cytoplasm is responsible for: (a) the shape of the cell, (b) filling out the cell, (c) keeping organelles in their place.

Cytosol This part of the cytoplasm that does not contain membrane bond organelles is composed of water (~70 %), salts and a complex mixture of cytoskeletal filaments and dissolved organic molecules.

Cytoskeleton The filaments (microfilaments, intermediate filaments, microtubules) make up the cytoskeleton. The structure and function of cytoskeleton are given in Sect. 1.8.3. The cytosol maintains a relatively high concentration of soluble molecules and proteins, granular structures (ribosomes, proteasomes) causing macromolecular crowding. In spite of the convergent evolution, the osmotic pressure of the cytosol in land vertebrates (mammals, reptiles, snakes, lizards, turtles, and birds) remained nearly identical (≈0.3 Osm). This osmotic pressure is still within the range of dilute aqueous solutions, which by the definition of dilute solutions has at least a 100-times higher molar excess of solvent over solutes and an osmolarity less than 0.555 Osm.

1.7.2 Fluid Mosaic Bilayer

The idea that cell membranes are made of lipids came from Overton (1895). That red blood cell membranes are formed by a fatty layer was described by Gorter and Grendel (1925). Danielli and Davson (1935) proposed that the lipid membranes contain proteins and these pore-like structures are responsible for the selective permeability of membranes. The thickness of the "unit membrane" based on several estimations is about 3–10 nm. The "unit membrane" hypothesis was established by Robertson's electron microscopy studies who stated that all biological membranes including plasma and organellar ones consist of phospholipid bilayers (Robertson 1959). The phospholipid bilayer without intra and transmembrane molecules (cholesterol, protein) was demonstrated in Fig. 1.14d. The fluid mosaic model of Singer and Nicolson (1972) replaced the Davson and Danielly model, and characterized the cell membrane as a two-dimensional oriented solution of integral proteins and lipoproteins in a viscous phospholipid bilayer solvent (Fig. 1.16). The recognition that certain head groups by altering the surface of the bilayer can serve as signals and anchors established the pathway of phospholipid signaling (Divecha and Irvine 1995).

The fluid mosaic model of the lipid phase behaves like an interface between two communicating aqueous compartments and as a matrix for a large variety of molecules, e.g. membrane transport proteins, receptors, adhesion/recognition molecules, etc.

The study of phospholipids in the 1970s led to the demonstration that lipid molecules can freely diffuse within synthetic lipid bilayers. The schematic view of a micelle, spherical lipid vesicle called liposome and its cross-section were shown in Fig. 1.14. The size of liposomes is between 25 nm and 1 μm in diameter depending

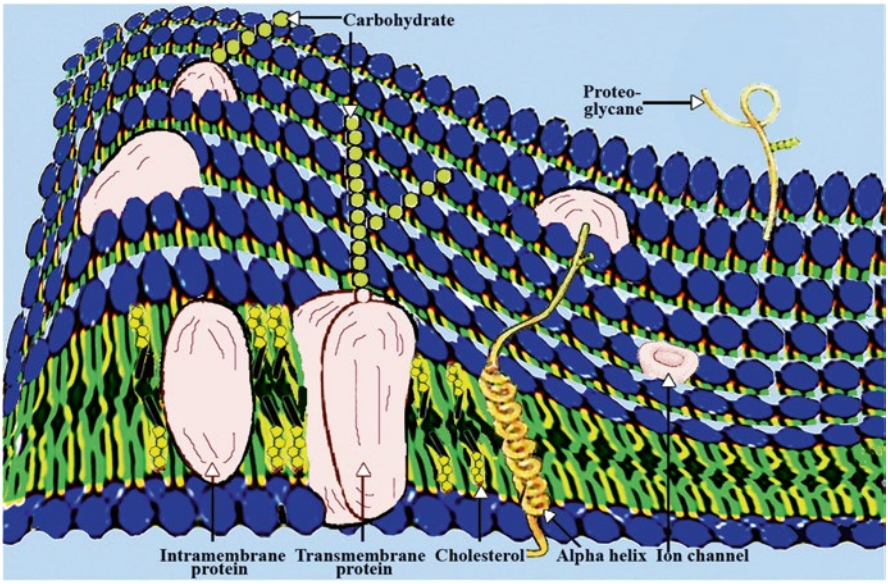


Fig. 1.16 Fluid mosaic structure of phospholipid bilayer with embedded intramembrane and transmembrane proteins, cholesterol, alpha helix, ion channel, glycoproteins containing carbohydrates

on the conditions used for their production (Bangham 1975). The polar heads of the synthetic lipid constructs containing an unpaired electron ("spin label") with paramagnetic property was used as a signal to follow the migration of incorporated and spin labeled lipid molecules. As a result three types of movements in lipid bilayer were verified: lateral movement (lateral diffusion), free rotation of individual lipid molecules along their long axis and less frequently the movement of lipid molecules jumping from one side of the bilayer to the opposite one ("flip-flop"). As an exception rapid flip-flop movement was detected in the endoplasmic reticulum membrane (Marx et al. 2000). The physical proximity of endoplasmatic reticulum to the mitochondria-associated ER membranes also allows for higher incidence of flipping between these bilayers (Osman et al. 2011).

Relative to its enormous lateral spread the lipid bilayer is thin, not much more than 10 nm in diameter. The bilayer does not contain covalent bonds, but is held together by week intermolecular interactions known as non-covalent forces. These hydrophobic interactions among hydrophobic tails are responsible for the physical properties of the lipid bilayer known as fluidity. Biological membranes, in the form of cell membranes, normally consist of two phospholipid layers (phospholipid bilayer) containing embedded proteins serving communication and transportation between the two sides of the lipid bilayer. Transmembrane proteins may completely cross the lipid bilayer with each end performing its own function (Fig. 1.17a). Partially embedded proteins do not cross the whole membrane and are only partially

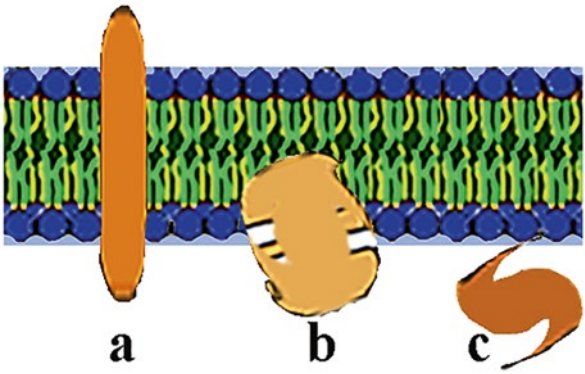


Fig. 1.17 Transmembrane (**a**), partially embedded (**b**) and peripheral protein (**c**)

inserted into the membrane (Fig. 1.17b). Some of the peripheral proteins are not embedded but are associated with the lipid bilayer (Fig. 1.17c).

Phospholipid units in the lipid bilayer of biological membranes provide the fluid matrix for integrated intramembrane and transmembrane proteins by rotation and lateral diffusion. The nuclear membrane (nuclear envelope, nucleolemma) is a double lipid bilayer surrounding the eukaryotic nucleus and consists of the inner and the outer lipid bilayers.

One of the most important elements of the phospholipid signaling is the hydrolysis of phosphatidylinositol (4,5)-bisphosphate (PIP_2) to inositol (1,4,5)-trisphosphate (IP_3) and diacylglycerol (DAG) with the synergistic action of protein kinase C and Ca^{2+}, mobilized by IP_3 (Fig. 1.18). However, due to the limited space no attempt will be made for the comprehensive coverage of phospholipid signaling with the participation of 3-phosphorylated inositol lipids. For the same reason neither the interactions of polyphosphoinositol lipids nor the cytoskeleton (Lassing and Lindberg 1985; Janmey and Stossel 1987), nor the nuclear inositide cycle (Divecha et al. 1993) will be discussed.

1.7.3 Bacterial Membranes

Similarly to eukaryotes (animals, plants, fungi, protists), all prokaryotic cells have at least one phospholipid bilayer membrane. This means that every cell on Earth has a cell membrane. In addition to serve as a permeability barrier and transport of molecules into the cell the bacterial plasma membrane plays role in energy conservation through the generation of proton motive force. With the notable exception of mycoplasma, methane utilizing bacteria (methanotrophs) and blue-green algae prokaryotic membranes do not contain cholesterol. Another notable exception relative to membranes of eukaryotic cells is the absence of glycoproteins in prokaryotic cell membranes. The structurally related compounds of cholesterol in bacteria are pentacyclic triterpenoids known as hopanes (Fig. 1.19) derived in the mevalonic acid pathway

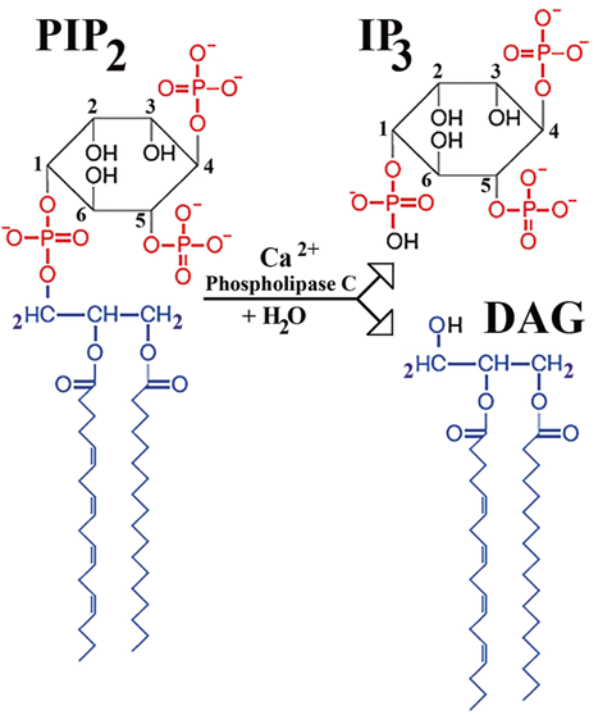


Fig. 1.18 Involvement of phospholipids in cell signaling. Enzymatic hydrolysis of phosphatidyl inositolbisphosphate (PIP_2) to inositol trisphosphate (IP_3) and diacylglicerol (DAG)

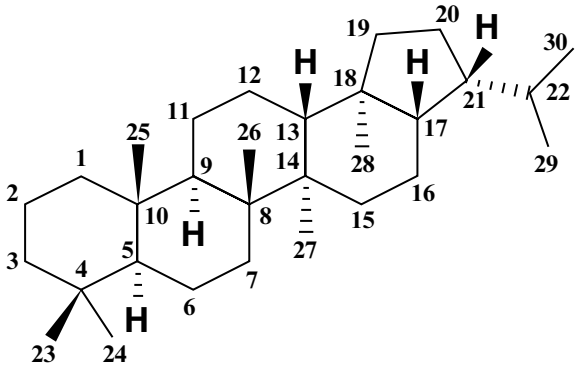


Fig. 1.19 Chemical structure of hopane and the numbering of its carbon atoms

similarly to the structure and function of cholesterol in the plasma membrane. In the hopane skeleton structure all four cyclohexane rings are in chair conformation and the cyclopentane ring in twisted conformation. All ring structures are rigid, planar and hydrophobic. Another notable difference in the bacterial membrane structure is the presence of a wide variety of fatty acids with methyl, hydroxy and cyclic side groups modulating the fluidity of the membrane upon temperature changes. The outer

bacterial lipid bilayer is impermeable to ions and charged molecules, but rendered permeable through porin channels for the passive transport of ions sugars, amino acids. Beside these molecules, the periplasmic space between the outer and inner cytoplasmic membranes contains high concentrations of peptidoglycans, proteins providing a viscous gel-like consistency. Signals are transported through the inner cytoplasmic membrane *via* the signaling proteins of the periplasmic space.

1.7.4 Eukaryotic Supramembrane Structures

Each eukaryotic cell is surrounded by the cell membrane. The intracellular components are separated by inner membranes. Fungi, bacteria and plants have in addition an external cell wall that provides mechanical support and prevents the diffusion of large molecules. Multicellular organisms (plants, animals, most fungi) are composed of many cells. The diverse group of eukaryotic microorganisms belonging to the kingdom of protists (algae, plankton, slime molds, etc.) is no more an official term, but remained in use. Although, protists are neither animals, nor fungi, nor plants, similarly to all eukaryotic cells contain cytoplasmic membrane, membrane-bond nuclei, endomembrane systems and several organelles. As protists evolved from prokaryotic cells some 1.7 billion years ago, the folding of their plasma membrane into the cell to form the nuclear membrane and other organelles of the endomembrane system represent the archetype from which the same kind of eukaryotic nuclear envelope, organellar membranes and supramembranes of higher eukaryotes evolved.

One would expect that the basement membranes opposed to supramembranes are simple forms of structures. However, the name of basement membrane is misleading since it is not a membrane, but a specialized form of extracellular matrix composed of three laminae: the electron-lucent (*Lamina lucida*), electron-dense (*Lamina densa*, formerly known as basal lamina) and the reticular lamina (*Lamina reticularis*) (Fig. 1.20).

Caveolae In vertebrate cells, particularly endothelial cells and in adipocytes the plasma membranes may give rise to higher, so called supramembrane structures visible by electron microscopy. Among these supramembrane structure are small (50–150 nm) invaginations resembling the shape of the Greek letter omega (Ω) that form little caves (*caveolae*). These supramembrane structures are often grouped one after the other in the plasma membrane. Caveolar membrane structures enriched in proteins, cholesterol and sphingolipids play role in cell division, cholesterol homeostasis, signal transduction, endocytosis of tumor cells, uptake of bacteria and viruses (Anderson 1998; Frank and Lisanti 2004; Li et al. 2005; Pelkmans 2005; Anchisi et al. 2012). In the caveolar structures one can find lipids (gangliosides, sphingomyelin, ceramide, diacylglycerol, cholesterol), acylated proteins (heterotrimeric G, Src, Fyn, Hck, Lck, E-NOS, CD-36, caveolin), multiple glycosylphosphatidyl (GPI)-anchored proteins (folate receptor, Thy1, urokinase, Rec, 5′-nucleotidase, CD14), prenylated proteins (Rap1A, Ras), membrane receptors (platelet-derived

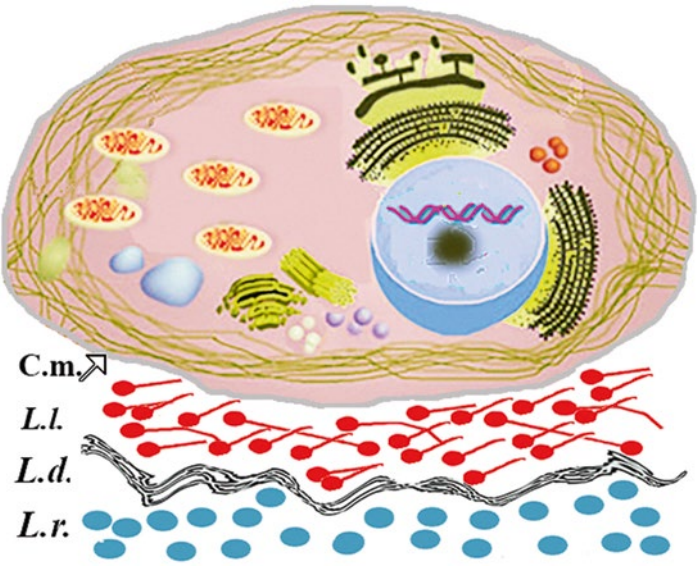


Fig. 1.20 Three distinct layers of extracellular basement membrane. Parts of the basal lamina: Electron lucent *Lamina lucida* (*L.l.*), electron dense *Lamina densa (L.d.*), and reticular fibres *Lamina reticularis* (*L.r.*). C.m., cellular membrane

growth factor, PDGF; epidermal growth factor, RGF), insulin receptor, cholecystokinin receptor, M2 muscarinic acetylcholine receptor, tissue factor, ß-adrenergic receptor, bradykinin, endothelin, SR-B1 receptors), membrane transport proteins (porin, IP3 receptor, Ca^{2+}-ATPase, aquaporin, H^{+}-ATPase), structural proteins (actin, proteolipids, annexin II, ezerin, myosin, the vesicular-fusion protein NSF), just to mentioned the best known ones.

Lipid rafts Large associations of proteins in the lipid bilayer may cause lateral interactions and aggregations to special lipid structures known as the lipid-rafts. Caveolae are invaginations of giant lipid rafts into the cytoplasm stabilized by caveolin oligomers, the main components of caveolar plasma membrane and by cholesterol. Lipid rafts are present in each eukaryotic cells, caveolae occur only in cells that express caveolin. In caveolar lipid rafts the membrane proteins generate rigid clusters instead of allowing free bilateral movement. Unlike the classical Singer-Nicholson plasma membrane model, in lipid rafts the cell surface is less organized, becomes inhomogeneous, separates to distinct phases resembling ice-floes. The rigidity of the rafted membrane is secured by the presence of cholesterol and sphingolipids (Fig. 1.21).

Postsynaptic Densities These supramembrane structures are specialized thickened parts of postsynaptic membranes in postsynaptic neurons. These electro-dense regions of the plasma membrane are thought to be responsible for the temporal changes in synapses to weaken or strengthen the electric signals and correspondingly

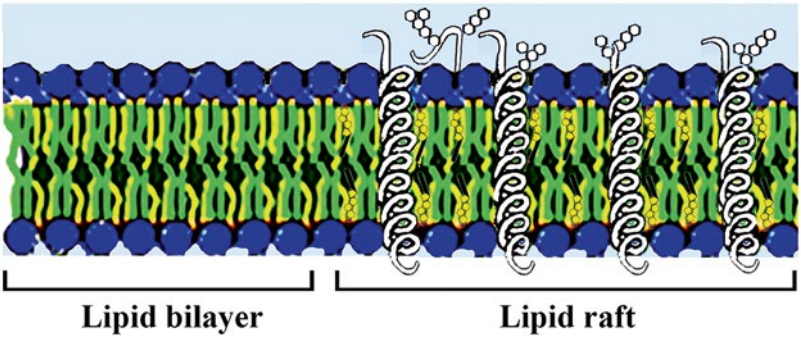


Fig. 1.21 Schematic view of a lipid raft. Regular lipid bilayer is contrasted by the lipid raft. Lipid raft contains transmembrane proteins surrounded by increased amount of imbedded cholesterol molecules. Polar head groups of cholesterol are oriented toward the polar heads of phospholipids

to decrease or increase their action potential (Hughes 1958). Many proteins have been found among the postsynaptic densities, primarily glutamate receptors, scaffold proteins and signaling molecules (Purves et al. 2008; Gerrow and Triller 2010; Renner and Specht 2008).

Invadosomes The cellular membrane can form "supramembrane" structures known as podosomes and invadopodia collectively termed invadosomes. Invadosomes are actin-based functional modules responsible for special cell-matrix contacts, able to degrade the extracellular matrix and regulate major invasive mechanisms (Tarone et al. 1985; Linder and Aepfelbacher 2003; Gimona et al. 2008). Macrophages, dendritic cells, and osteoclasts utilize the specialized adhesive array of podosomes composed of regulatory proteins most commonly occurring in focal adhesions in order to migrate (Calle et al. 2006; Gimona et al. 2008). The podosome size falls between 0.5 and 2.0 μm in diameter and depth. Podosomes are conical, actin-rich structures found on the outer surface of the membrane of animal cells (Rottiers et al. 2009). The lifetime of podosome is only minutes in duration, much shorter than observed in invadopodia. The short-lived (few minutes) podosome membrane structures are not to be confused with porosomes (Fig. 1.26) nor with the long-lived visceral epithelial cells known as podocytes that are found only in the kidney, wrapped around the capillaries of the glomerulus and are structural components of the blood-filtering system. In comparison, the size of the wild type murine podocytes is 19.2 ± 5.6 μm (Oshima et al. 2011).

Focal Adhesion The attachment and generation of focal adhesions are initiated by integrin-containing filopodia in cell migration. The radial projections of integrin-containing filopodia form the initial matrix contacts and recruit further cytoplasmic adhesion proteins (Partridge and Marcantonio 2006). Integrins are transmembrane receptors that are responsible for cell-cell bridging and extracellular matrix interactions. Filopodia are microspikes and as cytoplasmic projections form the leading

edge of lamellopodia in migrating cells (Mattila and Lappalainen 2008). Focal adhesions are assembled in a highly regulated manner (Zaidel-Bar et al. 2004) giving rise to structures by:

– cell to matrix binding,
– binding to the actin cytoskeleton machinery that powers cell migration,
– cell to cell molecular connections or movements by cell matrix adhesion initiate signal transduction pathways,
– regulated actin polymerization.

Focal adhesions foster the physical connection between the actin network and extracellular matrix, transmit chemical and mechanical signals, maintain the balance of adhesion assembly and disassembly, coordinate recycling and directed transport of components.

1.7.5 Endomembrane System: Membranes of Cellular Organelles

The endomembrane system consists of different intracellular membranes present in the cytoplasm and divides the eukaryotic cell into subcellular compartments known as organelles. The endomembrane system has been evolved from the intrusions of the cell membrane bringing about the nuclear membrane (nuclear envelop), the endoplasmic reticulum, the Golgi apparatus, lysosomes, peroxisomes, vesicles and endosomes. Mitochondrial membranes developed independently from prokaryotic cells. Their mitochondrial membranes will be dealt with separately.

The endomembrane system has three major subdivisions:

– secretory pathway,
– lysosomal pathway and,
– endocytotic pathway.

These pathways will be overviewed in Chap. 2 under Protein targeting.

The membranes of organelles are summarized in Table 1.2.

Nuclear Membrane (Nuclear Envelope, Nucleolemma, Karyotheca) The nucleus of the eukaryotic cell is the largest and densest organelle normally situated in the center of the cell or at the site corresponding to its function (Fig. 1.22).

Most of the cells have only one nucleus, occasionally they are binuclear, but some cells may have many nuclei (e.g. osteoclasts, muscle cells) (Fig. 1.22). The size of mammalian nuclei ranges between 5 and 10 μm, but tumor cells, particularly leukemic cells may have larger nuclei (Trencsenyi et al. 2012). Nuclei are mostly round organelles, but they can adapt flattened, rod- or string-like, bean-shaped, granular, lobular, segmented, lobular, so called Jugend forms, etc.

The view that the nucleoplasm is organized into a number of morphologically distinct and functionally significant domains was supported by an increasing number

Table 1.2 Names and functions of organelles of animal cells

Name of organelle	Major function
Nucleus	Information center, control gene function and integrity, regulation of gene expression
Rough endoplasmic reticulum	Transport and storage. Synthesis and export of proteins and glycoproteins. Liver cells secrete serum proteins, endocrine cells peptide hormones.
Smooth endoplasmic reticulum	Synthesis of lipids. In liver cells: biosynthesis of phospholipids and cholesterol, synthesis and repair of membranes, detoxification of metabolic waste products, foreign substances (biotransformation), and alcohol. In muscle cells: as sarcoplasmic reticulum stores and releases calcium ions needed for muscle contraction.
Golgi apparatus	Synthesis, packaging and releasing proteins and lipids
Peroxisomes	Catabolism of long chain and branched chain fatty acids, D-amino acids, and polyamines, reduction of reactive oxygen species, synthesis of plasmalogens and ether lipids
Lysosomes	Contain hydrolytic enzymes for digestion
Ribosomes	Sites of protein synthesis
Centrioles	Organizing chromosomes, anchoring chromosomes to the membrane
Mitotic apparatus (spindle)	Spindle-shaped structure developed during mitosis pulling chromatids apart toward opposite poles
Vesicles	Small organelles consisting of fluid enclosed by a lipid bilayer membrane are playing role in secretion (exocytosis), uptake of substances by endocytosis (phagocytosis, pinocytosis), intracellular transport of materials
Mitochondria	Generation of energy by oxidative phosphorylation

of evidence for subnuclear localization of processes including DNA replication (Banfalvi et al. 1989; Mills et al. 1989; Hozak and Cook 1994; Hutchison et al. 1994), DNA repair (Jackson et al. 1994), transcription (Jackson et al. 1993; Wansink et al. 1993), RNA splicing and processing (Huang and Spector 1991; Xing and Lawrence 1993). Although, these functional units and structures are not surrounded by membranes as cytoplasmic organelles, but function similarly to organelles (e.g. small ribonucleoprotein particles) in the cytoplasm (Spector 1990; Moen et al. 1995), and contain deep, dynamic tubular membrane-bound invaginations of the nuclear membrane. These invaginations are probably distinct from other topological features of the nuclear envelope, such as lobes or folds (Fricker et al. 1997) and could be transitory forms between cytoplasmic channels and organellar or vesicular forms.

The nuclear envelop itself is different from the cytoplasmic membrane. The membrane of the nucleus is a double lipid bilayer with an outer and an inner nuclear membrane bilayer. The gap between the two membranes surrounding the nucleus is the intermembrane or perinuclear space, which is in most cells 20–40 nm wide and results in a 30–60 nm thickness of the double bilayer nuclear membrane. As a consequence of the physical linkage of endoplasmic reticulum with the outer nuclear membrane, the perinuclear space is also contiguous with the lumen of the endoplasmic reticulum. The cross section of a nucleus is schematically viewed in Fig. 1.23. The small holes of the nuclear membrane are known as nuclear pores. Nuclear pores are multiprotein complexes forming aqueous

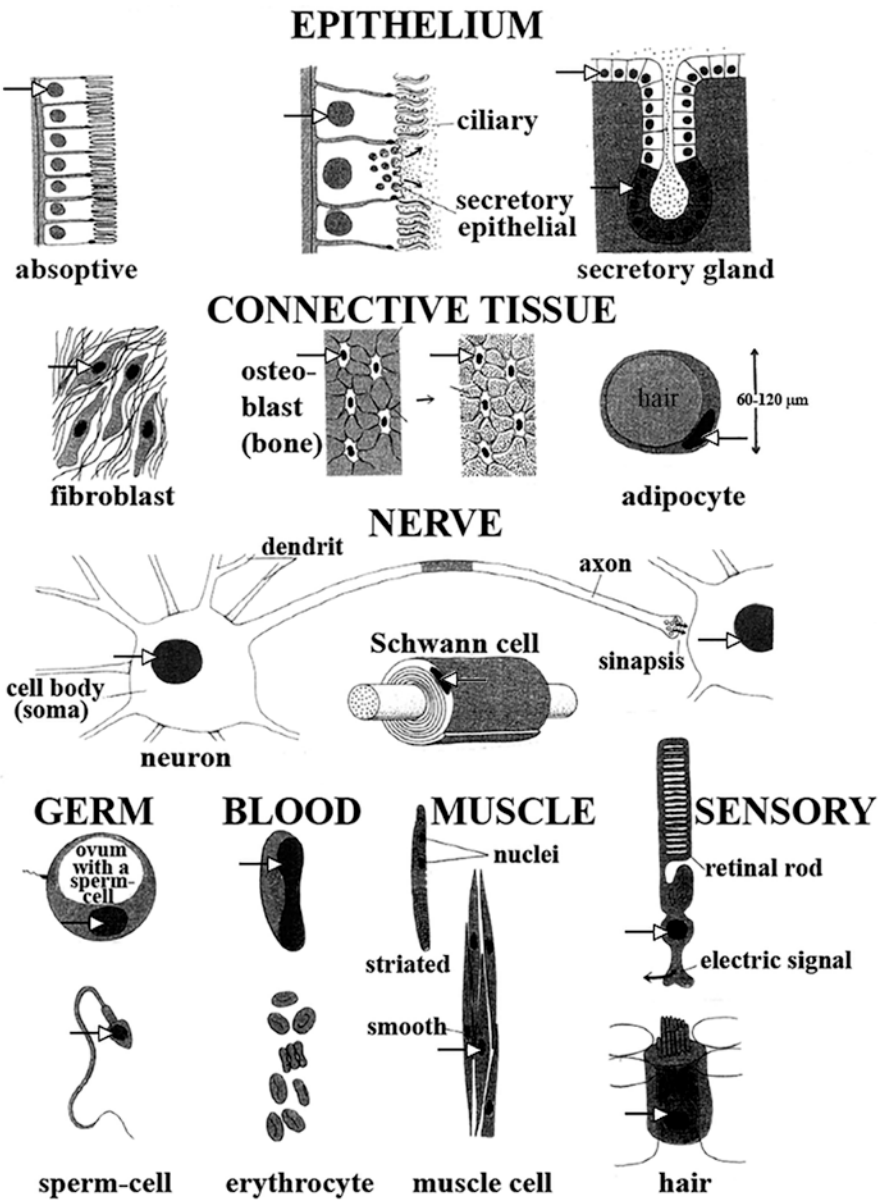


Fig. 1.22 Major types of tissues and nuclei. Nuclei are indicated by *white headed arrows*. Erythrocytes have no nuclei (Modified with permission Banfalvi 2009, p. 6)

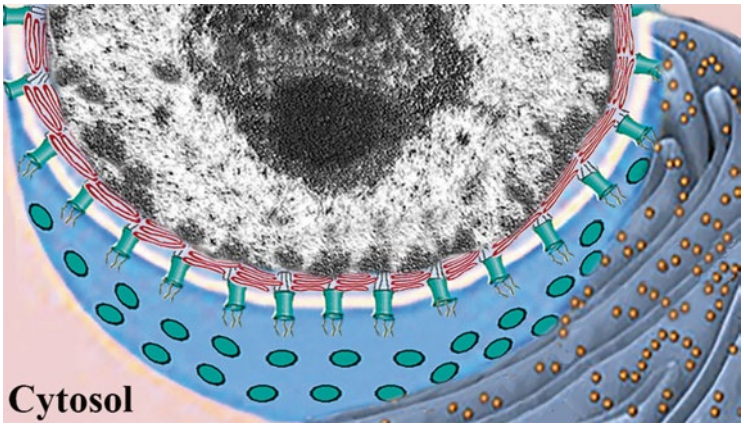


Fig. 1.23 Cross section of nucleus. Color coded structures: *Center dark black*, nucleolus; euchromatin, *light gray*; heterochromatin, *black spots* around the euchromatin; *red*, lamin bundles connecting nuclear pores; *green*, nuclear pores; *dark blue*, endoplasmic reticulum; *yellow dots* on endoplasmic reticulum are ribosomes. The lamin layer is located under the inner bilayer, binds to the nuclear pore complex, nuclear envelope proteins and transcription factors, helps to maintain the round shape and stability of the nucleus and organizes chromatin

translocation channels. Nuclear pore complexes are embedded in the nuclear envelope and connect the nucleus with the cytoplasm (Suntharalingam and Wente 2003). Nuclear pores allow the free diffusion of small molecules and ions, as well as receptor-mediated transport of large macromolecules (Grossman et al. 2012). Nucleopores consist of more than 30 different proteins known as nucleoporins (NUPs). Due to their controlling function it was generally believed that the nucleocytoplasmic transport is conserved and carried out by structures of ubiquitous composition. However, recently evidence was presented that the protein composition of NUPs varies among cell types and tissues (Raices and D'Angelo 2012). The double membrane of the nucleus is penetrated by large proteinaceous assemblies embedded in pores (Fig. 1.23). The composition of several nucleoporins has been described (Rout and Wente 1994; Heuser 2000). NUPs form a ring structure consisting of the cytoplasmic ring with extruding cytoplasmic filaments, thin ring, star ring, spoke ring complex, nucleoplasmic ring, basket, nuclear ring, inner spider ring.

The traffic system through the nuclear pores is based on a size and chemoselective gate-transport. The entrance of small (<5 kDa) molecules is fast, for larger molecules up to 17 kDa takes a few minutes. Large (>60 kDa) do not enter unless they are part of the nuclear transport system. The participants of the nuclear translocation are: (a) karyopherins (importins and exportins) that are small GTP binding transport protein molecules, (b) nuclear localization signal (NLS) or nuclear export signal (NES) on molecules to be transported, (c) nucleoporin proteins of the nuclear pore and Ran protein (Ras-related nuclear protein) referred to as GTP-binding nuclear protein Ran. The small G protein Ran is essential for the export of ribosomal

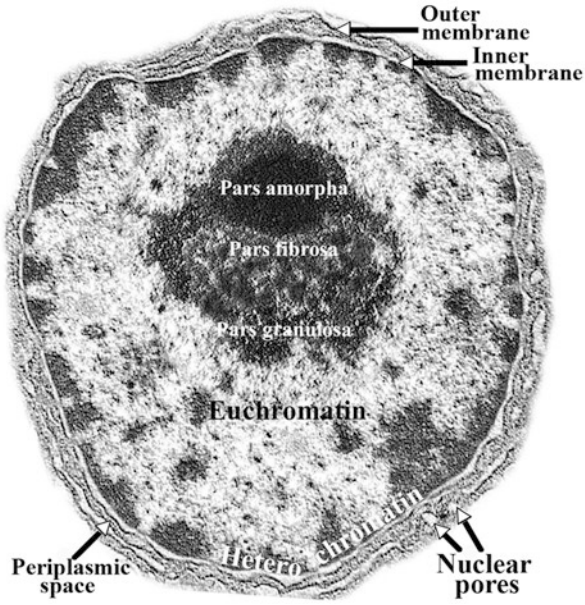


Fig. 1.24 Major structural elements of the nucleus. Double membrane consisting of outer and inner lipid bilayer. Periplasmic space between the inner and outer membrane. Euchromatin (*light gray*) sites of active transcription. Parts of the dark central nucleolus: *Pars amorpha, Pars fibrosa, Pars granulosa.* Heterochromatin as dark chromatin patches primarily around the inner nuclear envelop. Nuclear pores spanning the nuclear membrane and inside connected to heterochromatin

subunits, mRNA and tRNA to the cytoplasm and for the nuclear import of proteins involved in replication, repair, recombination, transcription and posttranscriptional modification of the primary transcript RNA into mature RNA.

Nucleolus The nucleolus is the largest structure contained inside the nucleus known as the site of transcription of ribosomal genes and the site of assembly of ribosomal subunits. It is not surrounded by membrane structures, nevertheless three parts of the nucleolus can be markedly distinguished: (i) the amorphous and dense *part amorpha* containing the ribosomal genes, (ii) the fibrous *part fibrosa*, where the transcribed fibrillar RNA molecules are present, (iii) and the granular *part granulosa* with the granular ribosomal subunits (Fig. 1.24). The ribosomal subunits are separate entities and are united only in the cytoplasm for protein synthesis after they have been translocated through the nuclear pores.

Endoplasmic Reticulum This organelle is the major manufacturing and packaging system of the cell in cooperation with other membranes such as the nucleus, Golgi apparatus, mitochondria and ribosomes. The close relationship of endoplasmatic reticulum with the nucleus is reflected by its presence in cells that have nuclei, but absent in cells lacking nuclei (e.g. prokaryotes, red blood cells of mammals). Structurally the endoplasmic reticular membrane is connected to the nuclear membrane. Indeed the supply of nuclear membrane during the growth of nucleus is attributed to the expansion of the endoplasmic reticulum. The double membranes of the endoplasmic reticulum contain sacs named cisternae. Protein molecules synthesized on the surface of the endoplasmic reticulum enter the cisternal space referred

to as lumen. The endoplasmic reticulum that surrounds the nucleus resembles sheets and disks. Due to the presence of bumpy ribosomes the surface of the endoplasmic reticulum is rough. This rough endoplasmic reticular membrane system forms large membrane sheets located in close proximity and continuous with the outer layer of the nuclear membrane (Shibata et al. 2006). The enormous membrane producing capacity of endoplasmic reticulum is understandable by considering that newly synthesized proteins that leave the endoplasmic reticulum are not moving freely and unattended, but are transported to the Golgi wrapped in membrane vesicles. The composition of the smooth endoplasmic reticulum is the same as that of the rough endoplasmic reticulum, but it does not contain ribosomes and its shape and function differ significantly. The packaging function of rough endoplasmic reticulum is contrasted by the storage function of the tubular smooth endoplasmic reticulum. The smooth endoplasmic reticulum is associated with the production and metabolism of fats and steroid hormones (Table 1.2).

Golgi Apparatus Similarly to the endoplasmic reticulum, the Golgi apparatus (Golgi complex, Golgi body) consists of a series of multiple compartments. These compartments store and package molecules for delivery to other cell components or for secretion from the cell. Upon synthesis of proteins on ribosomes they are translocated into the endoplasmic reticulum, then exported to the Golgi apparatus that consists of two major networks. The incoming vesicles enter the so called "*cis*" Golgi entrance and leave the multicompartment organelle of Golgi complex after posttranslational modification and packaging at the *trans* Golgi exit. The *cis* Golgi network is a collective term comprising up to 100 flattened membrane discs, sacs and cisternae, budded off the endoplasmic reticulum (Duran et al. 2008). It is not known how the variable number of cisternae is related to function, neither is the protein transport through the Golgi completely understood. Before their distribution proteins are packaged into vesicles in the *trans* Golgi, these vesicles are destined to lysosomes, to the cell membrane or they are excreted as secretory vesicles. These Golgi membrane–bound micro-sized particles are termed membrane vesicles. Ancestral endosome formation may have taken place in the trans Golgi network of yeast and plant cells (Suda and Nakano 2012).

The membrane of organelles (ER, Golgi, transport vesicles) that compose the secretory pathway is primarily synthesized and assembled in the endoplasmic reticulum, contributed by Golgi and mitochondria. An example of such complementation is the phosphatidylserine synthesized in the endoplasmic reticulum, transported from ER to mitochondria, transported from the outer membrane of mitochondria to the inner one, decarboxylated in separable reactions of phosphathidylethanolamine synthesis. After its synthesis, a portion of phosphathidylethanolamine is transported back to the endoplasmic reticulum (Tamura et al. 2012). Another example could be the retardation of Golgi disassembly by short chain ceramides leading to the conclusion that the Golgi apparatus could be stabilized by sphingolipids which are known as potential membrane components.

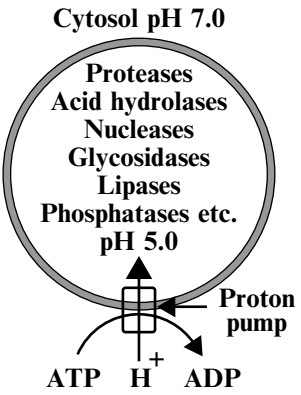


Fig. 1.25 Enzymes and proton pump of the lysosome

The distribution of proteins by the Golgi apparatus is regarded the last step in the transfer of genetic information. To convert the genetic information of DNA to communication, the proteins have to reach their targets. Correspondingly, this last step of transfer of genetic information is named protein targeting. Newly synthesized proteins destined for secretion gain entry into the secretory pathway by translocation across the ER membrane. This translocation apparatus integrates proteins into the membrane and establishes their topology with respect to the lipid bilayer. Proteins synthesized on free ribosomes follow their own intracellular targeting pathway.

Lysosomes These are "sack like" membrane-enclosed spherical organelles that contain hydrolytic enzymes able to break down biomolecules such as proteins, lipids, nucleic acids, carbohydrates produced by the cell or taken up from outside (Fig. 1.25). Lysosomes were supposed to be present only in animal cells. Earlier protein degradation in cells was thought to rely exclusively on membrane-bond lysosomes. However, in reticulocytes, which lack lysosomes ATP-dependent protein degradation was observed that suggested the presence of a second intracellular protein degradation mechanism. This lead to the discovery of the ubiquitin proteasome system (Peters et al. 1994) and was acknowledged in the award of the 2004 Nobel Prize in Chemistry to Aaron Ciechanover, Avram Hershko and Irwin Rose. Proteasomes are not membrane-bond structures and fall outside the scope of this book.

Although, many cell biologist doubt it, emerging evidence pointed to the existence of lysosomes in plant cells (Swansona et al. 1998). The size of lysosomes is between 0.1 and 1.2 μm (Kuehnel 2003). The phospholipid membrane of lysosomes protects the cytosol from the hydrolytic enzymes of lysosomes. Enzymes of lysosomes are synthesized in ribosomes of the rough endoplasmic reticulum subjected to modifications in the endoplasmic reticulum and Golgi, then released at *trans* Golgi as small vesicles. Protein containing vesicles carrying the mannose 6-phosphate tag differentiates them from other proteins and directs them to the endosomes. The acidity of endosomes is between pH 5 and 5.5 (Geisow and Evans

1984). After fusion, endosomes become functional lysosomes containing enzymes that are activated by the low pH (4.5–5.0) in the organelles' interior.

Lysosomes generate and maintain their pH gradients by a primary proton-pumping V-type ATPase, utilizing the energy of ATP to pump protons into the lysosome lumen (Mindell 2012). Lysosomes affect cellular homeostasis by their involvement in secretion, repair of plasma membrane, cell signaling, and by supplying the energy of their degradation products for basic metabolism (Friedman and Friedman 2012; Settembre et al. 2013). Endosomes and lysosomes are involved in three major related processes: phagocytosis, endocytosis and autophagy. The function of lysosomes is interlinked with the breakdown of unwanted extracellular material taken up by phagocytosis or pinocytosis. Macromolecules and microbes engulfed by endocytosis are degraded to basic molecules (monosaccharides, amino acids, fatty acids) in lysosomes (Luzio et al. 2007). Lysosomes are regarded as the recycling units of cells (Appelqvist et al. 2013). The basic substances are not only building blocks in recycling, but can be further degraded, reused as precursors or participate in the regulation of cell function.

Drug release, particularly those of lysosomotropic agents (e.g. chloroquine, cysteamine) from the endosomal-lysosmal organelles into the cytoplasm represents a significant risk of cytotoxicity (Xiong et al. 2010; Lu et al. 2002). Inhibitors of protein synthesis (cycloheximide, emetine, puromycin) also inhibit lysosomal proteolysis (Thoene et al. 1985). Lysosomal proteases (cathepsins) may induce cell death pathways after they have been released to the cytosol by a so far unclarified process termed as lysosomal membrane permeabilization (Reinheckel 2013).

Mitochondrial Membranes Mitochondria (0.5–1 μm) are membrane-bound organelles present in most eukaryotic cells (Henze and Martin 2003). These organelles function not only as "power plants" of the cell, by producing most of the ATP by oxidative phosphorylation under aerobic conditions (in the presence of molecular oxygen), but attributable to their own genome their replication takes place independently of the cell division. Beside supplying energy, mitochondria are involved in cell signaling, cellular differentiation, cell death, cell cycle control and cell growth (McBride et al. 2006).

The endosymbiontic theory explains the origin of mitochondria, plastids and probably other organelles by the uptake of free-living bacteria into other cells and became permanent residents of eukaryotic cells (Embley and Martin 2006; Cavalier-Smith 2006; de Duve 2007). How bacteria settled through the cell membrane into eukaryotic cells is not known. The symbiogenesis has been supported by molecular and biochemical evidence suggesting that mitochondria developed from proteobacteria and chloroplasts from filamentous cyanobacteria (Thrash et al. 2011; Deusch et al. 2008). Rickettsia as Gram-negative, highly polymorphic bacteria are the putative closest extant relatives of mitochondria (Gray and Spencer 1996; Gray 1998).

As a consequence of the Gram-negative origin of mitochondria, they share several common features with bacteria, including DNA organization, metabolism, and the double-membrane architecture. The outer membrane of mitochondria is smooth, the inner membrane is folded inwards and forms finger like compartmentalized

intrusions better known as cristae. Unlike Gram-negative bacteria, mitochondria did not maintain the mesh-like peptidoglycan layer deposited in the periplasmic space between the outer and inner membrane and has lost its structural importance to protect against mechanical stress. Nevertheless, studies in cyanelles fulfilling the function of chloroplasts in photoautotrophic protists have revealed the presence of peptidoglycan similar to that of Gram-negative bacteria (Aitken and Stanier 1979; Giddings et al. 1983). Peptidoglycan proteins derived from penicillin-binding proteins have been found in vertebrates (Peitsaro et al. 2008). To summarize the origin of organelles in context of membrane function it can be concluded that it was probably not the impermeable phospholipid bilayer that allowed the engulfing of foreign bacteria. Channels called porins present in the outer membrane allow the passive transport of ions, sugars amino acids and their accumulation in the periplasmic space. Although, the periplasm of organelles contains only a limited number of peptidoglycans, mitochondria can receive signals to be transported across the organellar membrane using transport and signaling molecules.

Outer Mitochondrial Membrane This membrane covers the mitochondrion. Electron microscopy of the outer membrane showed the presence of small elementary particles as regular arrays of hollow cylindrical subunits (60 by 60 Å) (Parsons 1963). The protein/phospholipid ratio (1:1) of the outer mitochondrial membrane corresponds to the cytoplasmic membrane and mitochondrion specific protein channels known as porins. These channels allow the free diffusion of molecules that are less than 5000 Da. Channel forming proteins of bacteria and related mitochondria and chloroplasts have relatively large and unselective pores. In the cytoplasmic membranes of animals and plant cells porins are smaller and highly selective (Alberts et al. 1989). As animal and plant channel proteins are concerned with ion transport, they are preferentially referred to as ion channels. Ion channels will be discussed with the permeability of cytoplasmic membrane. Larger than 5000 Da proteins can enter the mitochondrion through the outer membrane by binding to the subunit of its specific translocase by their signaling N-terminal signaling sequence (Herrmann and Neupert 2000). The integrity of the outer membrane of mitochondria is vital, disruption and leakage to the cytoplasmic space result in cell death. Mitochondrial permeability is responsible for engaging the apoptotic cascade governed by the proapoptotic proteins of the Bcl-2 family and the release of death-inducing proteins including cytochrome c (Chipuk et al. 2006). Another characteristic feature of mitochondrial outer membrane is its association with the endoplasmic reticulum contributing to the calcium signaling and transfer of lipids between these two membranes (Hayashi et al. 2009). The estimated mitochondria-associated endoplasmatic reticulum membrane may amount to 20 % of the outer mitochondrial membrane held together by protein complexes (Rizzuto et al. 2009; de Brito and Scorrano 2010). The lipid content of mitochondria is reflected by its chemical composition showing that based on dry weight, proteins comprise 65–70 %, lipids 25–30 %, RNA ~0.5 %. The proportion of DNA is less than 0.1 % due to the small genome of mitochondria.

The perimitochondrial space between the outer and inner membrane contains small molecules, primarily sugars and ions the concentrations of which correspond to that of the cytosol, due to the high permeability of the outer mitochondrial membrane. This is not the case with large protein molecules that need specific signaling sequences to be transported from the cytosol to the intermembrane space. One of the most important molecules present in the perimitochondrial space is cytochrome c (Chipuk et al. 2006).

Inner Mitochondrial Membrane This mitochondrial membrane separates the inner space of the mitochondrion known as the mitochondrial matrix from the intermembrane space. The ultrastructure of neuronal mitochondria was similar across species and neuronal regions. The outer and inner membranes were each approximately 7 nm thick, with a total thickness of both membranes approximately 14 nm (Perkins et al. 1997). The invaginations of the inner membrane are the cristae, juxtaposed to the outer membrane and increasing the total membrane surface area several times relative to the outer membrane. The inner membrane/outer membrane ratio is related to the ATP consumption of cells. Striated muscle cells, which are known for their high ATP demand contain more cristae than the cells of smooth muscle. The increased surface of cristae provides the metabolic area of mitochondria containing the enzymes for the Krebs cycle.

The folding of christae has been enhanced further resulting in the formation of minute structures, round elementary bodies, the F_1 particles also referred to as oxysomes. These oxysomes contain the base, the stalk and the spherical head and act as the ATP synthase enzyme. The majority of cellular energy in the form of adenosine triphosphate (ATP) is synthesized by the ubiquitous F1F0 ATP synthase (von Ballmoos et al. 2009). The F1 ATPase is the catalytic core complex and F0 ATPase complex is a membrane embedded proton channel. The mitochondrial matrix is the metabolic site of beta oxidation of fatty acids, anabolic site of protein biosynthesis on ribosomes closely resembling bacterial ribosomes (70 S), granular structures and enzymes. Beside the dominant function of ATP production, mitochondria are involved in heat generation, storage of calcium ions, NADH and $FADH_2$ related electron transport chain, energy conservation by switching from aerobic respiration to the process of fermentation that is independent of mitochondria (Voet et al. 2006). As far as energy conservation is concerned, there are two major energetic factors in the citric acid cycle originating from the combustion of hydrogen atoms of acetate to water and the oxidation of carbon atoms of acetate to carbon dioxide. Energy saving is achieved by converting the oxidation energy of carbon atoms to reducing equivalents ($NADH + H^+$ and $FADH_2$), resulting in a twofold increase of reducing power. This conserved energy is then channeled into the respiratory chain where the coupled oxidative phosphorylation liberates it in the form of ATP (Banfalvi 1991). Similar energy conservation has been described in the ß-oxidation of the fatty acids (Banfalvi 1992a) and during the carbon skeleton oxidation energy of amino acids to reducing equivalents (Banfalvi 1992b). These energy saving mechanisms increase the reductive power and ATP energy obtained from food molecules (carbohydrates, lipids, proteins). It was concluded from these chemical reactions taking place in mitochondria

that the hydrolytic oxidation of the carbon skeleton of fuel molecules is a recurring motif seen throughout the extraction of oxidation energy derived from fats, polysaccharides and proteins (Banfalvi 1992b), testifying the fundamental importance of aerobic oxidation in all cells containing mitochondria.

1.7.6 Vesicular Membranes

Vesicle is a small organelle in the cells formed by a lipid bilayer that contains fluid inside. Besides naturally occurring vesicles taking part in processes such as secretion and uptake of materials, they can be formed artificially as *unilamellar* or *multilamellar* liposomes by phospholipids. Artificial model lipid bilayers have been shown in Fig. 1.14. Vesicle formation is used as a tool to study the involvement of membrane organization in metabolism, membrane transport, storage of enzymes and other molecules. Naturally occurring vesicle types are: vacuoles, phagosomes, autophagosomes, chlatrin coated vesicles. Coatomer (COPI-coated) vesicles are involved in the retrograde transport carrying proteins back from Golgi apparatus to the endoplasmic reticulum. COPI-coated vesicles are responsible for creating small sacs originating from endoplasmic reticulum (Lee et al. 2004; Barlowe 2003), transport vesicles, secretory vesicles (synaptosomes, acrosomes, melanosomes, chromaffin granules) and other types. Vesicles similarly to different types of other membranes have diverse lipid and protein compositions.

Vesicles have also been found in *Archaea* bacteria, cyanobacteria, plankton, etc. Vesicles can fuse when their membranes are brought to ~1.5 nm vicinity. Secretory vesicles may dock at cup shaped supramolecular structures of cell membranes named porosomes by vesicle fusion (Jena 2004; Anderson 2006). The fusion is mediated by SNARE (Soluble NSF Attachment Protein Receptor) proteins. NSF is the abbreviation of *N*-ethylmaleimide-sensitive factor, and is an ATPase enzyme involved in membrane fusion. The schematic view of porosome mediated secretion is shown in Fig. 1.26. The molecular machinery of SNARE complex that mediates the vesicular fusion consists of a large group of membrane bound proteins. After the secretion is completed, the fusion pore of the porosome is sealed. The porosome secretory machinery is supposed to be of universal importance.

Synaptic vesicles may not be completely fused with presynaptic membranes to release their neurotransmitter content into the synaptic cleft. There are different types of membrane vesicle trafficking in eukaryotic cells:

- intracellular trafficking between subcellular compartments, e.g. Golgi cisternae and endosomes,
- budding of membrane vesicles and release from secretory cells,
- exosomes forming internal compartments by fusion with plasma membrane,
- viruses (e.g. retroviruses) may use the exosomal machinery for budding inside and secreted as exosomes.

The prokaryotic trafficking of vesicles is less known, but was revealed as nanoscale particles by electron microscopy (Ellis and Kuehn 2010).

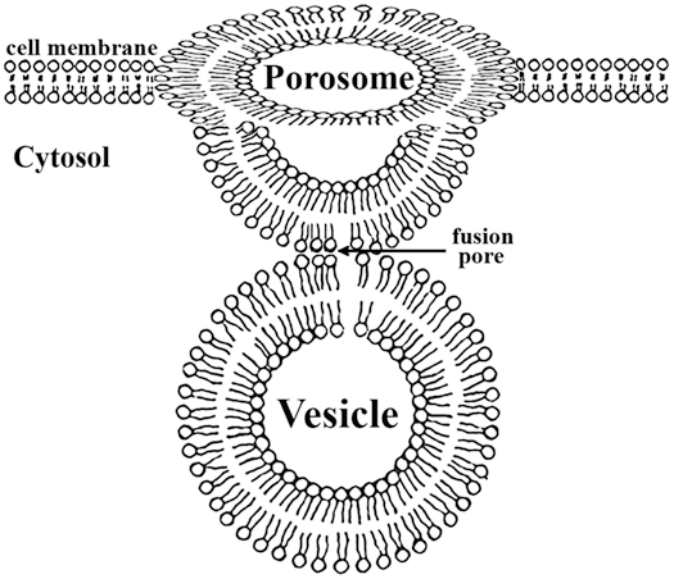


Fig. 1.26 Porosome mediated secretion. Transient fusion between secretory vesicle and porosome takes place through the fusion pore at the base of the porosome *via* the SNARE proteins. After the content of the vesicle is released to the porosome, the continuity of porosome membrane is restored

1.8 Function of Biological Membranes

1.8.1 Mechanical Function

The cell membrane defines the physical integrity of the cell and its function by:

– mechanically holding together its content by enclosing it in the cytoplasm,
– forming a barrier between the cell (internal milieu) and external environment.
– separating the intracellular components (organelles in eukaryotes).

1.8.2 Formation of Compartments

The fact that all eukaryotic cells possess the same basic set of membrane enclosed organelles indicates their common evolutionary origin. The evolution of internal membranes accompanied the specialization of membrane function. From evolutionary viewpoint four families of internal membranes can be distinguished (Alberts et al. 2002):

(a) topologically continuous but functionally distinctive nuclear and cytosolic communication through the nuclear pores,

(b) organelles of the secretory and endocytic pathways: endoplasmic reticulum, Golgi apparatus, lysosomes, transport vesicles and probably peroxisomes,
(c) mitochondria,
(d) plastids (in plants).

Viral Envelopes A special type of internal membrane that does not belong to organelles is represented by enveloped viruses. Human pathogen enveloped viruses were found among DNA viruses (Herpes-, Pox-, Hepadnaviruses), RNA viruses (Toga-, Corona-, Hepatitis D, Orthomyxo-, Paramyxo-rhabdovirus, etc.) and retroviruses. The genome of these viruses is enclosed in a protective protein capsid that is surrounded by a lipid bilayer membrane derived from the host cell membrane, but also includes viral glycoproteins.

1.8.3 Function of Cytoskeleton

The cytoskeleton is the intracellular matrix of all cells. The shape of the prokaryotic cells, except the genera of *Mico- and Thermoplasma,* is determined by their cell wall.

The three major components of cytoskeleton are

– microfilaments,
– intermediate filaments and
– microtubules.

These structural elements consisting of proteins form and support the shape of cells, mediate the inner movement of cellular components and are responsible for the cellular movement in their environment. Not only eukaryotes, but also prokaryotes possess a cytoskeleton. Actin-related and tubulin-related bacterial proteins have been described as cytoskeletal elements. It was speculated that constituents of eukaryotic cytoskeleton (tubulin, actin) could have evolved from prokaryotic cytoskeletal precursor proteins (Mayer 2003). This idea was confirmed by finding further bacterial cytoplasmic elements including homologs of actin, tubulin and intermediate filament proteins and a fourth group unique to bacteria (Shih and Rothfield 2006).

Microfilaments Actin is the major cytoskeletal protein that is synthesized to thin F-actin filaments by the polymerization of globular G-actin monomers. Microfilaments of ~7 nm in diameter with several micrometers in length consist of two F-actin filaments helically turned around themselves (Cooper and Hausman 2013). The broad range of processes actin is involved (muscle contraction, amoeboid movement, cellular motility, cell division, cytokinesis, organellar movement, formation of filo- and lamellopodia, maintenance of cell junctions, receptor signaling) indicate the extreme importance of microfilaments. Elements of the cytoskeleton, particularly actin interact intimately and communicate bidirectionally with cellular membranes (Doherty and McMahon 2008).

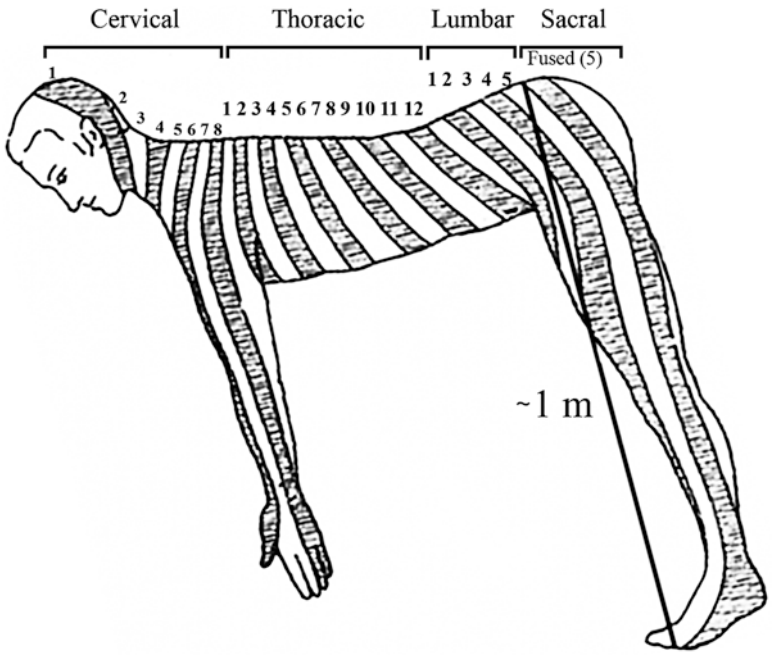


Fig. 1.27 Neurofilaments corresponding to the length of axons. In the major regions of the human spine the last, coccygeal spine (tailbone) is not indicated. The longest possible intermediate filaments corresponding to longest axons in the human body run in the sciatic nerve from the sacral spine to the big toe of the foot

Intermediate Filaments The second major group of cytoskeletal components are intermediate filaments that occur frequently in animal cells (Herrmann et al. 2007). The diameter of these filaments of about 10 nm in diameter is intermediate between microfilaments (~7 nm) and microtubules (~25 nm). The basic structural importance of intermediate filaments is to provide mechanical strength to cells and tissues, rather than being elements of cellular movements. Major types of intermediates filaments are classified into six types:

- Type I and II keratin is synthesized by epithelial cells forming either hard (hair, nails, horns) or soft (cytoplasmic) keratin.
- Type III filaments contain vimentin and desmin as their most characteristic proteins present in fibroblasts, white blood cells. Glial cells and neurons of the peripheral nervous system contain special subtypes of type III filamental proteins.
- Type IV neurofilamental (NF) light, medium and heavy (NF-L, NF-M and NF-H) proteins are forming long, thin processes to support axons of motor neurons and their early embryonic development. These filaments correspond to the length of the axons of nerve cells that can reach about 1 m in man (Fig. 1.27), ~4.5 m in

the neck of giraffe's recurrent laryngeal nerve cell, ~25 m in blue whale and could have been an estimated 40–50 m for nerves innervating the tail in the longest sauropods.

- Type V intermediate filament proteins are known as nuclear lamins forming the nuclear lamina (*lamina fibrosa*) and are components of the nuclear membrane. The nuclear lamina is missing in plant and single-cell eukaryotic organism. The cell size and form in these cells are protected by the cell wall. The central domain of intermediate filament proteins is an α-helix uniform in size of ~310 amino acids, the N terminal head and the C-terminal tail are variable in size and shape. In the nuclear lamin proteins the α-helix is longer (~350 amino acids). Lamins are components of the nuclear membrane. Lamin A is responsible for maintaining the characteristic meshwork and the round shape of the nucleus by connecting the nuclear pores. The genetic mutation of lamin A leads to the production of the mutant protein progerin that is missing a cleavage site. The mutation of lamin A known as progerin affects nuclear blebbing. The insertion of progerin in the nuclear membrane, results in the bulging of the nuclear membrane and causes the premature aging disorder, *progeria* (Gilford and Shepherd 1904; Booth-Gauthier et al. 2013). The phenotypic characteristic of progeria is nuclear blebbing. Progeria is not regarded as a genetic disorder, not inherited, or passed down in families. Neither parent is a carrier as the mutation is thought to be caused by a gene change affecting a single sperm or egg cell before conception. The protein progerin appears in many cells and causes rarely children to grow old quickly (Brown 1992).
- Alternative splicing of lamin B transcripts followed by translation results in two protein variants. Duplication of lamin B gene is associated with autosomal dominant adult-onset of leukodystrophy.
- The single type VI intermediate filament protein (nestin) is expressed during the early development of neurons, in stem cells of the central nervous system.

The many types of intermediate filaments indicate that there are many more, yet to be discovered.

Microtubules The third major component of the cytoskeleton is represented by microtubules present all over the cytoplasm in both animal and plant cells. The highly dynamic tubular polymers about 24 nm in outer and 12 nm in inner diameter are formed by heterodimer subunits consisting of an alpha and beta tubulin. Microtubules as part of the cytoskeleton are the "conveyor belt" of the cell. They are involved in maintaining the structure, in mitosis, in cell division, intracellular transport, formation of the internal structure of cilia and flagella. Cilia and flagella are connected to the basal bodies formed from centrioles that are anchored to the cytoplasmic face of the plasma membrane. In flagella the length of microtubules may be two to three-times longer (up 10 μm) than the bacteria.

1.8.4 Extracellular Matrix

The extracellular matrix consists of extracellular molecules secreted by cells and provide mechanical stiffness and elasticity, biochemical support and connection to neighboring cells.

The animal extracellular matrix includes the interstitial matrix and the basement membrane. The layers of the basement membrane were shown in Fig. 1.20. The interstitial matrix is connecting various animal cells. From evolutionary viewpoint the development of interstitial matrix led to cell-to-cell interactions, adhesion and differentiation of tissues. The cell membrane of single-celled organisms often interacts with the cell membrane of neighboring cells. In animal cells the extracellular matrix fills the interstitial space and acts as a compression barrier and forms sheet-like depositions on which various layers of epithelial cells are deposited.

The major structural components of extracellular matrix are: proteoglycans (heparan sulfate, chondroitin sulfate, keratan sulfate), hyaluronidic acid, collagen and elastin fibers, fibronectin, and laminin. The composition of components define the properties of the extracellular matrix, various cell types and contributed to the development of different connective tissues. Characteristic cell types in animal connective tissues are fibroblasts, chondrocytes in cartilage, osteoblasts blast in bone formation. In plant cells the cell wall comprises laminate layers of cellulose fibers embedded in the matrix of glycoproteins containing hemicellulose, pectin and extensin. The evolution of the extracellular matrix utilized the regrowth, regenerative, healing, anti-inflammatory and differentiation capabilities of isolated extracellular matrix proteins.

1.8.5 Selective Permeability

Size, charge, chemical properties of substances determine whether or not they can cross the selectively permeable structure of biological membranes. The mechanical, elastic and chemical properties of membranes influence shape, form and movement of cells. Selectivity of biological membranes is essential to differentiate between materials that attempt to cross the membrane, to separate cells and organelles from their surrounding environment. Detailed description of permeability of biological membranes will follow in Chap. 2.

1.8.6 Specialization

To perform several different activities towards their environment and adjacent cells, plasma membranes differentiate. Membrane specializations have been studied primarily by freeze-fracture studies based on high fidelity replicas made on the surface

of frozen hydrated biological materials. The freeze-factured membranes revealed two distinct faces, the internal protoplasmic face (P-face) and the external face (E-face) of the plasma membrane. Freeze-fracture observations also provided microscopic evidence that proteins and intramembrane particles 10 nm in diameter are localized at certain regions of the plasma membrane (Dallai 2005). Freeze-fracture electron microscopy demonstrated earlier the structural specialization of luminal membrane compared to its basal membrane (Wade et al. 1975). Indeed, modifications of the cell membrane seem to be structural adaptations for particular cellular functions.

Specializations of Cell Surfaces The separation of cells from their environment can involve specialized plasma membrane structures. Specialized cell surfaces occur in:

- apical and basolateral membranes,
- presynaptic, postsynaptic, myelin and dendritic spine membranes of neurons,
- sarcolemmas of muscle cells,
- membranes of flagella, cilia,
- filopodia and lamellopodia serving the movement of cells,
- microvilli, which are tiny hair-like membranes to increase the surface of the cells and involved in absorption, secretion, cellular adhesion, mechanotransduction.

Beside specialization there are other complex membrane structures that also develop from the plasma membrane and differ in their lipid and protein composition. These differences define the physical and chemical properties of biological membranes and are taken into consideration when these properties are exploited for medical purposes. Complex membrane structures are: caveolae, postsynaptic membranes, podosomes, desmosomes, hemidesmosomes, focal adhesions, cell junctions consisting of multiprotein complexes and providing cell-cell contacts particularly between epithelial cells and connections between cells and extracellular matrices.

The host plasma membrane serves also as a defense barrier against pathogens that invade from the extracellular environment. The lipid bilayer composition modulates the scaffold function. As a counteraction pathogen infections also cause numerous changes in plasma membrane composition, organization, and structure that can influence the regulatory role of membranes in cellular responses. Local changes in membrane microdomains such as lipid rafts can create signaling platforms for receptor signaling in response to pathogen perception (Faulkner 2015).

The luminal surface of the absorptive epithelium of the intestine (*striated* or *brush border*) examined with electron microscope has shown that it is composed of many small cylindrical processes (*microvilli*) that vary in size. From these studies it was concluded that they increased the cell surface enormously and contributed to the absorptive function of the intestinal epithelium (Fawcett 1962). Another example of surface expansion is the presence of numerous pinocytic vesicles formed along the lunimal and basal surfaces of cells of the endothelial capillary wall and the intervening cytoplasm. At the junctions of these endothelial cells large desmosomes are formed. The interior of these cells become crowded with vesicles either by the local formation of new membranes or obtained from the plasma membrane.

Similarly to pinocytosis, phagocytosis generates vacuoles that envelop bacteria or other large particles that are larger but fewer in number than pinocytic vesicles. To avoid overproduction, considerable amount of membrane generated during the formation of granules of pancreatic secretion in the endoplasmic reticulum and in the Golgi region could be recycled.

Specialization of Cell Base Shallow incursions of basal enfoldings take place in the ciliary body, choroid plexus, proximal convoluted tubules of the kidney. Deeper penetration of membranes are likely to include mitochondria in the distal convoluted tubule of the kidney or in the striated ducts of the parotid gland (Fawcett 1962).

Cell Shape Related Specializations Fibrillar and tubular components influence the shape of epithelial cells possessing microvilli at their apical surface termed earlier as terminal web (Leblond et al. 1960). The terminal web is composed of actin filaments stabilized by spectrin and is anchoring the terminal web to the apical cell membrane. The contractibility of the terminal web is accounted for by the presence of myosin II and tropomyosin molecules (Hirokawa et al. 1982; Keller et al. 1985; Ross and Wojciech 2011).

Cell-cell interaction refers to direct connections between the surfaces of different cells. These interactions are common in differentiation and in the development of different functions in multicellular organisms. Small, so called microenvironmental changes are sensed and communicated by cellular interactions by sending and receiving signals essential for the survival of cells. These communications take place *via* cell junctions. Stable interactions between cells are communicated through: tight junctions, anchoring junctions, gap junctions, direct-contact signaling receptor proteins, other (plant) cell-cell interactions. Stable interactions are communicated by transient interactions, between cells of the immune system, by factors of blood coagulation, bacterial interactions. Loss of equilibrium among interactions has pathological consequences e.g. uncontrolled cell growth, cancer, metastasis, bacterial infections, other diseases (Banfalvi 2014).

1.8.7 Metabolic Activities of Membranes

The maintenance of barrier function of cell membrane is demonstrated by the paradoxical problem of maintaining the unique internal electrolyte composition and uniform osmolarity while at the same time rapid ion exchange and leakage of ions are taking place in each cell. This problem is resolved by the function of the sodium-potassium ATPase, using up a significant proportion of the ATP pool.

Active muscle is one of the major consumers of metabolic energy of the body. For the contraction of animal muscle ATP energy is necessary. Glucose can be transported to the muscle by the blood synthesized in the liver and stored as glycogen reservoir of the body. Energy is also stored in muscle cells as glycogen and broken down to release glucose, metabolized under reductive conditions to pyruvate

which is converted to acetyl-coA under oxidative conditions. Glucose is the major source of ATP generated by cellular respiration in mitochondria in slow muscle generating a large amount of ATP. Much more ATP is consumed but less efficiently obtained by anaerobic glycolysis in the fast striated muscle. The activity of several plasma membrane transporters, among them the most important glucose transporters (GLUT1-4) play a crucial role in glucose metabolism (Zorzano et al. 2000). Energy requiring transport processes through the plasma membrane transport also play an important role in hormone metabolism (Hennemann et al. 2001).

"*Homeoviscous adaptation*" refers to the metabolic adjustment of membrane lipid adaptation at different temperatures. It was demonstrated in fish that the percentage of unsaturated fatty acid lipogenesis was elevated after an acute drop in temperature but declined with continued cold exposure (i.e. cold acclimation). Both acute and chronic cold elevated the incorporation of polyunsaturated fatty acids into phosphatidylserine. The conversion of phosphatidylserine to phosphatidylethanolamine was activated at cold temperatures (Hazel 1984). Evidence was presented that the diversity of membrane lipids is maintained through cross-regulatory effects, where classes of lipids activate the activity of enzymes operating in different metabolic branches (Nohturfft and Zhang 2009). The extreme importance of membrane lipids in metabolism was underlined by the suggestion that membrane fatty acids are the pacemakers of animal metabolism (Hulbert 2007).

Differences in biochemical function of membranes are demonstrated by their enzymatic composition (Korn 1968). Plasma membranes of animal cells contain many enzymes (esterases, phosphatases, peptidases, dehydrogenases, hydrolytic enzymes) (Emmelot and Benedetti 1967). Red blood cells are rich in enzymes related to glycolysis (Green et al. 1965). The inert lipid shield of myelin is probably an exception, it has no significant metabolic activity.

1.8.8 Localization of Membrane Function

The cellular function of electroneutral K^+-Cl^- cotransport (KCC) is to regulate epithelial ion transport and osmotic homeostasis. Potassium-chloride cotransporters play a crucial role in the function and development of both peripheral and central nervous systems (Karadsheh et al. 2004). KCC4 is perhaps the least abundant KCC in the adult mammalian brain, where its localization is unknown. Glycosylation is essential for the surface expression, stabilization, and bioactivity of KCC4 (Weng et al. 2013). The expression of KCC3 in esophageal squamous cell carcinoma may affect cellular invasion. The investigation of the role of K^+-Cl^- cotransporter 3 in the regulation of cellular invasion pointed to the clinicopathological significance of its expression in esophageal squamous cell carcinoma (Shiozaki et al. 2014).

Cation-coupled chloride transporters belong to the solute carrier 12 (SLC12) family and are known to contribute to ion fluxes across a variety of tissues, particularly in the kidney and choroid plexus of the brain. K^+-Cl^- cotransporters, which belong to the SLC12 family act as electroneutral symporters of K^+ and Cl^- ions

across the plasma membrane (Dunham et al. 1980). K^+-Cl^- cotransport, which can be activated by cell swelling, is necessary for the regulation of cell volume, the transepithelial transport of ions, and the maintenance of intracellular Cl^- concentration (Lauf and Adragna 2000). The four isoforms of KCCs identified as KCC1, KCC2, KCC3, and KCC4 (Weng et al. 2013), are the major determinants of osmotic homeostasis. KCCs are also emerging players in the field of tumor biology (Shen et al. 2004).

1.8.9 Regulation of Membrane Transport

The basic regulation of passage of electrolytes through the cell membrane is maintained by selective membrane permeability. Non-electrolyte hydrophobic substances normally pass through the membrane by dissolution in the lipid membrane *via* passive diffusion. Membrane transport proteins involved in the membrane transport include voltage-gated potassium and calcium channels, ion channels that mediate sensory and nociceptive responses, epithelial transport proteins and ionic exchangers (Gamper and Shapiro 2007). Ubiquitin is one of the major regulators of protein transport between membrane compartments by serving as a sorting signal on protein cargo and by controlling the activity of trafficking machinery. Other functions of ubiquitination include the transport of key endocytic proteins, regulation of internalization into the endocytic pathway, sorting of cargo into vesicles for delivery and degradation in the lysosome, ubiquitin ligation, control of timing and specificity of plasma membrane protein down regulation (Hicke and Dunn 2003). Phosphoinositides are known to regulate the membrane traffic and protein function (Krauss and Haucke 2007; Falkenburger et al. 2010). Rab proteins are members of the Ras superfamily of GTPases and key regulators of intracellular vesicular transport (Goody et al. 2005).

1.8.10 Membrane Receptors

External signaling molecules generally bind to transmembrane proteins known as receptors and transmit the signal through molecular switches to internal signaling pathways. Such signaling molecules are certain hormones, neurotransmitters and immune proteins received from the external environment. The transmission of these signaling molecules through the cellular membrane is prevented due to their large size and/or polarized nature. Based on the mechanisms that transmit their signals into the cell, three major classes of membrane receptors have been distinguished:

- receptors coupled to G-proteins
- ion channel receptors
- enzyme-bound receptors

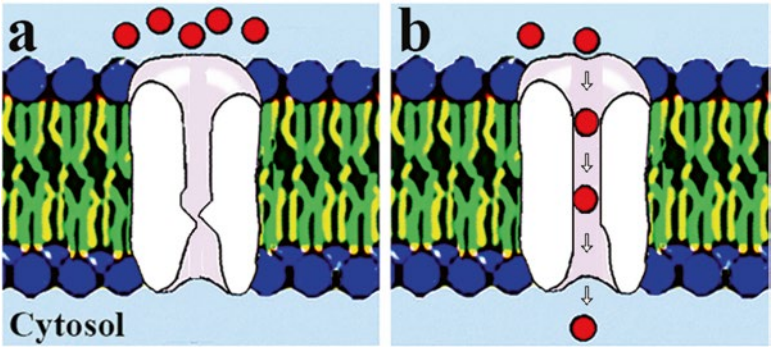


Fig. 1.28 Ion channel activation. The receptor forms a gated ion channel embedded in the plasma membrane. The transmembrane protein receptor has an aqueous pore inside that allows soluble molecules (e.g. acetylcholine) to cross the open plasma membrane. (**a**) Closed receptor. (**b**) Binding of ions (*red*) induces conformational change, opens the aqueous pore and allows the ions to flow into the cell

Not only external signals, but also internal molecules interact with membrane receptors and affect cellular functions. As an example of signal transmission an inactive (closed) ion channel (Fig. 1.28a) and its activated (open) conformation is shown (Fig. 1.28b).

After the binding of a signal molecule (first messenger) to its membrane protein receptor, conformational changes take place. These changes in turn initiate a series of biochemical reactions starting at the inner surface of the receptor leading to signaling pathways where each step in the transduction cascades amplifies the next one. Activation can trigger the synthesis of seconder messengers such as cAMP, cGMP, Ca^{2+} ions, phosphatidylinositol trisphosphate (PIP_3), diacyl glycerol (DAG), gases such as nitric oxide (NO), carbon monoxide (CO), hydrogen sulfide (H_2S). Examples of secondary messenger systems are the cAMP, phosphatidylinositol, arachidonic acid, cGMP and tyrosine kinase systems. The plasma membrane is not involved actively in the non-ionic, hydrophobic signal transduction pathways (e.g. steroid hormone pathway).

1.8.11 Cell Surface Markers

Animal cell membranes carry special molecular tags known as markers. **S**urface protein markers are embedded in the cell membrane to identify the cell, and to allow communication with other cells. It is the immune system that can differentiate between "self" and "non-self" and distinguish between the body's own cells that carry the molecular "flags" and those of invaders that have no self-markers. The immune system is able to destroy invading pathogens, but does not attack our own

cells in spite of the fact that own cells and invaders contain closely related cellular structures. This is known as the self/non self-discrimination of the immune system. Although, it seems to be a simple concept, but comprises a mechanistically complex system. Immune cells attack foreign agents but not own (self) cells of the body. Foreign agents are known as antigens. Antibodies locked onto antigens serve as the molecular tags marking the invader for destruction. Beside of recognizing foreign cells, the immune system can remember those disease causing viruses, bacteria or pieces of germs that attacked earlier the organism. Owing to the immune memory antibodies produced by B lymphocytes of the immune system invade pathogens (viruses, bacteria) and form memory cells that remember the same pathogen and produce the same antigen faster in future infections.

The immune system particularly the activation cascade of the complement system, which is a cell-killing membrane complex, is inherently linked to the biological membranes. Cluster of differentiation (CD) protocols are utilized to identify specific surface protein molecules for immunophenotyping (Chan et al. 1988), as these markers are often associated with certain immune functions. CD molecules may signify receptors or ligands, but may also have other functions such as cell adhesion. Several hundred human CD markers have been described (Zola et al. 2007). For immunophenotyping of leukocytes the several CD molecules have been useful cell surface markers:

- CD34 stem cells,
- CD45 all types of leukocytes
  - CD45, CD15 granulocytes
  - CD45, CD14 monocytes
  - CD45, CD3 T-lymphocytes

    CD45, CD3, CD4 T-helper cell
    CD45, CD25 T-regulatory cell
    CD45, CD3, CD8 Cytotoxic T cell
- CD45, CD19, CD20, CD24, CD38, CD22 B lymphocyte
- CD45, CD61 Thrombocyte
- CD16, CD56, CD3, CD31, CD30, CD38 Natural killer cell
  - CD45, CD19 B-lymphocytes
  - CD45, CD61 thrombocytes, T cells, B cells

Without going into immunological details, CD markers, especially the two commonly known CD4 helper and CD8 cytotoxic T cell markers are important molecules in antigen recognition, as well as CD135 as a surface receptor for growth factors. Other cell surface markers proved to be useful in the characterization of stem cells, pluripotency, differentiation, terminal differentiation, cell lineages and activation status (Zola 2000). The tumor grade is known to influence the expression of membrane-associated glycoproteins, e.g. CA19-9 in colorectal cancer (Plebani et al. 1996). The serine/threonine protein phosphatase level turned out to be high in endometrial cancer (Sugiyama et al. 2003). Cancer antigen CA-125 has been applied as a tumor

biomarker due to its elevated level in the blood of some patients with specific types of cancers (Bast et al. 1998). The potential panel of markers for molecular imaging by immunohistochemistry includes the expression of growth factor receptors (GFRs), membrane-associated tumor markers such as EGFR, IGFR1, FGFR2, CD44v6, CAXII, GLUT1, HER2. CD166 has been used as a marker for cancer stem cells (Kute and Quadri 1991; Grimm et al. 2011; Yan et al. 2013; Vermeulen et al. 2013a, b). Membrane-associated proteins represent a growing number of candidate biomarkers for early tumor diagnosis, prognosis and therapy.

1.9 Cell-Cell Interaction

These interaction refer to direct connections between cell surfaces i.e. cell membranes and allow the communication between neighboring cells in response to changes in their microenvironment. Stable interactions are transmitted through cell junctions forming multiprotein complexes that provide the contact between neighboring cells. Transient (temporary) interactions take place between cells of the immune system and cells of tissues involved in inflammation. Interactions among cluster forming membrane proteins regulate cellular processes such as cell growth, cell differentiation and apoptosis (Nohe and Petersen 2004).

Types of cell-cell interactions:

Stable Interactions

- *Tight junctions* as multi-protein complexes hold cells together tightly, preventing even the movement of water and water soluble molecules between cells. Tight junction forming transmembrane proteins are occludin, claudin, adhesion molecules.
- *Gap junctions* are composed of transmembrane pore or channel forming proteins called connexins, which allow the passage of ions, sugars or other small molecules. The hemichannel on each cell consists of six connexins that interact with the other hemichannel on adjacent cell membrane.
- *Anchoring connections* involve adherent junctions and desmosomes serving the formation of shape and tension of cells and tissues as well as cell-cell signaling. Characteristic adhesion proteins are cadherins. As their names suggest subtypes of cadherins are expressed at the highest levels in distinct tissue types: E-cadherin is expressed in all epithelia and is important for establishing and maintaining apico–basal polarity. N-cadherin is expressed in neural tissue and muscle; R-cadherin is expressed in the forebrain and bone; P-cadherin is present in the basal layer of the epidermis, and VE-cadherin is expressed in endothelial cells (Takeichi 1988; Hirano et al. 2003; Halbleib and Nelson 2006). The most abundant E-cadherins play pivotal role in epithelial cell behavior, tissue formation, function as invasion supressors and down regulators of most carcinomas (van Roy and Berx 2008). N-cadherins act as invasion promoters, being frequently up regulated. Expression of N-cadherins in epithelial cells induce changes in

morphology to a fibroblastic phenotype, rendering the cells more motile and invasive (Derycke and Bracke 2004). The expression of P-cadherins is highly associated with undifferentiated cells in normal adult epithelial tissues, as well as with poorly differentiated carcinomas. P-cadherin is frequently overexpressed in high-grade breast cancer tumors, being a well-established indicator of poor patient prognosis (Albergaria et al. 2011).

– *Direct-contacts between receptor proteins.* These proteins on the cell surface are able to bind specific signaling molecules secreted by distant (endocrine) and by nearby (paracrine) cells. These proteins are involved in signal transduction and cell signaling pathways not to be discussed in detail.

Transient Interactions

– *Interactions between bacteria.* Among these interactions are chemical factors known as bacterial pheromones or autoinducers that freely penetrate both the outer and inner plasmatic membranes and reach their intracellular targets. Quorum sensing is coordinating biofilm formation, virulence, antibiotic resistance within a bacterial population and controls many essential processes in prokaryotes including sporulation, conjugation, bioluminescence, virulence, cell division, etc. (Williams et al. 2007). Essential processes such as division, genetic transformation, sporulation, synthesis of antibiotics and secondary metabolites, are regulated in bacterial populations by intercellular communications mediated by secretion of signaling molecules, such as homoserine lactones and peptides. Physical contact (cell aggregation) between cells, plays a key role in formation of biofilms or cellular consortia and in cell proliferation under unfavorable conditions (Voloshin and Kaprelyants 2004)
– *Blood coagulation.* The negatively charged phosphatidylserine has long been recognized for its involvement in numerous biological processes including blood coagulation, complement activation and removal of senescent cells from the blood (Zwaal 1978; Martin et al. 1995; Szebeni 1998). The information that membrane lipids, anionic phospholipid, especially phosphatidylserine, were required for the assembly and optimal function of most of the coagulation complexes (Zwaal 1978) was critical to understand coagulation reactions. Studies performed with artificial liposomes containing incorporated negatively charged liposomes have been used to characterize blood coagulation as these liposomes turned out to bind high amounts of plasma proteins (Chiu et al. 2001). The cell-based model of hemostasis suggests that blood coagulation is regulated by the properties of cellular surfaces and initiation occurs on tissue factor bearing cells, followed by amplification where platelets and cofactors are activated for thrombin production and by propagation where large scale thrombin production takes place on the platelet surface (Hoffman and Monroe 2001). Despite of the critical importance of protein-membrane interaction in blood clotting, the proper understanding of how blood clotting proteins interact with the phospholipid surfaces remained relatively understood (Morrissey et al. 2009).
– *Involvement of immune system in transient interactions*. These interactions take place between cells of the immune system and those involved in tissue

inflammation. The ability of white blood cells (leukocytes) to attack and destroy abnormal and foreign cells is crucial from immunological viewpoint, but transitory in nature regarding the formation and breaking off cell-cell interactions. These interactions are accounted for by the cell adhesion molecules of selectins. Selectins are transmembrane glycoproteins representing a special type of lectins that as cell adhesion proteins bind sugar polymers (Parham 2005). The smallest L-selectins of lymphocytes are expressed on granulocytes, monocytes and leukocytes. The endothelial E-selectin is expressed in cells of the microvessels of the skin. The largest P-selectins are stored primarily in platelets and endothelial cells. Selectins are involved in lymphocyte homing to lymphoid tissues and sites of inflammation (Arbonés et al. 1994). T-cells remain functional in the absence of stable interactions e.g. immunological synapses and membrane bridges. Evidence was provided that transient interactions of naive T-helper cells with antigen presenting dendritic cells did not form a stable synapse, rather brief contacts with one dendritic cell being sufficient to stimulate T-helper cells to proliferate (Gunzer et al. 2000). Similarly, to helper T cells, cytotoxic T cells did not need stable synapse formation for their cytotoxic effect (Purbhoo et al. 2004).

1.10 Membrane Potential

Development of Transmembrane Potential Difference In multicellular organisms (plants, animals) the tissues are not in direct contact with the environment, but are protected by an internal environment constituted by fluids surrounding the cells of the body. Claude Bernard recognized the stability of this internal environment (*milieu intérior*) that provides free and independent life for multicellular organisms (Gross 1988). Every cell of a multicellular organism participates in the regulation and maintenance of the relatively stable internal environment, which is in fact the external microenvironment of cells, not to be confused with the internal cytosolic environment. In this book the external environment signifies the extracellular fluid (*milieu intérior*) and the internal environment refers to the conditions inside the cell. Blood is the largest circulating tissue consisting of extracellular fluid known as blood plasma and formed elements, red and white blood cells and platelets. While the extracellular osmolarity in land vertebrates is nearly identical (0.3 Osm) (Table 1.3) and corresponds to that of inner osmolarity, the ionic makeup and pH of the inner environment of cells are different. In virtually all cells — including microbial, plant, and animal cells — the cytosolic pH is kept constant with a much higher cytosolic concentration of K^+ relative to the intracellular concentration of Na^+.

The movement of ions across biological membranes is mediated by several types of secondary active transporters (symporters and antiporters cotransporting ions simultaneously along with specific small molecules), by ion channels, ion pumps. The mammalian cytosolic concentration of K^+ and the negatively charged protein concentrations are high (~140 mM each), with low Na^+ (12 mM), Cl^- (4 mM), Mg^+ (0.8 mM) and extremely low Ca^{2+} (0.2–0.3 μM) concentrations. Plasma concentration

Table 1.3 Mean osmotic values of blood plasmas of terrestrial vertebrates and seawater

Vertebrates	mOsm	Osmoregulation
Birds	317	Kidneys, salt-glands, intestines, internalization of respiratory surfaces to reduce evaporative water loss, uric acid excretion (in uricothelic organisms)
Lizards	307	Kidneys, specialized salt glands
Snakes	300	Kidneys, specialized salt glands
Mammals	295	Kidneys, nasal passages and lungs to reduce water loss
Turtles	287	Kidneys, specialized salt glands
Reptiles	286	Kidneys, salt-glands, tongue, intestine, uric acid excretion
Amphibians	160–240	Kidneys, gills, bladder, skin, intestine
Seawater	1094	Multifactorial global regulation

Modified with permission (Banfalvi (1991)

of Na^+ (~140 mM), Cl^- (~115 mM), HCO_3^- (~30 mM), Mg^{2+} (1.5 mM), Ca^{2+} (1.8 mM) are high, K^+ (4 mM) and protein concentrations (<10 mM) are low.

Beside the differences in ion concentrations on the two sides of the plasma membrane maintained by ion pumps that transport ions, the plasma membrane also contains channel proteins, which also move ions (Na^+, K^+, Ca^{2+}, and Cl^-) across at different rates down their concentration gradient. In most neurons ion gradients and selective movements generate a voltage difference corresponding to an intracellular resting potential. The resting potentials differ significantly in various cell types, e.g. in erythrocytes, photoreceptor cells, neurons, astrocytes, smooth muscle and striated muscle cells these values are −9, −40, −60 to −70, −80 to −90 and −95 mV, respectively. Due to these transmembrane potential differences the cell membrane acts like an electric battery determined by the vital imbalance of major ions (K^+, Na^+, Ca^{2+}, H^+) separated by a semipermeable membrane that allows the free movement of water molecules but maintains the electric potential between the two poles of the battery. The lower positive charge inside the cells (negative pole) relative to its microenvironment (positive pole) provides the driving energy for the transport across the cell membrane.

Diffusion Potential Although, the diffusion of ions through the cell membrane is low, there is a significant difference between the leakiness toward different ions. The diffusion potential of potassium ions across the cell membrane is the highest. The diffusion potential of sodium ions is about two orders of magnitude lower than those of potassium ions. Chloride ions have a low tendency to diffuse through the cell membrane. Consequently, the diffusion potential becomes entirely dominated by the concentration gradient of potassium ions alone, and the resulting potential will be the derivation of Nernst potential of potassium which can be extended to other ions. The derivation explains why in the calculation of the diffusion potential (E_D) by using the Nernst equation only the potassium ion concentrations was taken into consideration while other concentrations (Na^+, Cl^-) have

been neglected. The formula for the determination of the free energy (ΔG) from the chemical gradients is:

$$\Delta G = RT \ln \frac{[K^+]_0}{[K^+]_i}$$

where ΔG is the free energy from the chemical gradients, R is the universal gas constant, F is the Faraday constant, $[K^+]_o$ the potassium ion concentration outside and $[K^+]_i$ the K^+ concentration inside the cell.

The free energy can be also calculated from the electrical gradient as:

$$\Delta G = zFV$$

where ΔG is the free energy, F the Faraday constant, z is the valence of K^+ (+1) and V the voltage. By substituting the electrochemical ΔG with electrical ΔG:

$$zFV = RT \ln \frac{[K^+]_0}{[K^+]_i}$$

and solving for V we arrive to the formula known as the Nernst equation and the potential expressed in V (voltage) is referred to as the Nernst potential:

$$V = \frac{RT}{zF} \ln \frac{[K^+]_0}{[K^+]_i}$$

When there is no net movement of K^+ ion across the membrane the Nernst potential is said to be the equilibrium potential for K^+.

Data of average ion distribution (mmol/l) are given in Table 1.4. From the external and intracellular concentrations by using the Nernst equation, the diffusion potential of K^+ ions corresponding to the resting Nernst potential (−90 mV) can be calculated:

Nernst equation:

$$E_{K^+} = E^o - \frac{RT}{nF} \ln \frac{[K^+ \text{outside}]}{[K^+ \text{inside}]} = 2.3026 \frac{RT}{nF} \log_{10} \frac{[K^+ \text{outside}]}{[K^+ \text{inside}]}$$

$$E_{K^+} = \frac{2.3026 \cdot 8.3144621 \cdot 298}{1 \cdot 96{,}485} \times \log \frac{150}{15} = 0.060 \cdot \log 30 = 0.06 \cdot 1.5 = 0.09\text{V} = 90\text{mV}$$

Table 1.4 Averages of extra- and intracellular plasma concentrations in mammalian cells

Ions	Extracellular concentration mgion/l (mM)	Intracellular concentration mgion/l (mM)
Na^+	150	15
K^+	5	150
Cl^+	125	10

E_{K^+} = diffusion potential (mV) of K^+ under specific conditions
E^o = potential under standard state conditions (25 °C = 298 K, 1 atm pressure, 1 M)
R = ideal gas constant = 8.3144621 J/mol·K
n = number of moles of electrons transferred (number of charges, e.g. 1 for K^+, 2 for Ca^{2+})
F = Faraday's constant, electric charge per mole of electrons = 96,485 coulombs × mol^{-1}

To measure the membrane potential of a tissue, earlier silver microelectrodes were used. The microelectrode corresponds to a Galvanic (voltaic) cell consisting of a small glass with a metal wire inside. The micropipette with an end-hole contains potassium chloride solution into which a silver plate is inserted at its upper end. The tip of the silver chloride electrode is implanted to the tissue and the other silver metal end connected to the positive pole of the voltmeter. The negative pole of the voltmeter serving as an "indifferent electrode" has been placed in the extracellular fluid. Similar tiny, but more sophisticated microelectrodes are recently in use made from inert metals (tungsten, stainless steel, platinum, iridium) coated with glass or polymer insulators and conductive tips. Microelectrodes used in electrophysiology are able to record or to induce small voltage (mV) neuronal signals in nervous tissues.

Nerve Resting Potential As already referred to it, the extent of leakage through the cell membrane for potassium and sodium ions is not the same. The emphasis is on potassium leakage particularly in nerve cell membranes because their potassium channels are far more (>20 times) permeable to potassium than to sodium ions. As the cell membrane has a relatively high permeability to potassium, but is only slightly permeable to sodium, the logical consequence is that the diffusion potential of potassium will contribute much more significantly to the membrane potential than the diffusion of sodium. This would result in an internal membrane potential of −86 mV. In the Na^+/K^+ pump more sodium ions are pumped to the outside than potassium to the inside (3 Na^+ *versus* 2 K^+) causing a further loss of positive charges inside and generates an additional degree of negativity corresponding to an additional −4 mV. The resting potential of these neurons will be the summary of −86 mV and −4 mV giving a net resting membrane potential of −90 mM. It deserves repeated mention that the value of the resting potential depends on cell type.

Nerve Action Potential In response to appropriate nerve signals the cell membranes of nerve cells undergo a sequence of rapid changes in the membrane potential termed action potential. Action potential occurs in excitable animal cells (neurons, striated and cardiac muscle, and endocrine cells) and in some plant cells when a stimulus suddenly increases the permeability of the cell membrane to sodium ions. External stimulus to excitable plant cells generates action potentials in response to mechanical stimulation such as touch, injury, or direct electrical stimulation. In "firing" nerve cells the action potentials are known as "nerve impulses" or "spikes" that play a major role in cell-cell communication. In other cells the action potential activates metabolic processes. In muscle cells the action potential serves muscle contraction. The islets of Langerhans in the pancreas contain endocrine (i.e. hormone-producing) cells. In these cells the main function of action potential is to provoke the release of hormones: insulin and amylin by beta cells, glucagon by alpha cells, somatostatin by delta and pancreatic polypeptide by gamma cells. The sequence of action potential starts with depolarization from the resting stage by special types of ion channels. Upon stimuli first the so called slow Na^+ channels open. These channels, common in the cell membrane of excitable cells, open and allow the inflow of sodium ions resulting in the reduction of excess inner negative charge. The sequel depends on the presence of voltage-gated (fast) Na^+ ion channels.

The main excitable cells are the neurons, with a relatively simple mechanism of action potential. After stimulus first the slow Na^+ channels open and the inner negative charge falls from −90 mV below the threshold level between −30 and −50 mV. This initial phase of the action potential is followed by the opening of fast voltage-gated Na^+ ion channels and the inflow of Na^+ ions in such amount, that the cell loses its negative charge i.e. it is depolarized and may become positively charged due to an overshoot in the potential. This depolarization closes the fast Na^+ channels and opens the voltage-gated potassium channels. During the repolarization phase the high inner concentration of potassium drives K^+ ions out of the cell and the cell's membrane potential begins to return to the resting (equilibrium) potential. The repolarization typically "undershoots" the negative resting potential. This phenomenon known as hyperpolarization: (a) prevents the neuron from receiving a second stimulus within a short period of time, (b) raises the level of the threshold potential to avoid a new action potential and (c) assures signal processing in one direction.

Nerve Conduction Velocity The faster transmission of electric signals in the nervous system of vertebrates and in few invertebrates is achieved by myelination and saltatory conduction. Primarily peripheral afferent neuronal axons of the central nervous system are covered by a myelin sheath that is a multicellular membrane enwrapping the often very long axon in segments interrupted by nodes of Ranvier. The myelin sheath is formed by specialized Swann cells that cover axons of peripheral nerve cells and oligodendrocytes. The conduction of electricity in axons is directly proportional to the voltage applied to it and follows Ohm's law:

$I = V/R$, where I is the electric current, V is voltage and R the resistance.

The velocity of conducting the action potential ranges between 1 and 100 m/s and increases with the diameter of the axon (Hursh 1939).

Graded Potential Action potentials are preceded by graded potentials causing local depolarization in neurons to the threshold level before the action potential would step into full swing. It depends on the cell type and the stimulus whether or not a graded potential leads to an action potential. Those graded potentials that upon adequate stimuli can generate action potential are called synaptic potentials in neurons, generator potentials or receptor potentials in sensory cells, end-plate potentials or synaptic potentials in skeletal muscle cells. Graded potentials result from the slow passive diffusion through excitable membranes, while action potentials are orchestrated responses to depolarizing stimuli, and involve the coordinated activity of fast voltage-gated ion channels. Malfunctions including those of ion channelopaties impacting depolarizations have been implicated in diseases (Abraham et al. 1999).

1.10.1 Energy Transduction

Before biological energy transduction is discussed the units of energy have to be defined:

Biological energy (E) is given in units expressed in calorie (cal), joules (J) or in electron-volts (eV).

Calorie was formerly defined as the quantity of heat required to raise the temperature of 1 g of water by 1 °C under standard conditions. Calory has now largely been replaced by joule.

Joule is the standard International System (SI) unit of electrical, mechanical and thermal energy equal to the work done when force of one Newton acts on an object and moves it to a distance of 1 m in the direction of force (1 newton meter = 1 Nm).

Newton is the standard unit in the International System equal to the force that produces an acceleration of 1 m per second on a mass of 1 kg.

Electronvolt is the amount of energy gained (or lost) by the charge of a single electron moved across an electric potential difference of one V.

Conversion of energy units: 1 cal = 4.184 J. 1 eV = 1.6×10^{-9} J

The energy transduction in biological membranes follows the rules of thermodynamics:

1. The first law of thermodynamics states that the energy of an isolated system is conserved and is known as the low of conservation of energy. Energy is a state function the value of which is unique for a given state and its change between two states is independent on the pathway that links the states.
2. The second law of thermodynamics is known as the law of increasing entropy (randomness, disorder). As the useful energy is consumed, disorganization, (randomness) increases.

3. The third law of thermodynamics refers to a state known as “absolute zero”. The Kelvin temperature scale is absolute, i.e. 0° Kelvin would be the possibly lowest temperature in the universe. The absurdity of “absolute zero” corresponding to −273.15 °C is indicated by the fact that this state is physically impossible to reach and is only a mathematical limit of the universe. For example in a pure perfect phospholipid lipid membrane at “absolute zero” every unit would be identical and their molecular alignment would be perfectly even in each direction. In non-perfect i.e. real membranes neither the units are uniform nor are their alignments perfect, which means that there will be some energy associated with these imperfections and their entropy cannot decrease to zero level.

Thermodynamic systems including biological membranes can be described by intensive and extensive state properties (variables). Intensive variables are: density, chemical potential, electrical potential, temperature that are independent of the size of the membrane. Extensive variables such as mass, energy, enthalpy, entropy, volume depend on the size of the membrane.

The membrane system would be in equilibrium when the state variables would be independent of time and there would be no flux with respect to mass or energy moving across the membrane. This is never the case as the membrane proteins enable the specific transport of selected substances, consequently the membrane is not in equilibrium, but in a steady-state. Membrane proteins are not only sensors and receptor of signals that are received from the outer surface of the membrane and are transduced across the membrane, but are central elements of biological energy conservation. Due to the selective impermeability and compartmentalization of biological membranes, gradients are formed that represent energy storage and are used for thermodynamic work and energy production. Ion gradients produce differences in chemical concentrations at the two sides of the membranes and similarly to voltaic cells store the energy of electrical gradients. This energy is used to maintain osmolarity, pH, temperature, to produce action potential and after conversion to ATP, this biological energy unit serves motion, biosynthesis and transport across the membranes (Bradley 2008). In the first step of energy conservation of the two most important biological energy producing processes, namely in photosynthesis and cellular respiration, electrons and protons are transported through the membranes. The term respiration with breathing in the lungs, is not to be confused with cellular respiration. The photosynthetic reactions are centered on using the energy of light to separate and transport electrons through the thylakoid membrane.

In the general mechanism of cellular respiration ubiquinone and cytochrome c diffuse freely through the membrane and shuttle electrons form one to the next complex. These inner membrane bound enzymes of complexes generate three major steps (Complexes I, III and IV) (Fig. 1.29):

1. Electron donors (NADH, FADH) are oxidized and the reduction of quinone by complex I (NADH dehydrogenase, also known as quinone oxidoreductase) is coupled to charge translocation across the membrane.
2. Complex II is the only membrane-bound component of the Krebs cycle. Complex II is a linear electron transport chain that extends from the flavin and iron-sulfur

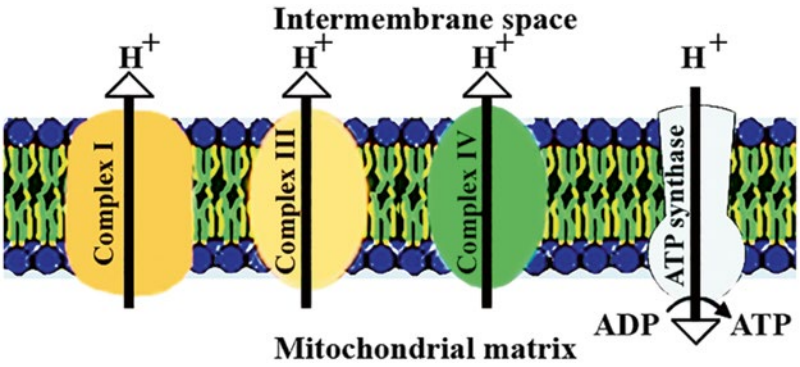


Fig. 1.29 Mitochondrial chemiosmosis. Respiratory complex I: NADH dehydrogenase. Respiratory complex III: cytochrome c reductase. Respiratory complex IV: cytochrome c oxidase. Terminal oxidation coupled to oxidative phosphorylation by the flow of protons from the intermembrane space into the matrix driving the ATP synthase to produce ATP from ADP

redox cofactors in the membrane extrinsic domain to the quinone and b heme cofactors in the membrane domain (Cecchini 2003).

3. Quinol – electron acceptor oxidoreductase activity performed primarily by the cytochrome (bc) complex family (complex III).
4. Heme-copper oxygen reductase (complex IV, cytochrom c oxidase) reduces molecular oxygen to water in the respiratory chains.

In mitochondria the respiratory complexes (I, III, IV) pump protons (H^+) against their concentration gradient i.e. by active transport from the matrix to the intermembrane space. As a result the pH decreases outside and increases inside (up to about pH 8). This produces ion gradients and membrane potentials that drive the:

(a) ATP synthase
(b) uptake of nutrients
(c) removal of waste products
(d) cellular and flagellar motion

The concentration gradient tends to be equilibrated through the ATP synthase. Protons flow down their gradient and produce ATP. This facilitated diffusion process is called chemiosmosis. In chloroplast the same chemiosmotic process takes place with the notable difference that the direction of chloroplast H^+ ion flow is in the opposite direction compared to oxidative phosphorylation in mitochondria (Wise and Hoober 2006; Reese et al. 2011).

The produced energy of ATP is consumed in ATP-dependent processes:

(i) biosynthetic processes
(ii) primary active transport driven by ATP
(iii) movement of myosins along actin filaments (muscular, amoeboid movement) using equivalents, resulting in power.

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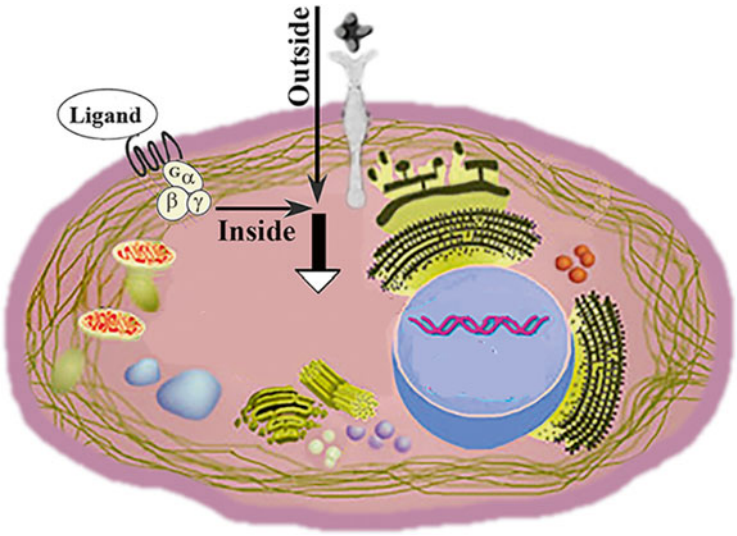
Chapter 2
Permeability of Membranes

Contents

G. Banfalvi, *Permeability of Biological Membranes*,
DOI 10.1007/978-3-319-28098-1_2



Abstract This chapter provides an overview of bilayer permeability and selectivity of permeabilization. Based on the hydrophobic nature of the lipid bilayer, cell membranes behave as diffusion barriers primarily for electrolytes, hydrophilic micro- and macromolecules in solutions. To overcome the permeability barrier different transport processes have been evolved for crossing the cell membrane. Small particles may be transported by passive transport, active transport or by carriers. To transport large particles through the membrane different strategies have been developed involving exocytosis, endocytosis and receptor mediated endocytosis. The importance of permeability is underlined by the integrity of cell membrane in maintaining the viability of cells. Different approaches have been applied to investigate membrane permeability as a measure of viability, by detecting permeability on account of hemolysis, turbidimetry and release of lactate dehydrogenase.

Keywords ABC transporter • Active pumps • Active transport • Adhesion • α-Helix • Bacterial membranes • ß-Barrel • Channel proteins • Chloroplast membranes • Cytotoxicity assay • Diffusion • Electrochemical gradient • Facilitated diffusion • Filtration • F1F0 ATPase • Hemolysis • Hydrophilic • Hydrophobic • Ion channels • Ionophores • Ligand-gated channels • Lipid bilayer • Membrane transport • Organellar transport • Osmosis • Overton's rule • Passive transport • Penetration • Permeability • Pore formation • Protein targeting • Receptors • Selectivity • Semipermeability • Signaling • Solubility • Transition temperature • Transmembrane proteins • Transporter • Vesicular transport • Voltage-gated channels • Water channels

2.1 Permeability of Biological Membranes

It was Overton who in 1900 compared the permeabilities of animal and plant cells and has found that the conditions are constant and characteristic to both cell types. All cells that were examined proved to be:

(i) ***freely permeable to***:

- monohydric alcohols, ethers, esters, aldehydes, substituted hydrocarbons, in general to substances able to dissolve fats or being dissolved in fats

(ii) ***difficultly permeable to***:

- dihydric alcohols, urea and glycerine

(iii) ***impermeable to***:

- sugars, polyhydric alcohols (erythrite and higher)
- amino acids (glycine, alanine, leucin, asparagine, taurin)
- neutral salts of alkali and alkali earth metals (Overton 1900).

The tendency of passing compounds through biological membranes from low to high permeability is summarized in Fig. 2.1.

In applying permeabilizing methods it should be kept in mind that only substances with lipid solvents/soluble groups may alter the normal permeability of the plasma membrane, other compounds have little or no influence (Lillie 1909). The permeability of molecules is closely related to their solubility in lipids known as Overton's Rule. This rule can be briefly summarized as substances dissolved in lipids pass readily the cell, while substances dissolved in water are excluded. This observation provided evidence that the cell is surrounded by a lipid membrane leading to the recognition that the modification of the phospholipid membrane may greatly impact permeability. The rate of diffusion is determined by the concentration and the properties of the substrate. The relationship between permeability and solubility of the diffusing molecule can be expressed by the permeability coefficient:

$$P = \frac{KD}{\Delta x}$$

where the variables are:

P permeability coefficient for a substrate (in cm/s)

K partition coefficient is a measure of the solubility of the substance in lipids. Low K value means low solubility in lipid

D diffusion coefficient (cm^2/s), a measure of the rate of entry into the cytoplasm depending on the molecular weight or size of a molecule

Δx thickness of the cell membrane (cm)

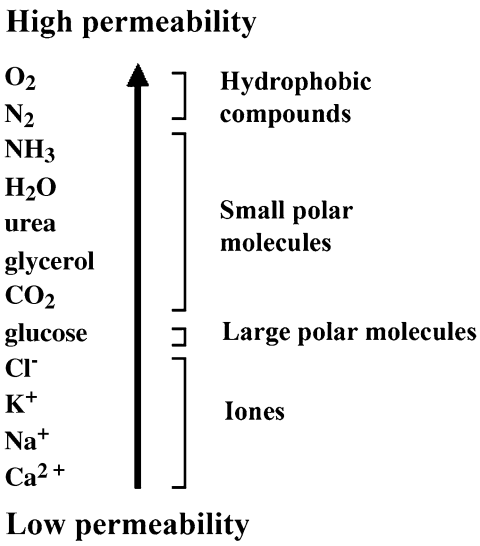


Fig. 2.1 Selective permeability of plasma membrane (With permission Banfalvi 2014, p. 28)

The permeability of molecules through a cell membrane is a linear function of the partition coefficient with a slope dependent on molecular size. In case of equal size, the molecule of greater solubility in lipids will pass faster into the cell. The basic rules of permeability can be summarized depending on: (1) diffusion coefficient (d), (2) thickness of membrane, and (3) solubility in lipids.

1. Permeability (P) depends in part on D (the diffusion coefficient). D is determined by the size of the particle diffusing and the solution being diffused through. When other parameters are equal, particles with a higher diffusion coefficient also have a higher permeability. P increases when D increases.
2. Permeability depends also on the thickness of the membrane. When anything else is equal, permeability is lower when the membrane is thicker. P decreases when Δx increases.
3. "Greasy" molecules like lipids, have higher K values. Charged particles (e.g. ions) have low K values. K can vary over a wider range, than P, Δx or D. P increases when K increases.

Exceptions to Overton's rule are the lipid layers of the brain. The brain is surrounded by four barriers consisting of lipid layers, which possess however, several inward and outward active transport systems. Overton's rule was able to explain only part of the differences in cerebrospinal fluid penetration of individual compounds. There are several other factors that influence the permeability of membranes such as the age of the patients, underlying diseases. Active transport and alterations in the pharmacokinetics by co-medications also appeared to strongly influence the penetration of the drugs studied in the cerebrospinal fluid (Djukic et al. 2012).

2.2 Transport and Signaling Across the Membrane

Although, the permeability of molecules is closely related to their solubility in lipids, but cells exist in an aqueous environment, thus permeability in cell biology refers to the aqueous transport or flow of molecules across biological membranes. The aqueous transport can be: (a) slow or (b) fast.

(a) The non-specific, non-mediated spontaneous diffusion is protein-independent with a relatively slow rate and depends exclusively on the differences of concentrations at the two sides of the membrane.
(b) The second class of membrane transport is fast, highly selective, protein-mediated, saturable, regulated by the metabolic need and cytokines, and can be influenced by drugs. Defects in transport cause diseases known as channelopaties.

Outside-in Signaling Ligand binding to the external domain of the receptor spanning the plasma membrane causes conformational changes that further increase ligand affinity and modulate interactions at the cytoplasmatic site of the receptor and initiate controlled changes in cell adhesion (i.e. integrin signaling).

Inside-out Signaling Intracellular signals received by other receptors can also initiate integrin activation by the so-called "inside-out signaling". The control of integrin function comes from signals that originate within the cell cytoplasm and are then transmitted to the external ligand-binding domain of the receptor (Faull and Ginsberg 1996; Shen et al. 2012) (Fig. 2.2).

Controlled changes in cell adhesion are hallmarks of a number of basic physiological processes that directly impact: (1) cell migration during leukocyte infiltration into inflammatory sites (glomerulonephritis, interstitial nephritis, renal transplant rejection) and during embryogenesis (development of the genitourinary system) and (2) platelet aggregation at sites of clot formation (blood coagulation) (Faull and Ginsberg 1996).

2.3 Permeability of Small Particles

The semipermeable plasma membrane of eukaryotic cells functions as a permeability barrier separating the cells from their environment and from each other. Semipermeability, selective permeability, partial permeability also mentioned as differential permeability means that certain molecules or ions may pass through the membrane mostly by passive and less frequently by other types of transport. The permeability of the lipid bilayer was shown in Fig. 2.1 decreasing from top to bottom. The lipid bilayer is permeable to water and few small, uncharged diffusible molecules (O_2, CO_2, N_2, NH_3, urea, glycerol). The diffusion of water through the plasma membrane is driven by osmosis. The membrane has a low permeability for small hydrophilic molecules (glucose) and very low permeability for positively charged cations (K^+, Na^+, Ca^{2+}), negatively charged anions (Cl^-, HCO_3^-), and informational macromolecules (DNA, RNA, protein).

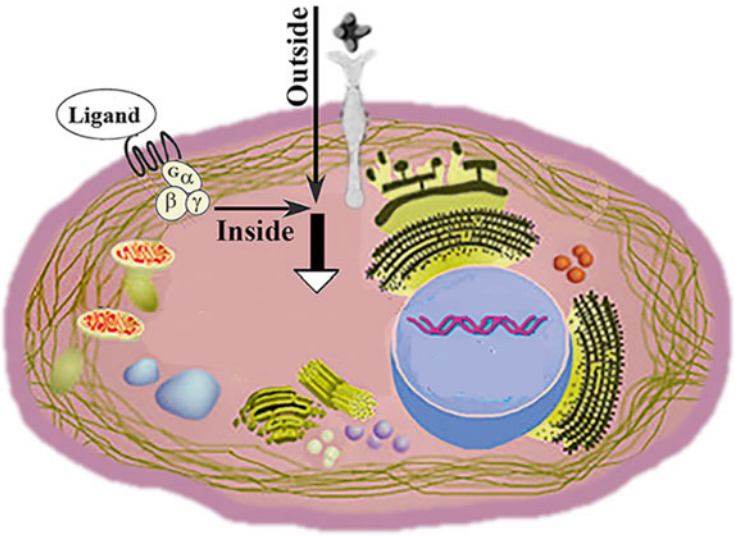


Fig. 2.2 Inside-out and outside-in signaling. In the inside-out pathway signals originate within the cytoplasm of the cell and are then transmitted to the external ligand-binding domain of the receptor. In outside-in signaling the ligand that approaches to the external domain of the receptor causes conformational changes and enhances the ligand binding affinity of the receptor, modifies protein interactions in the cytoplasmic domains of the receptor and contributes to the transmission of the signal

2.4 Bilayer Permeability

Depending on temperature lipid bilayers can either be in liquid (sol) or in solid (gel) phase. The characteristic temperature at which transition between these two phases occurs is known as melting. Transition prevents flip-flop movements across the bilayer. In liquid phase the thermal diffusion of phospholipid units is high and their propensity of exchanging locations is high. The position of lipids in gel phase is more stable. The sol-gel phases of lipid bilayers are determined by weak molecular interactions known as van der Waals forces exerting apolar attraction between neighboring molecules. Lipids with longer fatty acids have larger overlapping areas and represent stronger interactions, decrease the mobility and the permeability of the lipid bilayer (Rawicz et al. 2000). Short tailed lipids are likely to melt at lower temperatures. Similarly, the transition temperature is lowered by the presence of increasing amount of unsaturated fatty acids. The kink in the alkane chain generates more space between the lipid tails and lower density of lipid bilayer with concomitant increase in permeability (Fig. 2.3). An opposite effect is exerted by the presence of cholesterol that provides mechanical strength and reduces the permeability of the lipid bilayer.

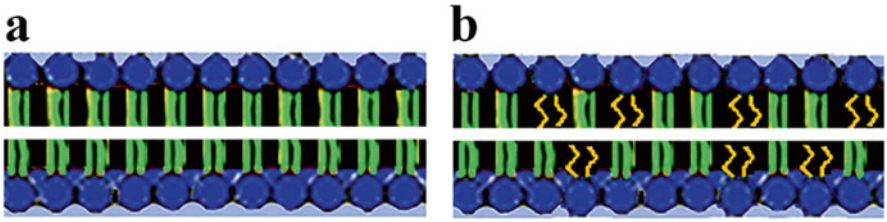


Fig. 2.3 Effect of unsaturated fatty acids on lipid structure and permeability. (**a**) Saturated fatty acid chains are more tightly packed and in closer proximity to each other than (**b**) unsaturated fatty acid tails that have less overlapping surfaces. The lack of reduced van der Waals forces make lipid bilayers with higher percentage of unsaturated fatty more permeable toward water and small molecules

2.5 Selectivity

Selective membrane permeability refers to the regulation of passage of specific substances of distinct molecules or ions, but remaining impermeable to others. For impermeable molecules the transport process may need the chemical energy of ATP. The selectivity rules of permeability apply to the two major groups of solutes:

– Small hydrophobic (lipid) molecules
– Molecules crossing the membrane by protein mediated transport

The passage of solutes will be detailed in subchapters devoted to passive transport, protein mediated transport, active transport, electrochemical potential-driven transporters, ABC transporters, organellar membrane transport, transmembrane proteins, and macromolecular membrane transport.

2.6 Passive Transport Processes

Small hydrophobic (lipid) molecules cross the membrane by simple diffusion. Water moves across membranes through special protein channels (aquaporins) driven by osmosis. The movement of water depends on whether the environment of cell is isotonic, hypotonic or hypertonic.

– Cells in isotonic solution: The movement of water in and out of the cell is balanced. Solutions corresponding to the osmosis of mammalian cells (0.3 Osm) are isotonic (e.g. saline, the 0.9 % w/v solution of NaCl; PBS, phosphate buffered saline; Ringer's buffered saline; 0.3 M glucose solution, etc.).
– Cells in hypotonic solution. The osmotic concentration of solution surrounding the cell is less (<0.3 Osm) than that inside the cell (0.3 Osm). Cells exposed to

hypotonic solutions swell, up to their osmotic resistance and burst at higher concentrations.
– Cells in hypertonic concentrations. The osmotic concentration of the solution is higher (>0.3 Osm) than the osmolarity inside the cell (0.3 Osm), causing the loss of water and shrinkage of the cell. Dehydration of cell may results in loss of function, rehydration may restore function.

Passive transport is the movement of atomic or molecular substances across the cell membrane. It does not require an input of chemical energy, and is driven by the difference of concentration between the two sides of the membrane termed as the *concentration gradient*. According to the second law of thermodynamics such a system will spontaneously proceed towards maximum entropy and to obtain thermodynamic equilibrium by passive transport.

The rate of passive transport depends on the permeability of the cell membrane, determined by the characteristics of the membrane lipids and proteins.

The following main passive transports are known:

– simple diffusion,
– osmosis,
– filtration and
– facilitated diffusion.

2.6.1 Simple Diffusion

Large molecules are unable to diffuse through membrane (Fig. 2.4a). Small solute molecules move from the higher concentration to the lower concentration (Fig. 2.4b) until the concentration of the solute becomes uniform and reaches equilibrium (Fig. 2.4c) without the involvement of transmembrane proteins. The substance penetrates the hydrophobic core of the phospholipid bilayer. Diffusible molecules that travel freely through the membrane are: O_2, CO_2, H_2O, NO.

2.6.2 Osmosis

Osmosis is similar to simple diffusion but describes the movement of water across a membrane until there is an equal osmotic concentration on both sides of the membrane. The difference in concentration at the two sides of the membranes can be caused by different solutes. The passive diffusion of water (osmosis) is not to be mistaken with the movement of water through water channels.

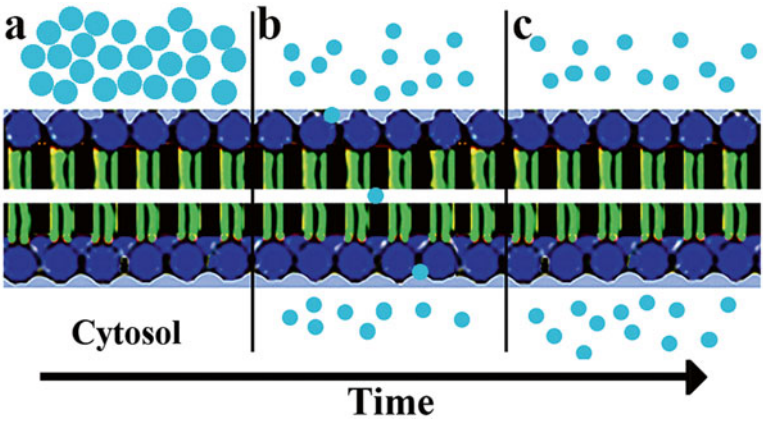


Fig. 2.4 Passive diffusion across the cell membrane. (**a**) large impermeable solute molecules, (**b**) diffusion through the membrane, (**c**) equilibrium solute concentration in the exterior and cytosol

2.6.3 Filtration

During filtration both water and solute molecules can be pressed through the membrane. The importance of filtration is exemplified by the hydrostatic pressure generated by the cardiovascular system and by the ultrafiltration of the Bowman's capsule of the kidney generating the glomerular filtrate that contains small molecules. The filtration barrier consists of a special fenestrated endothelium (pores in glomerular capillaries), and filtration slits of podocytes, that are visceral epithelial cells wrapped around glomerular capillaries holding back only large molecules such as proteins. Another major filtration occurs in hepatocytes, where large pores in their cell membrane allow passing various molecules.

2.6.4 Facilitated Diffusion

The movement through the membrane similarly to simple diffusion is dependent on the concentration of molecules, but aided by transport proteins embedded in the cell membrane. In simple diffusion the kinetics showing the relationship between the rate of uptake and the concentration in the external medium is linear, while in facilitated diffusion a seemingly biphasic saturation curve is observed resembling the Michaelis-Menten kinetics of enzymatic catalysis (ter Kuile and Cook 1994). The central channel of the transport protein acts as a selective corridor and helps molecules to cross the membrane (Fig. 2.5a). The other difference between the diffusion and facilitated diffusion is that in simple diffusion the relationship between the concentration in the external medium is proportional to the rate of uptake of the substance (Fig. 2.2b, line A), while the saturation curve of facilitated diffusion reaching

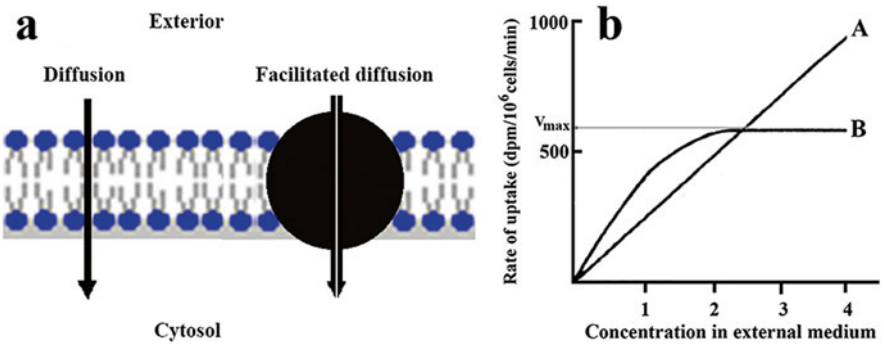


Fig. 2.5 Comparison of diffusion and facilitated diffusion. (**a**) Movement across plasma membrane by passive and carrier-mediated (facilitated) diffusion. (**b**) Rate of diffusion during passive (**A**), and facilitated diffusion (**B**) (Modified with permission Banfalvi 2014, p. 28)

Vmax in facilitated diffusion resembles that of the enzymatic catalysis (Fig. 2.5b, curve B).

2.7 Protein Mediated Transport

Particles required for cellular function but unable to diffuse freely across a membrane enter through a membrane transport protein either by facilitated diffusion that does not require energy, or by active transport that requires some kind of chemical energy. In this regard facilitated diffusion is also a protein mediated, but passive transport.

2.7.1 Transport Systems

Among the transport systems we distinguish:

- channel proteins, such as water channels, with water flowing in the direction of the osmotic gradient
- ion channels, permitting the passage of ions toward the electrochemical gradient
- facilitative transporters, working in the direction of concentration or electrochemical gradients
- primary active pumps working against gradients ("one bilayer, two compartments")

2.7.2 Channel Proteins

Channel proteins allow the movement of macromolecules, including proteins and RNA, ions, and small molecules, across the biological membrane. The macromolecular transport of proteins and RNA is better known as targeting and translocation to cellular compartments: nucleus, endoplasmic reticulum, Golgi apparatus, peroxisomes and mitochondria in animal cells, chloroplast is plants and periplasm in bacteria. The mechanism of macromolecular transport will be discussed separately after reviewing the transfer of small molecules and ions. The mechanisms regulating the transfer of small molecules and ions through the lipid bilayer membranes mediated by proteins are collectively termed membrane transport. Channels/pore types classified by Saier et al. (2009) include:

A	α-Type channels
B	β-Barrel porins
C	Pore-forming toxins (proteins and peptides)
D	Non-ribosomally synthesized channels
E	Holins (small proteins produced by dsDNA bacteriophages, to permeabilize bacteria for bacteriophage infection)
F	Vesicle fusion pores (e.g. transient fusion of secretory vesicle membrane at the porosome base *via* SNARE proteins, resulting in a fusion pore)
G	Paracellular channels (passing through the intercellular space between the cells)

Water Channels Without water there would be no life on Earth. The major component of all cells and tissues is water, the "solvent of life". One of the major problems in multicellular organisms is the movement of fluids across biological barriers primarily through the plasma membrane. After the discovery of the lipid bilayer it was assumed that water permeability occurs exclusively by simple diffusion through the plasma membrane. However, in certain tissues (red blood cells, renal tissues, secretory gland) the water permeability turned out to be much more higher than could be explained by diffusion, leading to the assumption that there must be a special water transporting mechanism, which turned out to be performed through integral membrane pore proteins forming water channels also known as aquaporins (Agre 2006).

> Peter C. Agre at the Johns Hopkins University School of Medicine, was the recipient of the 2003 Nobel Prize in Chemistry for his discovery of the aquaporin water channels.

Diffusion allows a slow and low-capacity bidirectional movement of water. To the contrary, water channels provide a highly selective, fast and high capacity conduction of water in and out of the cells, prohibiting the movement of ions and solutes. The high selectivity of aquaporins is indicated by the impermeability of protons present in very low concentration (10^{-7} M = pH 7) in water, a property

necessary to maintain the electrochemical potential difference of the membrane (Gonen and Walz 2006). There are other less selective aqueous channels that depending on size allow the crossing of uncharged solutes e.g. aquaglyceroporins that transport beside water solutes containing glycerol, carbon dioxide, urea, etc. The modulation of expression of aquaporin genes is essential and has been found in many organisms (bacteria, plants, animal cells) to protect against drought and is responsible for salt tolerance (Kruse et al. 2006; Xu et al. 2014).

2.7.3 Ionophores

These proteins help to reestablish resting membrane potential, control ion flow across the membrane and control the action potential by gating function and regulation of cell volume. Ionophores can be divided to ion channels and ion transporters.

– Ion channels can be further subdivided to:

  Voltage gated (sodium, calcium, potassium, proton) ion channels
  Ligand gated ion channels
  Other gating channels activated by second messengers

– Ion transporters correspond to ion pumps, among them active transporters as primary active and secondary active pumps such as sodium-potassium pump, sodium-calcium exchanger, sodium-glucose and other transporters.

The major transfer properties that differentiate ion channels from ion transporter proteins are:

– Ion channels allow a much lower transport rate than ion transporters
– As a function of ion concentration, ions pass through channels down their electrochemical gradient, but this "downhill" process does not need the direct or indirect metabolic energy of ATP or cotransport. Active transport processes to be discussed after the ion channels need the direct or indirect involvement of ATP energy.

Ion Channels Similarly to aquaporins ion channels are also pore-forming proteins but the crossing of ions generates electrochemical potential difference at the two sides of the membrane. The function of ion channels is to control the flow across the membranes of secretory and epithelial cells, to establish resting potential, to initiate action potential and to regulate cell volume. Ion channels are found in the plasma membrane of almost all cells and in many organelles. These water filled channels are selective for specific ions (Na^+, K^+, Ca^{2+}) and are normally as wide as the size of the specific ion or allow to pass through certain amount of charge (Fig. 2.6).

Voltage-gated Ion Channels Electric charge is carried through ion channels by ionic salts. Ion channels are selective to specific ions and determine membrane resistance. There are two major types of voltage-gated ion channels, voltage-gated sodium channels and voltage-gated calcium channels.

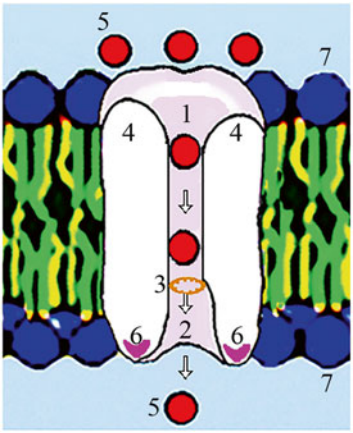


Fig. 2.6 Schematic view of ion channel in the cell membrane. *1* Outer vestibule, *2* inner vestibule, *3* selectivity filter of the pore, *4* channel domains, *5* ions, *6* sites of phosphorylation

Voltage-gated sodium channels are fast and the duration of their action potentials is shorter than 1 ms. Calcium-based channels are slow and their action potential may be longer than 100 ms. Voltage-gated Na^+ ion channels are present in considerable amount in the axons of neurons and are responsible for the fast action potentials involved in nerve conduction. The fast depolarization in response to triggering voltage channels may generate action potential. Sodium and potassium voltage-gated channels are depolarized in nerve and muscle cells. Beside the typical membrane depolarization voltage-gated ion channels can be activated by a specific subset of potassium selective ion channels (inwardly rectifying potassium channels) targeted by several toxins.

Voltage-Gated Calcium Channels Slower action potentials generated by voltage-gated calcium channels are characteristic to muscle cells and some types of neurons. These channels are present primarily in the membranes of excitable muscle, glial cells, and neurons, with a 1,000-fold greater permeability to the calcium ion Ca^{2+} than to sodium ions (Yamakage and Namiki 2002; Catterall 2011). They are occasionally called Ca^{2+}-Na^+ channels (Hall 2011). Voltage-dependence refers to channels closed at resting and opened (activated) at depolarized membrane potentials. Since the extracellular Ca^{2+} is more than 10,000-times higher than the intracellular Ca^{2+} concentration, the opening of Ca^{2+} channels causes the Ca^{2+} ions to rush into cells, activates calcium-sensitive potassium channels and initiates the contraction of smooth muscle (Wilson et al. 2005). The extracellular Ca^{2+} concentration in smooth muscle is an estimated ~1.2 mEq/L = 1.2 mM (Yang et al. 2004) and the intracellular Ca^{2+} concentration in the cells of the smooth muscle ~100 nM (Castro et al. 1994). Voltage-gated calcium channels also initiate the release of neurotransmitters in presynaptic nerve endings. The importance of activation of voltage-dependent Ca^{2+} channels is demonstrated by the excitation of neurons, up-regulation of gene expression, release of hormones and neurotransmitters. Overactivation of voltage-dependent Ca^{2+} channels results in high calcium conductivity, high intracellular Ca^{2+} load, activation of enzymes leading to self-digestion of essential cellular components, particularly the pathological damage of mitochondria known as exocitotoxicity.

Other Ion Channels Among other ion channels transient receptor potential channels, cAMP-, cGMP-gated channels and voltage-gated proton channels deserve mention. Transient receptor potential channels are less selective to cations and mediate sensations such as pain, taste, pressure, vision, coldness, warmth. Some of these channels are also activated by spices like allicin (from garlic), capsaicin (in chilli pepper), menthol, peppermint, acting as sensors (changes in osmosis, volume, pressure, stretch, vibration, etc.).

Cyclic nucleotide-gated ion channels show little voltage dependence. These channels are nonselective cation channels with limited discrimination between alkali ions and alkaline earth metal ions, in particular Ca^{2+} (Kaupp and Seifert 2002). These channels have been identified in retinal and olfactory neurons and are opened by the binding of cAMP and cGMP. Their function has been established in retinal photoreceptors, but yet to be confirmed in other sensory and nonsensory cells since their purported roles in neuronal path finding or synaptic plasticity are not well understood (Kaupp and Seifert 2002).

Voltage-gated proton channels are ion channels that open upon depolarization in a pH sensitive manner. These channels open when the electrochemical gradient is directed outward and allows protons to leave the cell. H^+ ions are conducted passively down their electrochemical gradient, proton permeation is not coupled to the movement of any other ion, ATP is not required, and the pH displays a time- and voltage-dependent gating (Cherny et al. 1995). The activation by membrane depolarization of proton selective conductance was observed in neurons (Thomas and Meech 1982) and in the phagocytosis of human neutrophils (DeCoursey and Cherny 1994) during respiratory burst. Bacteria engulfed by phagocytes (eosinophils, neutrophils, macrophages) activate NADPH oxidase that produces reactive oxygen species (ROS) to kill bacteria (Babior 1999).

Ligand-gated Ion Channels (Ionotropic Receptors) Ionotropic receptors form an ion channel pore. Ionotropic receptors, are directly linked to ion channels and do not use second messengers. Upon binding the chemical messenger (ligand), channel proteins open and ions (Na^+, K^+, Ca^{2+} or Cl^-) can pass through the plasma membrane. Ionotropic channel proteins have two major domains, after the binding of ligand to its binding site the other domain, the transmembrane ion pore opens and the ions pass through the membrane (Fig. 2.7). Ligand-gated ion channels are located at synaptic clefts and convert the presynaptic chemical signal (neurotransmitter) to postsynaptic electric signal. Among the ligands that activate ionotropic receptors are: nicotine, acetylcholine, glutamate, ATP-gated P2X purinoreceptor, $GABA_A$-gated (GABA = gamma amino butyric acid) ionic receptors.

Nicotinic Acetylcholine Receptor This is the prototype and the best studied ligand-gated ion channel. It consists of pentamer protein subunits, with two binding sites for acetylcholine. Acetylcholine binding alters the configuration of the receptor by moving the leucine residues, which block the channel and opens the pore. The inward flow of Na^+ ions depolarizes the membrane and initiates action potential that

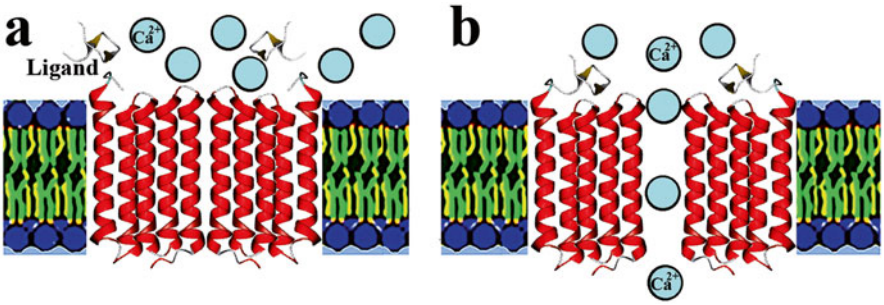


Fig. 2.7 Ligand-gated ion channel. (**a**) Closed membrane. (**b**) Ligand binding (e.g. neurotransmitter) opens a channel and allows the inflow of ions (e.g. Ca^{2+})

leads to muscular contraction. As their name would suggest the nicotinic receptors can be opened both by acetylcholine and by nicotine (Itier and Bertrand 2001).

Other inotropic receptors Ionotropic receptors form an ion channel pore and comprise: (a) several receptor classes: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate receptors that bind the neurotransmitter glutamate. Receptor heterogeneity arises from the assembly of distinct subunits into cation-selective tetramers. (b) ATP-gated P2X receptors, which form trimers and open in response to binding the nucleotide ATP. (c) PIP_2-gated channels (PIP_2 = phosphatidylinositol 4,5-bisphosphate) antagonize inward rectifying potassium channels (Hansen et al. 2011). (d) GABA receptors act at inhibitory synapses in the vertebrate brain, by the binding of GABA to transmembrane receptors of the plasma membrane. Besides playing role in pre- and postsynaptic neuronal processes the opening of ion channels allow the flow of chloride ions into the cell and positively charged potassium ions out of the cell Purves et al. 2008).

Metabotropic Receptors Beside the ionotropic receptors, metabotropic receptors represent the other major type of neurotransmitter receptors. In contrast to ionotropic receptors forming ion channels, metabotropic receptors are not directly linked to ion channels, but act through signal transduction mechanisms, primarily *via* G proteins. The activation of metabotropic receptor is linked to a series of intracellular reactions, which can also result in the opening of ion channels with the involvement of second messengers.

There are further gating mechanisms that can be activated both from the inside or outside of the membrane, by second messengers, such as inward-rectifier potassium, calcium activated potassium, two-pore-domain potassium channels, as well as the involvement of adhesion molecules, with particular attention to their role in tumor suppression.

Diseases of ion channels are caused by chemicals: tetrodotoxin, saxitoxin, conotoxin, lidocain, endrotoxin, etc., by genetic mutations (Shaker gene, hyperkalaemic periodic paralysis, epilepsy, ataxia, migraine, long QT syndrome, cystic fibrosis, mucolipidosis) with serious consequences for the individuals affected.

Channelopaties are diseases related to ionic imbalances, normally not involved in tumor formation. In patients with cystic fibrosis an increased risk of digestive tract cancer was reported (Neglia et al. 1995).

Ion channels have also been classified by the type of ions they allow to cross: Na^+, K^+, Ca^{2+}, H^+, and other non-selective cation channels. These and further classifications would result in unnecessary repetitions of ion channels already mentioned.

Membrane transport proteins (transporters) as integral membrane proteins support the movement of substances by two types of carrier mediated transport (Thompson 2007). The carrier proteins assist facilitated diffusion and active ion transport. Each carrier protein is specific and recognizes only its own substance or its group of chemically related substances. Facilitated diffusion belongs to one of the passive transport processes (simple diffusion, osmosis, filtration, facilitated diffusion) and has already been discussed under the subtitle: Passive transport.

2.8 Types of Active Transport Processes

Ion pumps or primary transporters move ions across the plasma membrane against their concentration gradient. Primary transporters are enzymes that can utilize chemical energy (ATP), light energy (sunlight) or turn the energy of redox reactions to potential energy stored as electrochemical gradient. The energy of electrochemical gradient is exploited by the secondary transporters of ion transport and ion channels. Ion transporters move ions against concentration gradient *via* active transport in contrast to ion channels that allow passive transport.

2.8.1 Primary Active Transport

The most important representative of primary active transport is the active antiport of Na^+ *versus* K^+ catalyzed by the sodium-potassium ATPase, that is pumping sodium out and potassium inside the cell. Beside this "housekeeping" ionic pump there are other active transport systems that need ATP, among them cellular and organellar Ca^{2+}/H^+ and H^+/K^+ ATPases (Fig. 2.8).

Primary active pumps are P-type ATPases (E1-E2). P-refers to the self-phosphorylation of pumps with ATP and E1-E2 to conformational transitions inside these pumps. The best known P-type ATPases are the Na^+/K^+-ATPase, the plasma membrane proton pump (H^+-ATPase), the proton-potassium pump (H^+/K^+-ATPase), and the calcium pump (Ca^{2+}-ATPase) (Fig. 2.8). P-type ATPases are present in bacteria, Archaea and eukaryotes, and in vacuoles of eukaryotic cells. In contrast to other vacuolar (V-type) ATPases the pumps of neurotransmitter vacuoles are not self-phosphorylated. The phylogenetic classification and importance of the five types of P-ATPases (Type I–V) are out of the field of interest.

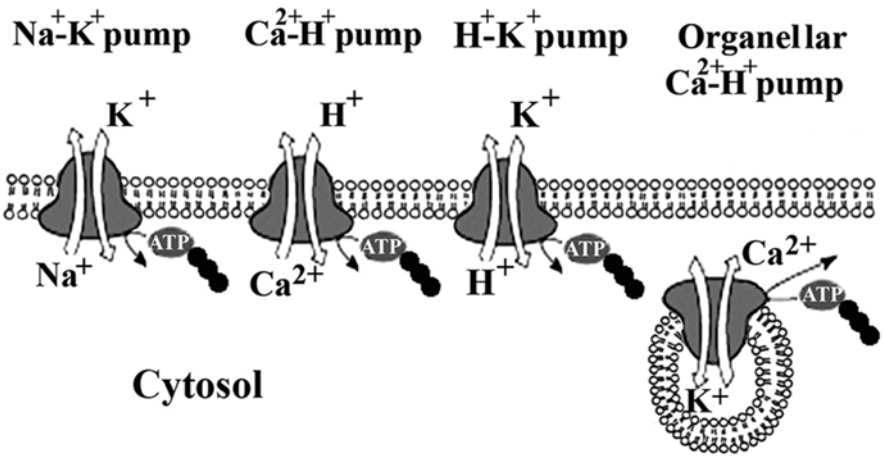


Fig. 2.8 Cellular and organellar active transport processes require the energy of ATP. Primary active pumps move ions across the plasma and organellar membranes against their concentration gradient (Modified with permission Banfalvi 2014, p. 30)

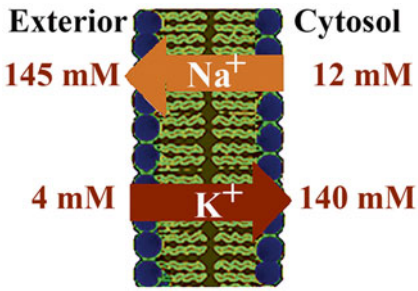


Fig. 2.9 Ion movements and concentrations maintained by the Na^+–K^+ transport ATPase. Data of ion concentrations in mammalian cells and in blood are from Darnell et al. (1986)

Na^+-K^+ Transport ATPase The most important P-type pump present in all eukaryotic cells is the Na^+/K^+ ATPase that is maintaining the basic (resting) membrane potential by keeping the concentrations of these ions constant and restoring their concentrations after activation. Inside the mammalian blood cells the K^+ concentration (~140 mM = gion/L) is high and the Na^+ concentration (~12 mM) low. The extracellular concentration of K^+ is ~4 mM and Na^+ ~145 mM (Darnell et al. 1986) (Fig. 2.9).

The canine and feline red blood cells have higher external Na^+ and lower inner K^+ concentrations. Among sheep there are both "small K^+" and "high K^+" concentrations in different species. The regular function of the Na^+/K^+ ATPases is necessary to regenerate the ionic gradients disturbed by action potentials and by the passive diffusion of Na^+ ions inward and K^+ ions outward that would dissipate the concentration gradients. The transport of Na^+/K^+ ATPases can be inhibited by quabain, a cardiotonic steroid that binds to the external surface of the membrane and inhibits the dephosphorylation of the pump. Vanadate (V^{5+}) ions inhibit from inside

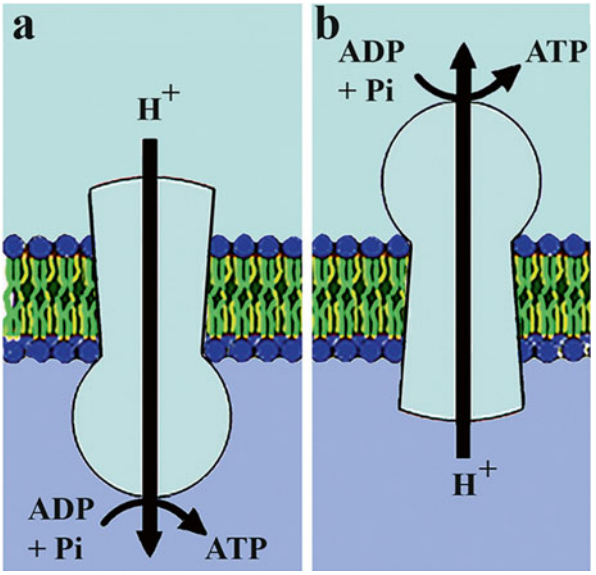


Fig. 2.10 Orientation of proton mump of F1 ATP synthase: (**a**) in mitochondrial ATP synthase and (**b**) in bacterial and chloroplast inner membranes

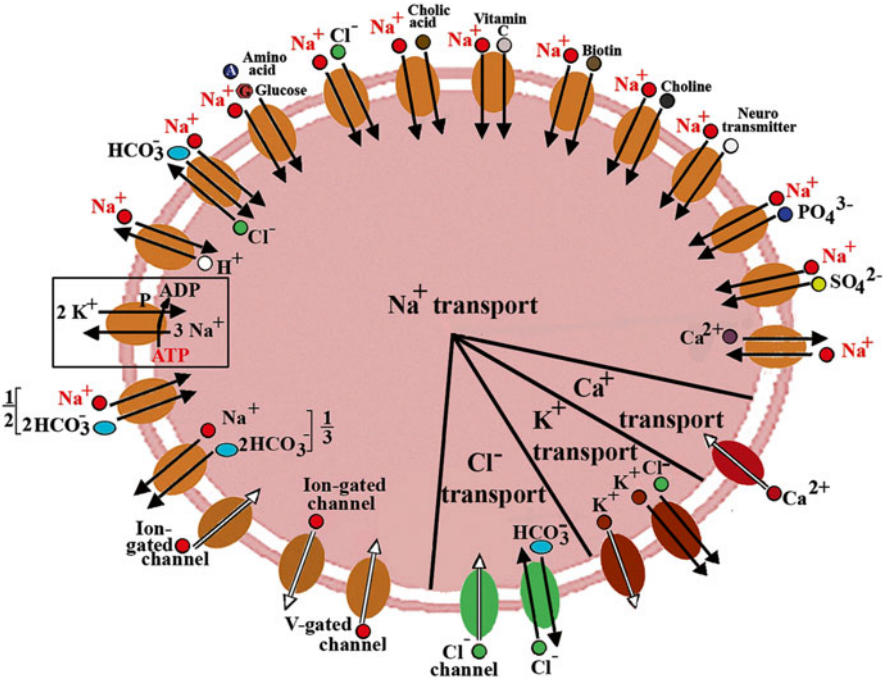


Fig. 2.11 Membrane-bound ion transporters are used to transfer the cargo between membranes of the secretory and the endocytic pathways. Primary active transport processes require ATP. In secondary active transport processes the high sodium concentration generated by the primary active transport is supplying the energy and can move together substrates with Na^+. Color codes: Na^+ exchanger: , K^+ exchanger: , Cl^- exchanger: , *Ca* transporter:

by locking the E2 conformation of the pump. The phosphorylation step of the Na^+/K^+ ATPase (E1) by ATP takes place in the presence of Na^+ and Mg^{2+} ions.

F-type ATPase (F-ATPase, ATP synthase) is present in bacterial plasma membranes, in the inner membrane of mitochondria and in the thylakoid membrane of chloroplasts. The letter "F" comes from "phosphorylation Factor". The major function of ATP synthase is to generate ATP in most organisms. However, occasionally the reverse reaction may be equally important and the transmembrane proton pumping may be driven by ATP, which means that ATP synthase may work in both directions: ADP + Pi <=> ATP. F-type ATP synthases are the major suppliers of chemically bound ATP energy in all cells. Under aerobic conditions the proton gradient drives the ATP synthase by the flux of protons across the membrane down the electrochemical gradient and catalysis the synthesis from ADP and inorganic phosphate (Pi). In mitochondria the proton (H^+) ions move from the intermembrane space to the matrix (Fig. 2.10a). In bacteria under aerobic conditions ATP synthase is oriented in opposite directions. Similarly to bacteria in chloroplasts protons move from inside (lumen) to the outward (stroma) direction (Fig. 2.10b).

2.8.2 Secondary Active Transport

In these transmembrane processes the sodium ions, accumulated in the primary active transport drive the crossing of ions through the membrane. In the primary active transport processes (e.g. K^+/Na^+ ATPase) the movement of substrate across the membrane is taking place against the concentration gradient (boxed in Fig. 2.11). Secondary active transport is driven by the high extracellular concentration of Na^+ generated by the primary active transport. The types of plasma membrane pumps depend on cell types. Ca^{2+} pumps are not present in each cell, but are important to maintain the low cytoplasmic concentration in many cells. The Ca^{2+} pump is pumping out Ca^{2+} ions from red blood cells, plays a crucial role in the Ca^{2+} absorption in intestinal cells. The H^+/K^+ is present in the distal tubule cells of the kidney and as gastric hydrogen-potassium ATPase in the stomach. Pumps of intracellular organelles help the reuptake of Ca^{2+} in the endoplasmic reticulum and sarcoplasmic reticulum (in muscle cells) by the Ca^{2+} -ATPase. The acidic internal environment of lysosomes and secretory vesicles is accomplished by proton pumps.

Secondary active ion transport processes connected to the primary active Na^+/K^+ pump are summarized in Fig. 2.11 and in Table 2.1.

2.9 Electrochemical Potential-Driven Transporters

Among the electrochemical potential-driven transporters are:

(a) ion-gradient-driven energizers (secondary active transporters)
(b) transport proteins (porters: uniporters, symporters, antiporters), and
(c) non-ribosomally synthesized porters (Saier et al. 2009).

Table 2.1 Secondary active transporters connected to the primary active transport of Na^+/K^+ ATPase

Transporter	Function
Na^+/Ca^{2+}	Ion exchanger
Na^+/H^+	Ion exchanger
Na^+/Cl^-	Symporter
Na^+/HCO_3^-	Symporter, glia cells
Na^+/PO_4^-	Symporter
Na^+/SO_4	Symporter
Na^+/amino acid	Symporter, small intestine and other tissues
Na^+/cholic acid	Symporter, small intestine
Na^+/folic acid	Symporter
Na^+/ neurotransmitter	Symporter, brain
Na^+/ascorbic acid	Symporter
Na^+/biotin	Symporter, liver
Na^+/cholin	Symporter

2.9.1 Uniporters

Carrier proteins recognize specific substances or a group of similar substances. Transport proteins or membrane transport proteins also referred to as transmembrane pumps, transporter proteins, escort proteins, acid transport proteins, cation transport proteins, anion transport protein or simply transporters acts as such carriers.

Transport proteins can be also classified as uniporters, symporters and antiporters. According to this classification *uniporter* is an integral membrane protein mediating the transport of a single solute and involved in facilitated diffusion (Fig. 2.12a). Uniporters can be either ion channels or carrier proteins. Uniporter channels may be gated by:

1. Voltage – difference in voltage across the membrane
2. Stress – physical pressure on the transporter
3. Ligand – binding of a ligand to either the intracellular or extracellular side of the cell

Uniporters are involved in the action potentials of neurons. These voltage-gated sodium channels are functional in the propagation of nerve impulses across the neuron. Calcium is transported into the presynaptic neuron by voltage-gated calcium channels. GLUT1 is a glucose uniporter. The ionophore valinomycin is an antibiotic uniporter, facilitates the movement of potassium ions through lipid membranes "down" the electrochemical potential gradient (Cammann 1985). Potassium channels, help to restore the resting membrane potential. Stress-regulated channels in the ear are opened by sound waves, sending impulses to the cranial nerve (VIII).

2.9.2 Symporters

Symporters or cotransporter carriers are ion-gradient-driven energizers. As integral membrane proteins symporters bind to two (or more) different molecules or ions and transport them together in the same direction across the phospholipid bilayer (Fig. 2.12b). The gradient of one substrate, normally an ion may drive the transport of the co-substrate even against the concentration gradient of the co-substrate. This type of cotransport is also regarded as secondary active transport. Examples of symport: glucose-Na^+ symport in the intestinal epithelium transports sodium ions (Na^+) and glucose across the luminal membrane of the epithelial cells leading to the absorption into the bloodstream. $Na^+/K^+/2Cl^-$ symporter in the loop of Henle in the renal tubules of the kidney transports sodium ion, potassium ion and two chloride ions. The H^+/K^+ symporter, also known as proton pump, is used to increase the acidity of stomach acid (HCl).

The secondary active transport is based primarily on the accumulation of sodium ions outside cells generated by the primary active transport. Na^+-coupled transporters are Na^+-K^+-Cl^-, Na^+-amino acid, Na^+-glucose, Na^+-fatty acid cotranporters, Na^+-PO_4^{3-}, Na^+-HCO_3^-, Na^+-SO_4^{2-}, Na^+-cholate, Na^+-folate, Na^+-ascorbate and Na^+-cholin symporters, etc. were shown in Fig. 2.11.

2.9.3 Antiporters

Antiporters or exchange diffusion carriers exchange one solute in return of another across a membrane. The antiporter "ping pong" kinetics is exhibited by binding and transporting the first substrate across the membrane, followed by the binding and transport of the other substrate in the opposite direction. Typical antiporters (exchanger or counter-transporters) are sodium-coupled co-transporting integral membrane proteins that are involved in the secondary active transport of two or more different ions across the plasma membrane in opposite directions, e.g. Na^+-H^+, Na^+-Ca^{2+} antiporters, HCO_3-Cl^- (Figs. 2.11 and 2.12c). The mitochondrial adenine nucleotide translocase (ADP/ATP exchanger), is exchanging ADP for ATP across the inner membrane.

Antibiotic Antiporters Nigericin acts as an ionophore, an antiporter of H^+ and K^+. It was used as an antibiotic against Gram-positive bacteria inhibiting the Golgi functions of cells. Ionomycin is an ionophore produced by the bacterium *Streptomyces conglobatus*, that preferentially induces Ca^{2+}/H^+exchange and raises the intracellular level of calcium (Ca^{2+}). Ionomycin also stimulates the production of cytokines, interferon, perforin, IL-2, and IL-4.

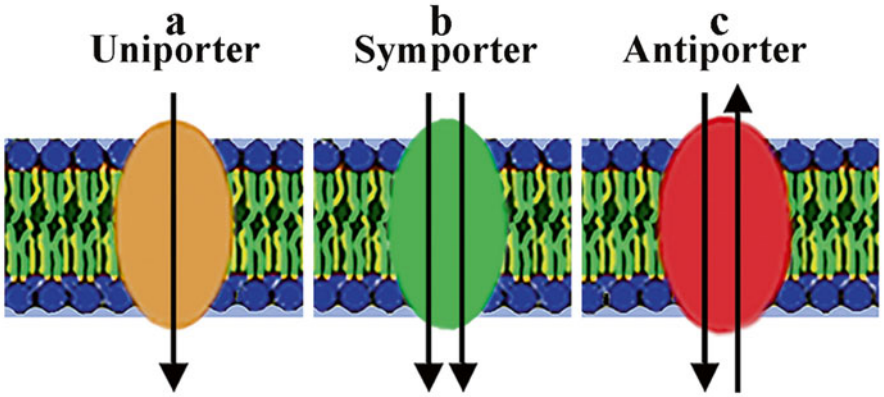


Fig. 2.12 Classes of carrier proteins: uniporter, symporter and antiporter

2.9.4 Non-ribosomally Synthesized Porters

These channels are formed from molecules, often chains of L- and D-amino acids and of the hydroxy acids lactate and b-hydroxybutyrate, forming oligomeric transmembrane channels. Voltage may induce channel formation by promoting assembly of the oligomeric pore-forming structure. These porters also mentioned as "depsipeptides" can be produced by bacteria and fungi and belong to the agents of biological warfare. Other substances, without amino acids, may also be involved in channel formation.

2.10 ABC Transporters

This is the largest transporter protein family, with several hundred membrane transport proteins that need the energy of ATP to transport substrates across the cell membrane and organellar membranes. Transporters of the ABC superfamily are ubiquitous in organisms from prokaryotes to humans (Choi 2005). Among the substrates are various ions, mono- and polysaccharides, amino acids, peptides, polypeptides, phospholipids, cholesterol, etc. (Scott et al. 2012). ABC transporters were first recognized as proteins involved in multiple drug resistance, especially in cancer cells that overexpress these proteins and generate tumor resistance toward drugs. The physiological importance of the **A**TP **B**inding **C**asette (ABC) Family is not completely understood, their most important role is probably their participation in the absorption and secretion of endogenous and exogenous substances. Nevertheless, the large number of proteins, involved in membrane transport including these ABC transporters, with nearly 48 ABC genes reported in humans, many of them related to diseases (Linton 2007), underlines the importance of these proteins, and more importantly the vital significance of transport through biological membranes.

2.11 Organellar Membrane Transport

This transport can be subdivided into:

(a) the uptake of foreign substances by the cell. The organellar membrane transport is represented by the endocytic transport pathway that starts with the endosomal internalization from the cellular membrane.
(b) intracellular movement of transport vesicles (e.g. targeting of proteins synthesized on free ribosomes)
(c) transport of vesicles ending up in secretion. Secretory vesicles mediate the transport of hormones, neurotransmitters, release locally acting paracrine substances from organelles. The secretory vesicle is a vesicle that mediates the vesicular transport of cargo – e.g. hormones or neurotransmitters – from an organelle to specific sites at the cell membrane, where it docks and fuses to release its content. It has been demonstrated that membrane-bound secretory vesicles dock and fuse at porosomes (see Fig. 1.26), which are specialized supramolecular structures at the cell membrane.

2.12 Transmembrane Proteins

Many transmembrane proteins serve as gateways or deny the transport of specific substances across the biological membranes. Transmembrane proteins as integral membrane proteins are discussed separately, as not all integral membrane proteins cross both faces of the lipid bilayer. Transmembrane proteins are spanning the entire biological membrane to which it is permanently attached. The topological classification of transmembrane proteins distinguishes four types: types I, II, and III are single-pass molecules, type IV are multiple-pass molecules. Here only type I proteins are mentioned that are anchored to the membrane with a stop-transfer anchor sequence and have their N-terminal domains targeted to the lumen of the endoplasmic reticulum (Lodish et al. 2007; Saier et al. 2009).

Integral monotopic proteins, are attached to only one side of the membrane. Polytopic proteins pass the entire biological membrane either only once (single-pass) or weave in and out of the membrane while crossing it several times (multi-pass proteins). Monotopic integral and polytopic multi-pass transmembrane proteins are schematically viewed in Fig. 2.13.

Corresponding to the shape or charge of substances the transmembrane proteins have developed special folding motives and bending patterns to move “freight” molecules through the membrane. The two basic types of transmembrane proteins are alpha-helical proteins and beta-barrels. Alpha-helical proteins represent the major category of transmembrane proteins present in the plasma membrane of eukaryotes and in the inner membrane of bacterial cells (Fig. 2.14). The structure of beta-sheet of proteins is shown in Fig. 2.15.

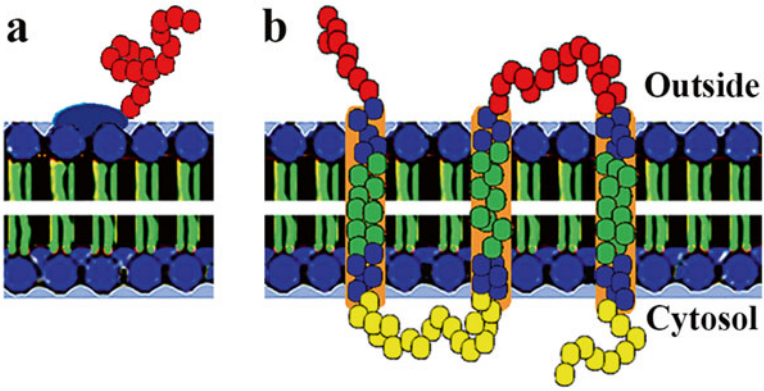


Fig. 2.13 Integral and transmembrane proteins. (**a**) Integral monotopic membrane protein not passing the entire membrane. (**b**) Polytopic multi-pass transmembrane protein crossing three times the entirety of the membrane to which it is permanently attached

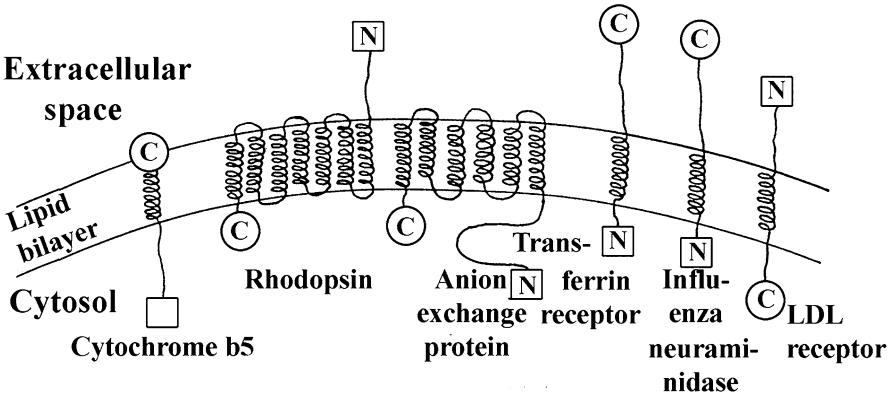


Fig. 2.14 Transmembrane alpha helices. The C and N terminal ends of transmembrane proteins can be at the extracellular or intracellular side of the lipid bilayer

A beta barrel is a large β-pleated sheet (beta-sheet) that twists and coils to form a closed structure in which the first strand is hydrogen bonded to the last one. Beta-barrels are typical transmembrane structures of the outer membranes, evolutionarily related to Gram-negative bacteria, mitochondria and chloroplasts. Characteristic beta barrel topologies include: (a) the simplest up-and-down beta barrels consisting of a series of beta strands, (b) Greek key barrels contain at least one Greek key structural motif linked to a beta hairpin or to another Greek key, (c) the jelly roll barrel (Swiss roll), is an even more complex structure with four pairs of antiparallel beta sheets, one of which is adjacent in sequence, "wrapped" in three dimensions to form a barrel shape.

Crossing the cell membrane is uniform in cells, which are not polarized. In oriented (polarized) cells the permeability at the basolateral pole (adjacent to the inner basement membrane) differs from that of the luminal (apical) membrane surfacing

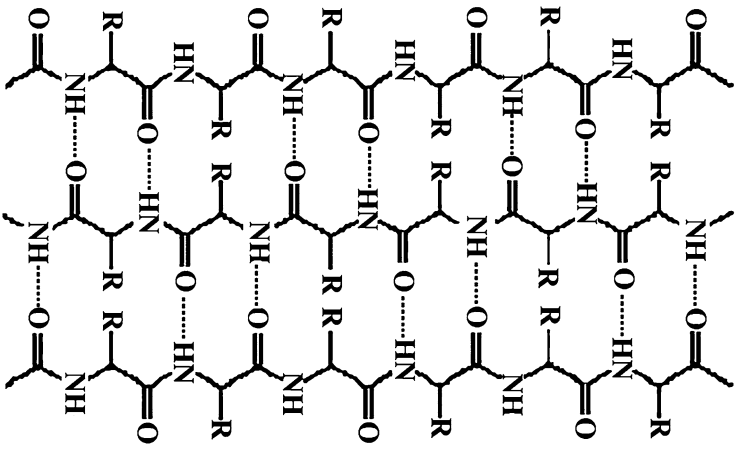


Fig. 2.15 Beta-pleated sheet secondary structure of proteins

the plasma membrane inward to the lumen. This polarization is characteristic to epithelial, endothelial and neuronal cells. The negative polarization of the cytoplasm relative to the other surface of the membrane is known as resting potential (resting voltage) (E_m) as opposed to the specific dynamic action potential and graded membrane potential.

There are three major types of integral membrane proteins:

- transport systems (ion pumps, facilitated transporters, channel proteins)
- integrins bound to proteins of the extracellular matrix
- receptors, recognizing extracellular signals

2.13 Macromolecular Membrane Transport

The macromolecular transport processes are more complex than the transport of small molecules and ions and require the combined action of many molecular components. Transported proteins encode specific sequences, so called targeting signals, signal sequences that dictate the final location of these proteins. The macromolecular transport of proteins and RNA also referred to as macromolecular targeting includes the translocation to and from cellular compartments. Target recognition and interactions between targeting sequences and their receptors determine whether the final location of the transported macromolecule will be in the nucleus, peroxisomes or mitochondria in animal cells, chloroplast is plants and periplasm in bacteria. The endoplasmic reticulum, Golgi apparatus, lysosomes, endosomes and secretory vesicles belong to the endomembrane system and are involved in the processing of proteins for export from the cell, destined for degradation in lysosomes and entering into the cell from outside. The three major subdivisions of the endomembrane system are the secretory, lysosomal and endocytic pathways.

Macromolecules such as DNA, RNA, proteins do not cross the cellular membrane. The exclusion of large dye molecules is generally used in cell biology to prove the viability of cells (e.g. trypan blue dye exclusion). However, membranes impermeable to dye molecule can be rendered transiently permeable by femtosecond laser beams that create small transient holes in the cellular membrane to inject nanomolar concentration of dye for staining the cell. Upon optical damage propidium iodide enters the cell and stains the nucleus (e.g. laser beam induced optoporation by flow cytometer) (Baumgart et al. 2008; Davis et al. 2013). Introduction of the impermeable rhodamine phalloidoine by optoporation has been used for staining filamentous actin in cells (Dhakal et al. 2014). Beside the introduction of fluorophores (fluorescent dyes, proteins, antibodies linked to fluorescent dyes), markers (actin, tubulin), several other physical methods have been developed for the injection of external factors through the plasma membrane to visualize cytoplasmatic elements. The most widely employed methods were electroporation (Neumann et al. 1982) and microinjection (Lacal 1998). These methods have been escalated by the introduction of vesicle fusion, chemical transfection, viral transduction (Lacal 1998), permeabilization of bacterial cells (Moses and Richardson 1970; Banfalvi and Sarkar 1983). Initial attempts to permeabilize mammalian cells applied different methods such as hypotonic solution (Berger and Johnson 1976; Nishio and Uyeki 1982), Triton X-100 (Hyodo and Suzuki 1980) and dextran sulfate (Kucera and Paulus 1982). These permeabilizations were followed by using reversibly permeabilized cells to study intracellular processes (Halldorsson et al. 1978; Banfalvi et al. 1984; Banfalvi 2014). Methods of permeabilization will be detailed in Chap. 3, applications of permeabilization will be given in Chap. 4.

2.13.1 Targeting Biological Information

Information is knowledge, but knowledge in itself is not information. By definition the real information is the weapon of material organized into a system to fight against randomness (chaos). The flow of information is called communication. The transfer of biological information is communicated *via*:

- processes involved in the transfer of cellular and subcellular (mitochondrial, plastid) information
- environmental information → signal transduction, viral transfection
- *in vitro* information transfer → gene manipulation

To speak about biological communication the genetic information has to take its proper form, needs material carriers and energy to reach its target. The flow of genetic information is regulated by several basic rules, among them the most important one known as the general idea stating that DNA makes RNA makes protein:

$$\text{DNA} \Rightarrow \text{RNA} \Rightarrow \text{Protein}$$

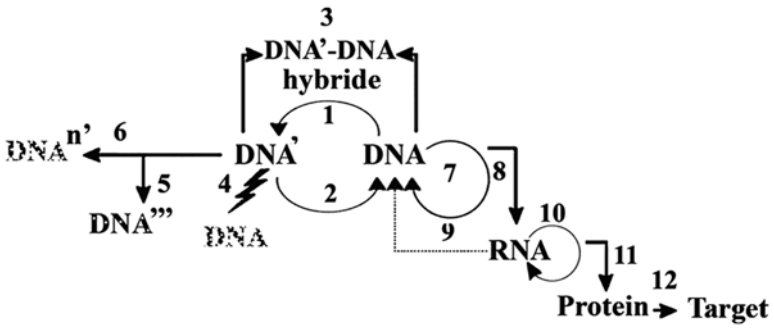


Fig. 2.16 Processes involved in the transfer of genetic information. *1* Mutation, *2* repair, *3* recombination, *4* apoptosis, *5* aging, *6* malignant transformation, *7* DNA replication, *8* transcription, *9* reverse transcription, *10* RNA (viral) replication, *11* translation, *12* protein targeting (With permission Banfalvi 2009, p. 17)

This basic rule applies to all cells. The transfer of genetic information involves several processes (Fig. 2.16):

From the point of view of targeting biological information the transfer of DNA, RNA and protein are to be considered, with particular attention to the most frequently occurring protein targeting. Protein targeting is the last step in the transfer of genetic information and completes the flow of information to become communication (Fig. 2.16).

2.13.2 Nuclear Targeting

Nuclear targeting can be (a) protein and RNA targeting (nuclear export and import) or (b) DNA targeting. Major molecular components of proteins involved in nuclear targeting are:

- Cargo proteins carrying the nuclear localization signals. Targeting to the nucleus requires a 6–20 amino acid long nuclear localization signal consisting mainly of highly basic amino acids (Lys, Arg). These sorting nuclear localization signals show little or no homology and are not cleaved off from the transported protein.
- Nuclear pore complex (NPC) consists of many distinct types of proteins known as nucleoporins or NUPs.
- Karyopherins also belong to the proteins of the nuclear pore complex family: *importins* help proteins to get into the nucleus, and *exportins* supporting proteins to get out of the nucleus.
- RanGTP/GDP small G-proteins possess GTPase activity that by hydrolizing GTP to GDP initiate translocation through the nuclear pore.

(a) Nuclear import and export

The nuclear envelope and the nuclear pore complex are the structures that both separate and connect the nucleoplasm and the cytoplasm. According to

the classical nuclear protein import pathway, the cargo protein contains a nuclear localization sequence. This sequence is recognized and bound by import proteins belonging to the members of importin superfamily. Importin mediates the passage of cargo protein through the nuclear pore complex into the nucleus where the binding of Ran-GTP to importin initiates the dissociation of the complex and releases the cargo protein (Quimby and Corbett 2001; Pemberton and Paschal 2005). Similarly to nuclei of animal cell protein targeting, the plant nuclei also use a Ran-cycle component named Ran-GAP (Ran GTPase-activating protein) (Meier et al. 2010).

The nuclear export of RNA species (mRNA, tRNA), ribosomal subunits, and nuclear import of proteins involved in DNA replication, DNA repair, recombination and ribosomal proteins are shown in Fig. 2.17.

(b) *Targeting viral cDNA to the nucleus*. Retroviruses such as HIV must integrate their cDNA into the host cell genome requiring the virus to be translocated through the nuclear envelop. HIV-1 integrase is involved in the importing of viral cDNA into the nucleus (Hearps and Jans 2006).

(c) *Gene therapy by nuclear targeting*. Nuclear targeting *via* nonviral delivery of DNA is one of the challenges in gene therapy. Targeting could be done by delivering plasmid DNA into the nucleus of cells *via* nuclear localization signal peptide conjugated to DNA. Such conjugates are expected to enhance transfection efficacy as well as nuclear localization of plasmid DNA (Shiraishi et al. 2005). Unlike several viruses, plasmids have not evolved

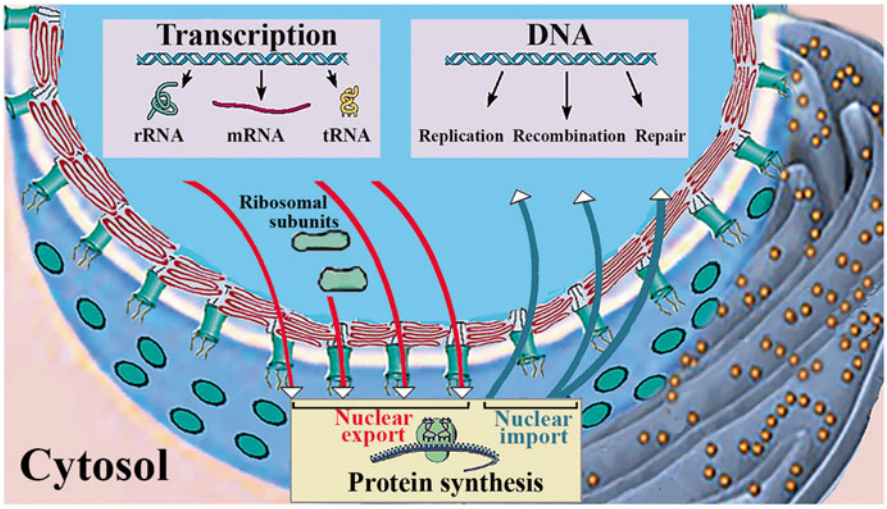


Fig. 2.17 Nuclear transport through the nuclear pores. Nuclear export: RNA molecules (rRNA, mRNA, tRNA) and ribosomal subunits are exported to the cytoplasm where proteins synthesis is taking place. Nuclear import: Proteins carrying signal sequences are synthesized in the cytosol on free ribosomes and directed into the nucleus. These include enzymes and proteins for DNA synthesis (DNA replication, DNA repair), for recombination and assembly of ribosomal subunits. *Red arrows* show the direction of nuclear export. *Blue arrows* indicate nuclear import

mechanisms to target to the nucleus of non-dividing cells. The ability to target DNA to the nucleus can increase gene transfer *in vivo* and inclusion of the SV40 sequence into plasmids could enhance nonviral gene delivery (Young et al. 2003). However, one cannot escape to mention the major problem of gene therapy, namely the inefficiency of gene transfer to slowly and non-dividing cells. The integration sites of viral vectors in human gene therapy can contribute to safety and efficacy (Miller et al. 2005). The efficiency problem could be overcome by integrating retroviral vectors, primarily the lentivirus family, because they can transduce nondividing cells. Human immunodeficiency virus type 1 (HIV-1) based gene transfer systems have been suggested for safe (not causing viral infection) and efficient gene therapy due to their ability to transduce terminally differentiated and non-dividing cells (Srinivasakumar 2001). HIV-based vectors have been favored for human gene correction (Cartier et al. 2009; Cavazzana-Calvo et al. 2000, 2010).

2.13.3 Protein Targeting to Peroxisomes

Organelles among them peroxisomes are defined as subcellular particles surrounded by lipid membranes containing proteins (Palade 1983). While the first genomic code (cellular DNA) and the second genomic code (mitochondrial DNA) are written in nucleotide letters, some of the organelles are derived exclusively from pre-existing organelles, referred to as the "Third genome" (Schatz 2006). Organelles can be differentiated and divided into two categories. The multiplication of autonomous organelles (perinuclear space, mitochondria and chloroplasts) are inherited by the genetic rule. In the second group of organelles the endoplasmic reticulum is regarded the autonomous donor for other compartments (endoplasmic reticulum, Golgi, lysosomes, vacuoles, endosomes, secretory granules, and plasma membrane). Earlier peroxisomes were considered to originate from endoplasmic reticulum (Novikoff and Novikoff 1972), but this concept changed to peroxisomes being autonomous organelles (Lazarow and Fujiki 1985; Subramani 1988). The recent view of how peroxisomes are formed and maintained in the cell returned to the original concept, and instead of being autonomous organelles, peroxisomes are formed from the endoplasmic reticulum and are part of the endomembrane system (Motley et al. 1994; Tabak et al. 2006). It was published earlier that glycosomes of plants also originate from the endoplasmic reticulum (Gonzalez 1986).

As peroxisomes have no DNA, all peroxisomal proteins are encoded by the nuclear DNA, synthesized in free ribosomes and imported from the cytosol into the peroxisomes (Rachubinski and Subramani 1995). Evidence was presented for peroxisome proteins (Pex3p, Pex13p, Pex14p, Pex16p) targeting and entering the endoplasmic reticulum after their synthesis on free ribosomes in the cytoplasm. Pex3p is the major peroxisomal membrane protein that is essential for peroxisome biogenesis, involved in the recruitment and stabilization of other proteins. Although, Pex3p contains a stretch of positively charged amino acids at the N terminal, it lacks a real signal sequence, consequently it is not known how the import of peroxisomes

is achieved (Baerends et al. 2000). Nevertheless, peroxisomal targeting signals are necessary and sufficient for targeting peroxisomes. In contrast to mitochondria and chloroplasts, peroxisomes are able to translocate not only monomeric but also oligomeric proteins across their membranes. This mechanism may utilize the association of targeting signals to polypeptide chains that do not contain such targeting signals (Glover et al. 1994; McNew and Goodman 1994). This could mean that cells have developed unique solutions to the problem of moving proteins from their initial site of synthesis on cytoplasmic polysomes to their final destination inside the peroxisome (Rachubinski and Subramani 1995).

2.13.4 Targeting to Mitochondria

According to the endosymbiontic hypothesis mitochondria are supposed to have originated from engulfed α-purple bacteria (Gray 1992). The two mitochondrial membranes divide these organelles into two aqueous compartments. The outer one separates the mitochondrion from its cytosol. The intramitochondrial compartment is the intermembrane space. The inner invaginated membrane separates the intermembrane space from the inner space defined as matrix. The inner membrane invaginations known as cristae harbor the enzymes of terminal oxidation and the coupled oxidative phosphorylation. Other energetically closely related oxidative processes also taking place in the mitochondrial matrix are central pathways of metabolism, such as the citrate cycle and the ß-oxidation of fatty acids. Mitochondria release amino acids (Alberti and Bartley 1963), and are essential sites of steroid hormone biosynthesis by the cholesterol side-chain cleavage enzyme P450scc, converting cholesterol to pregnenolone, a determining step of steroidogenic capacity in mitochondria (Miller 2013), leading to the formation of hem-ring starting with the condensation of glycine and succinyl-CoA originating from the citrate cycle. The small genome of mitochondria encodes only a limited number of proteins involved as subunits in the five respiratory enzyme complexes and in the composition of ATP synthase. Proteomic analyses estimated that mitochondria contain about 1500 proteins in mammals and ~1000 proteins in yeast (Reinders et al. 2006; Pagliarini et al. 2008; Pagliarini and Rutter 2013). About 700–800 mitochondrial proteins have already been annotated. The expected number of mammalian mitochondrial proteins has been estimated to be in the range of 1500–2000 proteins (Prokisch and Ahting 2007). The human mitochondrial DNA was reported to contain only 37 genes, coding for two rRNAs, 22 tRNAs and 13 polypeptides (Taanman 1999). Consequently, ~97–98 % of mitochondrial proteins are encoded by nuclear genes, synthesized on cytosolic ribosomes and then transported into the mitochondria. Cytosolic ribosomes that translate mRNAs to mitochondrial precursor proteins have been found in close proximity to the outer mitochondrial membrane (Kellems et al. 1975; George et al. 2002; MacKenzie and Payne 2004). A new perspective of pre-endosymbiontic hypothesis suggests that the majority of the mitochondrial proteome is of endogenous origin and evolved mitochondria into energy-generating

organelles, while the minority of genes are of exogenous origin and developed into protein import system and various ion and small-molecule transporters (Gray 2014).

Targeting to mitochondria involves proteins that carry an N-terminal presequence composed of charged and hydrophobic amino acid residues forming an amphipathic helix that facilitates protein translocation across mitochondrial membranes and targets the protein to the mitochondrial matrix. Additional presequences are necessary to secure sub compartmental localization. Proteins located in the intermembrane space are first directed to the matrix, then with their intermembrane-space-targeting sequence to the intermembrane space (Fig. 2.18). Outer membrane proteins possess the N-terminal presequence, and another one that localizes proteins in the outer membrane. The presequences of outer membrane proteins may or may not be cleaved off.

Protein Integration in the Outer Membrane As far as the mechanisms of protein integration are concerned proteins may get into the outer membrane (Dudek et al. 2013) by:

- insertion of α-helical proteins without the help of translocases
- insertion with α-helical membrane anchor
- several closely cooperating translocases

Most of the mitochondrial proteins are transported into the intermembrane space of mitochondria *via* the translocase of the outer membrane. Among the multiple pathways for sorting mitochondrial precursor proteins the sorting and assembly machinery (SAM) protein complex operates after the action of translocase of the outer membrane and mediates the insertion of beta barrel proteins into the outer mitochondrial membrane (Bolender et al. 2008). Beta barrels have been defined as large beta-pleated sheets arranged in an antiparallel fashion that twist and coiled to form open and closed structures. Sixteen- to eighteen-stranded beta barrel structures are typically forming porins and function as transporters for small molecules and ions that would not be able to diffuse through the cellular membrane. Beta barrel proteins are found in the outer membranes of endosymbiontic organelles of prokaryotic origin. Barrel structured porins are present in the outer membrane of mitochondria, in Gram-negative bacteria and in chloroplasts (Schleiff and Soll 2005; Dolezal et al. 2006). Beta barrel proteins of the outer mitochondrial membrane that form the porins are import channels. The other type of transmembrane proteins, namely the alpha-helical proteins are present in the inner membranes of bacterial cells, plasma membrane of eukaryotes and occasionally in the outer membranes. Among the proteins of the outer membrane are **t**ranslocases of the **o**uter mitochondrial **m**embrane such as the Tom40, the sorting assembly machinery 50 kDa subunit (SAM50), and **m**itochondrial **d**istribution and **m**orphology protein 10 (Mdm10). Mdm is not to be mixed up with **m**ouse **d**ouble **m**inute (Mdm) oncogene proteins. Porins are the most important translocases in the outer mitochondrial membrane.

Protein Import Across the Outer Membrane This process is executed with the help of protein translocases mediating the transport of precursor proteins carrying various import signals to the outer membrane and distributing these signals to down-

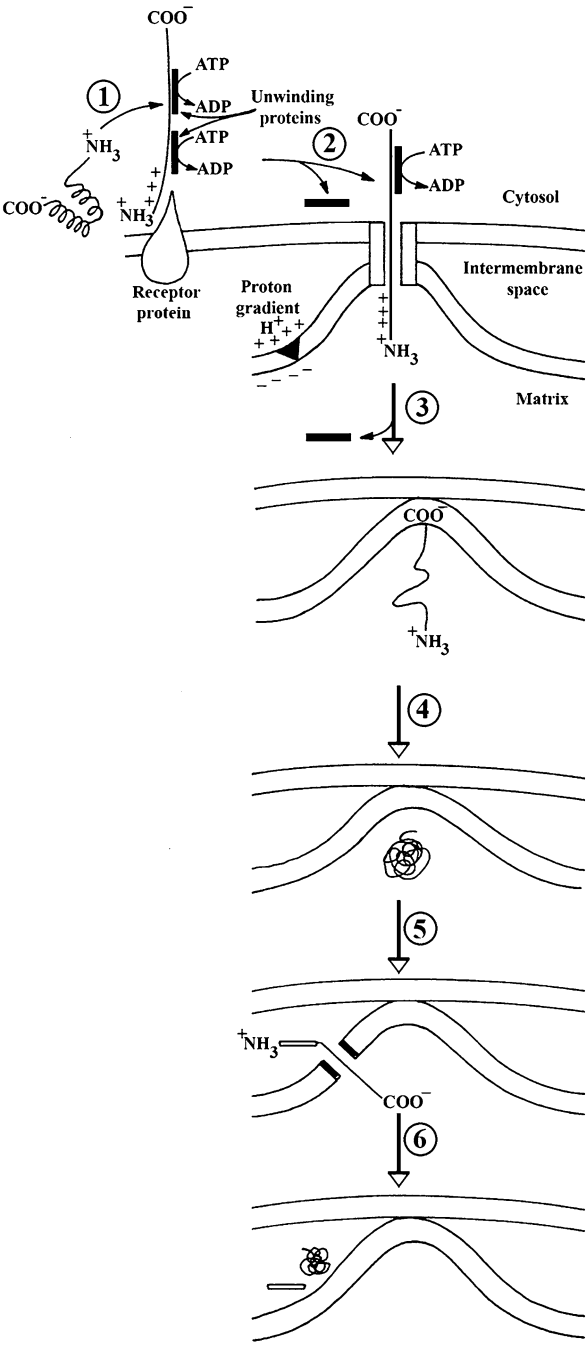


Fig. 2.18 Translocation of mitochondrial proteins. Proteins synthesized on free ribosomes do not contain endoplasmic target sequences. The direction of proteins into the matrix and intermembrane space is supported by N-terminal presequences. Steps involved in the translocations: *1* Energy-dependent (ATP) protein unfolding is aided by unwinding proteins. *2* Unfolded protein starts to cross the channel spanning the outer and inner mitochondrial membranes. N-terminal matrix presequence is cleaved off. *3* Target protein in the matrix begins to refold. *4* Protein targeted to matrix refolds into native conformation. *5* Proteins targeted to the intramembrane space use their second N-terminal presequence to cross the hydrophobic inner membrane channel and move to the intermembrane space. *6* The N-terminal presequence of preprotein is removed, protein targeting is completed

stream protein sorting complexes. The preprotein translocase containing a porin channel of the outer membrane of mitochondria (TOM complex) is a multi-subunit complex required for the recognition and membrane translocation of nuclear-encoded mitochondrial preproteins (Brix and Pfanner 1997; Künkele et al. 1998). The import machineries are in close connection with each other and other systems that function in the respiratory chain, in mitochondrial membrane organization, protein quality control and endoplasmic reticulum-mitochondrial junctions. It was thus proposed that the mitochondrial protein import should be regarded a network of cooperating machineries responsible for major mitochondrial functions, rather than independent pathways (Becker et al. 2012). The import of precursor proteins into mitochondria generally occurs in a post-translational manner.

Matrix Translocation of Preproteins The inner membrane creates the region between the inner and outer membrane, called the intermembrane space, and the more sequestered space inside the inner membrane, called matrix. The two major sites of the intermembrane space are the intercrystal and the peripheral spaces. The matrix contains small organic molecules and soluble proteins primarily membranes that catalyze oxidation. The mitochondrial matrix also contains the mitochondrial DNA and ribosomes.

Most of the studies related to mitochondrial preproteins were carried out first in yeast then confirmed in other organisms. Mitochondrial preproteins carry N-terminal, cleavable presequences that target these proteins into the mitochondrial inner membrane or matrix (Vögtle et al. 2009). Components of translocases of the inner mitochondrial membrane are protein complexes that facilitate the translocation of proteins into the matrix and insertion in the inner membrane and then function as mitochondrial carrier proteins. The import supported by translocases of the inner membrane complexes recruit mitochondrial heat shock protein 70 that makes the translocation of the precursor protein possible through ATP hydrolysis (Gabriel et al. 2003). Upon entry of preprotein into the matrix, the N-terminal presequence is cleaved off by the matrix processing signal peptidase and the inserted protein aided by heat shock protein 60 is folded into its active conformation (Liu et al. 2001). Sequence specific conformational changes in the ATPase domain and peptide binding of mitochondrial heat shock protein 70 provide the basis for the translocation of proteins into the matrix (Kang et al. 1990; Ungermann et al. 1994; Voisine et al. 1999; Liu et al. 2003; Mapa et al. 2010). The translocation of proteins into the mitochondrial matrix and intermembrane space is schematically shown in Fig. 2.18.

2.13.5 Targeting the Endomembrane System

Before the discussion of protein targeting *via* the endomembrane system, spatially distinct ribosomes have to be distinguished. Depending on their location ribosomes can be divided into two general groups based upon whether or not a signal peptide sequence of the produced protein is encoded at their N-terminal end. Proteins that

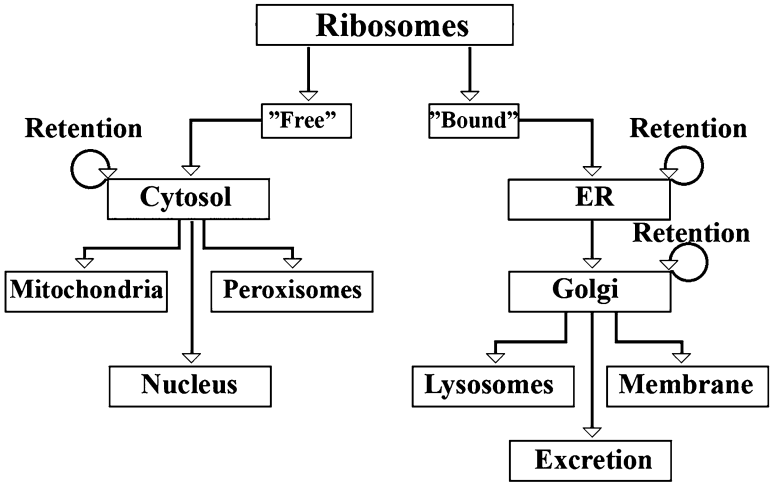


Fig. 2.19 Protein targeting in the absence and presence of signal peptides. Proteins synthesized on "free" ribosomes are either retained in the cytosol, or targeted to the nucleus, mitochondria or peroxisomes. Proteins synthesized on ribosomes "bound" to the endoplasmic reticulum (ER) are: (**a**) retained in the ER, (**b**) further processed and the retained in the Golgi, (**c**) transported to the lysosomes for degradation, (**d**) imbedded in the plasma membrane or (**e**) excreted from the cell

contain no signal peptide are translated on "free" ribosomes and may remain in the cytosol, are transported to the nucleus, peroxisomes, mitochondria or chloroplasts. Proteins carrying an N-terminal signal peptide upon translation on ER-attached ribosomes ("bound" ribosomes) either stay in the endoplasmic reticulum, in the Golgi or vacuoles, or are secreted to the plasma membrane, cell wall or extracellular matrix (Fig. 2.19). Household proteins of the cytosol are lacking any sorting amino acid sequence.

Targeting to Endoplasmic Reticulum To overview targeting to the endomembrane system it is logical to begin with the transport of proteins from the cytosol to the endoplasmic reticulum. There are two major groups of proteins that are targeted to the endoplasmic reticulum:

- Soluble proteins translocated into the lumen of the endoplasmic reticulum are not inserted in the membrane.
- Proteins inserted into membranes and only partially translocated into the endoplasmic reticulum. Beside the endoplasmic reticulum the destination of these proteins is the plasma membrane or another organelle.

After protein synthesis on "bound" ribosomes the N-terminal end of the ~20 amino acid long signal sequence consisting mainly of hydrophobic amino acids combines with the signal recognition particle, crosses the membrane and enters the endoplasmic reticulum. Protein targeting to the endoplasmic reticulum and the ribosome cycle are summarized in Fig. 2.20.

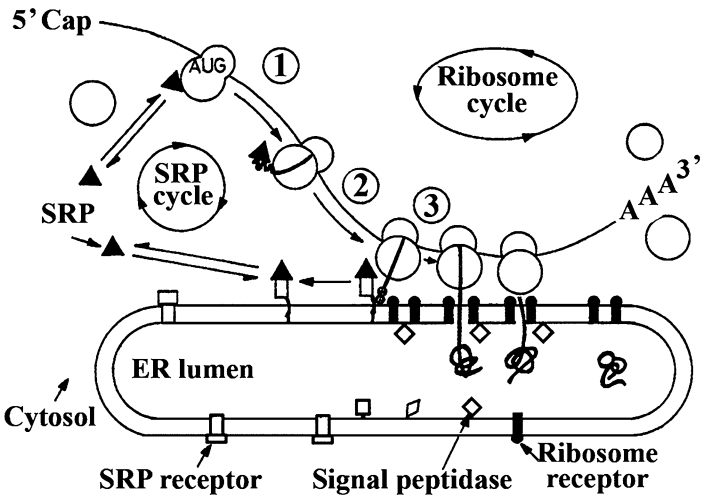


Fig. 2.20 Transport of proteins through the membrane of the endoplasmic reticulum. *1* The signal recognition particle (SRP) is bound to the signal-transfer complex containing the protein being synthesized and to the ribosome. *2* The SRP-ribosome complex moves to the endoplasmic reticulum and is anchored to the SRP receptor. *3* The ribosome binds to its endoplasmic receptor (riboforin channel) and the nascent protein enters the endoplasmic reticulum through the channel

Protein Targeting to the Golgi Apparatus The proteins and lipids of the Golgi apparatus originate in the endoplasmic reticulum. Cargo proteins and lipids from the endoplasmic reticulum are processed and sorted within the Golgi apparatus. The Golgi apparatus is the central organelle of secretory and protein sorting pathways. Of the two basic vesicular trafficking mechanisms that contribute to the secretory pathway of eukaryotic cells:

– the constitutive secretion delivers proteins to the cell surface through continuous exocytosis
– the regulated pathway responds to extracellular signals in order to trigger occasionally the release of vesicular content.

Most of the proteins and accompanying lipids of the Golgi apparatus cycle continuously between the Golgi and the endoplasmic reticulum (Storrie 2005). *De novo* synthesized proteins arriving from the endoplasmic reticulum to the Golgi are destined for secretion from the cell, follow the endocytic branches of the secretory pathway, to the plasma membrane (Banfield 2011). Resident Golgi proteins have localization signals to be targeted to the Golgi compartment and not swept further along the secretory pathway. There is a number of distinct groups of detained Golgi membrane proteins, including glycosyltransferases, recycling trans-Golgi network proteins, peripheral membrane proteins, receptors and viral glycoproteins (Gleeson et al. 1994). Beside Golgi retention oligomerization by glycosyltransferases, lipid-mediated sorting and intra-Golgi retrograde transport have been reported (Gleeson 1998). Resident proteins of the endoplasmic reticulum that have overridden their

retention mechanisms and appear in the Golgi are returned to the endoplasmic reticulum by retrograde transport to the endoplasmic reticulum (Glick and Nakano 2009; Jackson 2009). The retention process in the Golgi takes place through repeated cycles of forward and backward directions known as anterograde and retrograde transport.

In both secretory and protein sorting pathways there are components that are thought to support common mechanisms and serve analogous functions in vesicle budding, targeting and fusion between the endoplasmic reticulum and Golgi apparatus. Syntaxins are a family of vesicular transport proteins involved in endomembrane traffic. Particularly syntaxin 5 (human), a member of the syntaxin or t-SNARE (target-SNAP receptor) an acronym derived from "SNAP" (Soluble NSF Attachment Protein) family encoded protein that is known to regulate the movement from the endoplasmic reticulum to Golgi transport (Dascher et al. 1994). Soluble factors, N-ethylmaleimide sensitive fusion protein (NSF) and SNAPs are required in many membrane fusion events within the cell to interact with a class of type II integral membrane proteins termed SNAP receptors, or SNAREs. Interaction between cognate SNAREs on opposing membranes is a prerequisite for NSF dependent membrane fusion in the Golgi (Nichols and Pelham 1998).

In *Arabidopsis thaliana* plant cells the specific vesicle fusion during vesicular transport is also mediated by membrane-associated proteins called SNAREs (soluble N-ethyl-maleimide sensitive factor attachment protein receptors). The multiple localization patterns in different organelles, suggested that SNAREs shuttle between the organelles indicating the complexity of the post-Golgi membrane traffic in higher plant cells (Uemura et al. 2004).

Protein Targeting to Lysosomes The membrane, the endosomal fusion and enzymes of lysosomes have been described in Chap. 1, and the lysosomal proton pump viewed in Fig. 1.25. Here we describe the transport of a variety of soluble acid hydrolases and transmembrane proteins from the endoplasmic reticulum to Golgi apparatus and from trans-Golgi network to different destinations. Most of the targeting from trans-Golgi upon budding in the degradative compartment, is a unidirectional process. Budding vesicles wear coats consisting of triskelion units. Some of the transmembrane proteins, particularly acid hydrolases are retrieved to the trans-Golgi. The bidirectional transport involves vesicular intermediates into which cargo molecules are packaged selectively (Rouillé et al. 2000).

The anterograde, retrograde and bi-directional transport processes between the secretory and the endocytic pathway have been described in terms of protein-protein interactions, allowing the selective sorting of membrane proteins (Rouillé et al. 2000). Newly synthesized proteins are sorted at the trans-Golgi network and subsequently delivered to the endosomal/lysosomal system. These proteins including acid hydrolases are bound to two related transmembrane proteins in mammalian cells *via* a common mannose 6-phosphate recognition receptor (Kornfeld 1992; Ludwig et al. 1995). This receptor protein along with other proteins are in dynamic equilibrium with the trans-Golgi, endosomes and plasma membrane and generate multiple rounds of protein sorting (Mellman 1996). From the trans-Golgi mannose

6-phosphate receptors direct acid hydrolases to endosomes where upon dissociation from their receptors reach lysosomes (Rouillé et al. 2000). Receptors retrieved from endosomes for recycling return to trans-Golgi. Other transmembrane lysosomal proteins such as glycoproteins sorted in the trans-Golgi are not recycled (Mellman 1996; Honing and Hunziker 1995).

Nascent proteins in the endoplasmic reticulum transported to the Golgi complex, can be either targeted directly to endosomes, or first to the cell surface from where they can be rapidly internalized into the endocytic pathway for delivery to lysosomes specified by sorting motifs in the cytoplasmic tails of the proteins that are recognized at the trans-Golgi or plasma membrane (Sandoval and Bakke 1994). The clathrin triskelion has three kinked 'legs' that radiate from a central vertex. Clathrin is involved in coating membranes that are endocytosed from the plasma membrane and those that move between the *trans*-Golgi network (TGN) and endosomes (Kirchhausen 2000).

Major pathways of protein targeting from endoplasmic reticulum including intracellular sorting and extracellular exocytosis as well as endocytotic pathways are summarized in Fig. 2.21. The best studied pathway is endocytosis leading from coated pits to endosomes and further to lysosomes. The biosynthesis of lysosomal hydrolases and proteins taking place in ribosomes on endoplasmic reticulum is followed by their transport to the Golgi. These proteins are delivered from the trans-Golgi to the endosome then to the lysosomes. Lysosomes obtain material in a mannose 6-phosphate receptor mediated manner. Receptor proteins bind lysosomal enzymes and concentrate them in clathrin-coated vesicles then bud from the trans-Golgi network. These receptors carry lysosomal enzymes into endosomes and lysosomes. Mannose 6-phosphate receptors shuttle back and forth between endosomal and Golgi membranes.

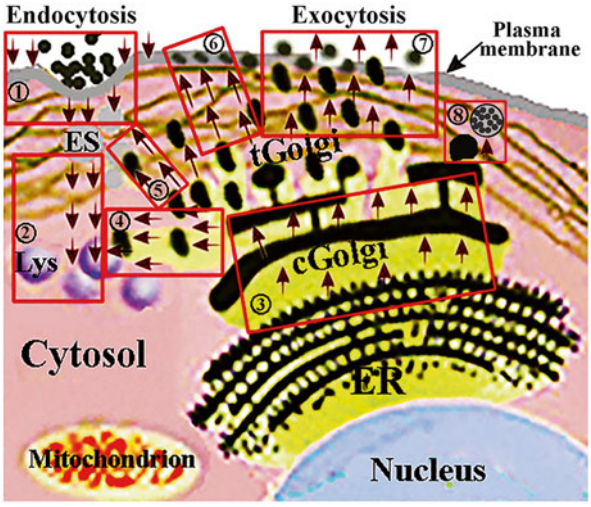


Fig. 2.21 Protein targeting processes related to the endomembrane system. Endocytosis: *1* Foreign substances targeted to endosomes (ES). *2* Endosomal → lysosomal (Lys) targeting. *3* Endoplasmic reticulum → Golgi targeting. Protein sorting: *4* transGolgi (tGolgi) → lysosomes, *5* tGolgi → endosomes, *6* tGolgi → plasma membrane (integral membrane proteins). Exocytosis: *7* tGolgi → protein secretion, *8* tGolgi → storage vesicle

Transport vesicles that leave the trans-Golgi network can immediately fuse with the plasma membrane in a continuous manner. The transport of lipids and intramembrane proteins characteristic to all cells secures the constant supply and renewal of plasma membrane. Soluble proteins carried by vesicles are secreted by the cells and produce the proteoglycan and extracellular matrix of cells. This constitutive membrane related transport of vesicles is opposed by a second and more specialized secretory pathway in which soluble components are released in a regulated manner. In this second, so called regulated secretory pathway proteins are modified and released as secretory vesicles that are stored until extracellular signal initiates secretion. The membranes of secretory vesicles is recycled after fusion with the apical side of cells (primarily epithelial cells) in a body tube or cavity. The apical side of the membrane faces the lumen with special features like cilia or microvilli of the brush border. The basolateral side of the membrane faces adjacent cells and the underlying connective tissue. As epithelial cells can also secrete proteins at their basolateral side it is likely that they either have distinct sorting signals or only one pathway requires the sorting signal and the other operates by default.

Viruses are known to exploit the sorting capacity of the host cell for their own benefit and contribute to the diversification of the intracellular transport. Enveloped animal viruses are enclosed in a lipid bilayer and mimic secretory vesicles. The enveloped RNA Semliki virus infection starts with the binding of virus to its receptor protein(s) on the plasma membrane of the host cell. By using the host's pathway the virus is delivered *via* receptor mediated endocytosis to the endosome, but escapes being delivered to lysosomes by one of the envelope proteins of the virus. The viral envelope fuses with the endosome membrane, but the nucleocapsid is released into the cytosol. After uncoating, the viral-RNA is translated to viral coded RNA polymerase that produces several copies of + RNA strands serving as template for the synthesis of viral structural proteins, capsid and viral envelope proteins. Capsid proteins bind to newly replicated viral RNA to form nucleocapsids. Envelope proteins are inserted in endoplasmic reticulum, transported to the Golgi apparatus for oligosaccharide modification and then transported to the plasma membrane. The nucleocapsids and envelope proteins are wrapped in plasma membrane and the free virus is released by the cell, and ready to start a new cycle of viral infection (Simons et al. 1982; Simons and Warren 1983).

2.13.6 Vesicular Transport in Protein Targeting

Vesicular transport is a major cellular activity, responsible for molecular traffic between a variety of specific membrane-enclosed compartments. Transport vesicles are referred to as vesicles carrying lipids and protein from the endoplasmic reticulum to Golgi apparatus and from Golgi to other destinations. Lipids and proteins are shuttled between compartments *via* small membrane-bound structures known as transport vesicles. Vesicles are formed by each compartment separately and fuse with the next compartment. Vesicular transport is not to be confused with the

vesicular transporter or vesicular transport protein that regulates, normally facilitates the movement of specific substrate molecules across the vesicular membrane. Distinction has also to be made between transport vesicles and secretory vesicles. Specialized endocrine, exocrine and neuronal cells were classified as secretory cells. Secretory vesicles can be distinguished based on their type of secretion mechanism: "constitutive" vesicles secrete their products as soon as they are produced, "storage" vesicles accumulate their products in the cytoplasm before they are released by the cell.

Transport vesicles are normally mentioned among those vesicles that transport material from the endoplasmic reticulum to the Golgi apparatus or from one part of the Golgi to another one. The transport of newly synthesized proteins from the endoplasmic reticulum to the Golgi apparatus starts with the clustering of tubular vesicles. After the budding of coated vesicles from the endoplasmic reticulum, they shed their coat and fuse with each other. The fusion of membrane originating from the same compartment is called *homotypic* fusion. In *heterotypic* fusion SNARE (Soluble NSF Attachment Receptor) transmembrane proteins present in organellar membranes and vesicles derived from them are involved. SNAREs exist as v-SNARE in the vesicle membrane that binds specifically to the complementary t-SNARE in the target membrane.

2.14 Bacterial Membranes

Similarly to the cell membrane of eukaryotic cells the bacterial cell membrane is a highly selective barrier preventing materials from simply diffusing into and out of the cell. It allows the entry of nutrient molecules needed for survival, but prevents the influx of vital components and noxious compounds from the environment. Although, the bacterial cell membrane is also known as a simple phospholipid bilayer, its surface is not smooth, with bulging proteins, sugars and complex transporter and transduction complexes. Additional proteins are anchoring points for extracellular extrusions such as flagella and pili. The embedded proteins contribute to about 60 % and reduce the lipid content to ~40 %. The major difference between eukaryotic and bacterial cell membranes is that the bacterial membranes normally do not contain sterols. The cell membrane of *Archaea* is functionally equivalent to the bacterial membrane, but their saturated and branched fatty acids are bound to the glycerine through ether linkages rather than through ester bonds. The presence of ether bonds of *Archaea* can be explained by their high resistance to extreme external conditions. The function of prokaryotic cell membrane can be summarized as:

(a) permeability barrier,
(b) transport of specific substances, primarily ions and nutrients,
(c) energy (ATP) production *via* oxidative and photophosphorylation using the proton motive force generated between the outer and inner membranes,

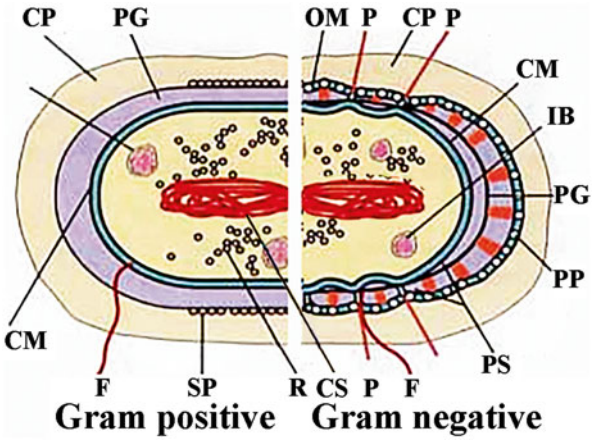


Fig. 2.22 Major differences in the composition of membranes of Gram positive and Gram negative bacteria. Abbreviations: *CP* capsule, *PG* peptidoglycan layer, *OM* outer membrane, *P* pilus, *CM* cytoplasmic membrane, *IB* inclusion body, *PP* porin proteins, *PS* periplasmic space, *SP* surface proteins, *R* ribosome, *CS* chromosome, *F* flagellum. Not all structures are present in all bacteria (Modified with the permission of Denis Kunkel, Medical Microbiology http://micro.digitalproteus.com/morphology2.php)

(d) membrane lipid and liposaccharide (in Gram-negative bacteria) biosynthesis,
(e) peptidoglycan cell wall synthesis,
(f) secretion of proteins,
(g) coordination of DNA replication, segregation and cell division. Mesosomes as coiled cytoplasmic membranes were supposed to bind and pull apart daughter chromosomes during cell division. Although, a few researchers continue to believe in mesosomes, but cryofixation and electron microscopy studies disproved their existence,
(h) movements directed by chemicals of the environment (chemotaxis),
(i) sites of specialized enzyme system.

Several pathways evolved that enable protein transfer across the outer membrane, to periplasm, and to the inner membrane.

Outer Bacterial Membranes Bacteria are protected against their environment by the lipopolysaccharide-phospholipid asymmetric bilayer of the outer membrane. Major differences in the composition of membranes of Gram positive and Gram negative bacteria are shown in Fig. 2.22. The cell wall of Gram-negative bacteria is composed of the outer membrane, the peptidoglycan layer and the periplasm between the outer and inner membrane. Roughly half of the outer membrane consists of protein in the form of integral membrane proteins, or as lipoproteins anchored to the membrane by N-terminally attached lipids (Blattner et al. 1997). Unlike other membrane proteins integral outer membrane proteins do not contain transmembrane alpha-helices, but are folded into antiparallel beta-pleated barrels.

Of the two structural motifs, alpha-helical bundles are most often found in the receptors and ion channels of plasma and endoplasmic reticulum membranes. Beta-barrels are restricted to the outer membrane of Gram-negative bacteria and to the mitochondrial membrane, and represent the structural motifs used by several microbial toxins to form cytotoxic transmembrane channels (Galdiero et al. 2007).

Gram-positive bacterial proteins are generally targeted across the plasma membrane and attached to the bacterial cell wall. The signal recognizing sortase enzyme cleaves off the C-terminal end of the target protein and transfers the protein onto the cell wall. Similar analogous so called exosortase systems exist also in Gram-negative bacteria, and as archaeosortase A system in *Archaea* releasing proteases, nucleases and other enzymes related to bacterial secretion.

Due to the explosion of information the discussion of porins has to be limited to selective permeability eliminating other functions of the outer bacterial membrane (Nikaido 2003). By accepting this concept, only the following membrane protein families related to membrane transport are mentioned: the OmpA and OmpX membrane proteins, phospholipase A, general porins (OmpF, PhoE), substrate specific porins (LamB, ScrY) and the TonB-dependent siderophore transporters utilizing ion translocation (FhuA, FepA) (Koebnik et al. 2000).

Inner Bacterial Membrane The major route of protein translocation in and across the cytoplasmic membrane is the general secretion pathway (Sec-pathway). This secretory (Sec) pathway serves the translocation of secretory proteins across the membrane and the integration of proteins into the cytoplasmic membrane. Newly synthesized proteins in the cytoplasm are immediately targeted to the Sec complex by the signal recognition particle or by the tetrameric SecB chaperon after translation. The trigger factor involved in protein export acts as a chaperon by maintaining the newly synthesized protein in open conformation and competes with the signal recognition particle for the binding of the nascent protein (Facey and Kuhn 2010). The insertion of bacterial membrane proteins into the inner membrane takes place by the Sec translocase system. The complex consists of the SecYEG protein-transducing channel and the SecA ATP-ase engine. The signal peptidase cleaves the signal sequence off from the preprotein at the outer side of the inner membrane (Facey and Kuhn 2010). The insertion is contributed and facilitated by the YidC insertase and phosphatidylethanolamine (Dalbey et al. 2011). The formation of α-helical bundles of membrane proteins was reported in the inner membrane during and after insertion. Chaperons may function as assembly factors in oligomer formation, but most of the subunits building homo- and hetero-oligomeric membrane complexes are folded and oligomerized spontaneously (Dalbey et al. 2011).

2.15 Chloroplast Membranes

Chloroplasts contain the most complex type of plastids, and unlike mitochondria have not only two but three compartments: (a) the intermembrane space, (b) the stroma and (c) the thylakoid lumen within the stromal and granular thylakoids. The

plastid genome encodes ~100 proteins (Sugiura 1989). The overwhelming majority of plastid proteins is encoded by nuclear genes and synthesized as precursors in the cytoplasm. Specific protein transport mechanisms direct these precursor proteins to their proper location within chloroplasts. Chloroplasts have three phospholipid bilayers: two membranes of the envelope (outer and inner membrane, with a ~10–20 nm intramembrane gap) and the compartmental membrane. The compartmental membrane is forming the inner thylakoid space of the chloroplasts and cyanobacteria. The thylakoid of chloroplasts may generate stacks of discs known as grana. Additional membranes serving secondary endosymbiosis may enclose chloroplasts in the best known groups of flagellates and in other small groups of photosynthesizing algae present in tropical oceans known for ingesting bacteria and protists (Kim and Archibald 2009). The outer lipid bilayer of the envelope membrane has a higher phospholipid content and is permeable to ions and metabolites, the inner membrane contains more galactolipids the selectivity of which is determined by specialized transport proteins (Heldt and Sauer 1971; Inoue 2007).

2.15.1 Protein Targeting to Chloroplasts

The vast majority of chloroplast proteins are encoded by the cellular DNA in the nucleus and synthesized on free, cytosolic ribosomes in precursor forms. Precursors containing an amino-terminal extension called recognition signals or transit peptides, direct the protein by the post-translational targeting pathway (Jarvis 2008). The targeting process directs proteins towards the chloroplast, usually using signals contained within the protein. Imported proteins are synthesized as cytosolic precursors containing uptake-targeting sequences that direct each protein to its correct subcompartment the recognition signal of which is removed upon arrival inside the organelle. Thousands of proteins are known to serve photosynthesis, expression of the chloroplast genome, and other processes. Similarly to mitochondria most of the chloroplast proteins are synthesized in the cytoplasm of the plant cell, then imported and targeted to the chloroplast compartment. The rest is encoded by the genome of the chloroplast, synthesized within the organelle, and targeted by mechanisms that are poorly understood. Preproteins may contain stromal import, stromal or thylakoid targeting sequences. For targeting to the chloroplast, proteins contain sequences, called transit peptides, which contain 40–50 amino acids at the N terminus. After translocation into the stroma, the transit peptide is enzymatically removed. Chloroplasts are composed of more multiple subcompartments than mitochondria. Proteins destined for the thylakoid lumen contain an additional targeting sequence, called a luminal targeting sequence, for correct protein sorting.

The protein import process is mediated by the coordinated action of two major multiprotein complexes of the envelope membranes known as the TOC (Schnell et al. 1997) and TIC complexes (Kessler and Blobel 1996; Lübeck et al. 1996). The stromal import sequence is also recognized, cleaved off and folded. The import of proteins from the cytosol into the chloroplasts consists of more than a dozen components located in and around the chloroplast envelope, including a pair of GTPase receptors, a β-barrel-type channel across the outer membrane, and an AAA^+-type (ATPases Associated with diverse cellular Activities) engine in the stroma (Li and Chiu 2010).

Known mechanisms such as posttranslational, cotranslational (particle-mediated signal recognition) and mRNA based mechanisms have been described for protein targeting of specific chloroplast proteins in the green alga *Chlamydomonas* (Uniacke and Zerges 2009). In the posttranslational mechanism, the import machinery of the organelle selects the correct proteins by virtue of their having a transit peptide or nuclear localization signal. In the cotranslational mechanism, the signal sequence of the nascent polypeptide binds to the signal recognition particle that represses further translation by docking the translational complex at the endoplasmic reticulum. The translation ends with insertion of polypeptide into the lumen of the endoplasmic reticulum membrane. In the third mRNA-based mechanism, the untranslated mRNA is localized by protein binding associated with target membrane, and translation is initiated only upon mRNA localization (Johnston 2005; Sanchirico et al. 1998). New studies using proteomics, revealed a significant number of chloroplast proteins transported *via* the pathway that involves the endoplasmic reticulum and Golgi apparatus (Jarvis 2008).

The targeting to primary plastids can be summarized as:

(i) most proteins targeted to primary plastids carry a transit peptide,
(ii) proteins to be targeted are transported post-translationally using Toc and Tic translocons,
(iii) most of the players of chloroplast protein import complex have been clarified.

Future research is likely to focus on functional and mechanistic aspects of individual components as well as to resolve conflicting ideas to get a complete picture of protein import in chloroplasts. However, several proteins with N-terminal signal peptides that are directed to higher plant plastids in vesicles are derived from the endomembrane system. These proteins inspired the hypothesis that all nuclear-encoded, plastid-targeted proteins initially carry signal peptides that are targeted to the ancestral primary plastid *via* the host endoplasmic reticulum (Gagat et al. 2013).

It is likely that RNA targeting by mRNA localization could be an efficient means of targeting gene products to specific intracellular regions not only in animal (Jansen 2001; Kloc et al. 2002; Van de Bor and Davis 2004), but also in plants cells (Fedoroff 2002; Okita and Choi 2002).

2.16 Measurement of Membrane Permeability

Overton, the pioneer in the field of membrane permeability classified some 500 chemical compounds, with respect to their ability to enter cells, by stating his conclusions in an essentially nonquantitative manner (Overton 1895, 1907). Later studies followed for a longer period of time the course of volumetric changes of cells exposed to solutions containing different solutes (Bärlund 1929).

Bärlund published curves showing the relative rates of penetration of individual substances which however, did not permit quantitative comparisons of the same cells under different conditions. The ideal method of defining cell permeability could be by means of constants expressing in numerical form the amount of material that would theoretically enter the given cell through unit surface in unit time with unit difference of concentration between the cell and its surroundings.

Scientist pursued the ideal method to define permeability by means of a constant that would express permeability of materials in a numerical form (Northrop 1927; Jacobs 1927; McCutcheon and Lucké 1928; Lucké et al. 1931). Here the difficulty emerged that obtaining true permeability constants for such substances by the usual method of osmotic volume changes is complicated by the simultaneous penetration of both the solute and solvent. In fact, the solvent and a penetrating solute influence each other's behavior in a complicated manner. To avoid the measurement of simultaneous penetration of solvent and solute Jacobs and Stewart focused on cases where the entrance of the solvent was so rapid in comparison with that of the solute that approximate osmotic equality between the cell and its surroundings was maintained. The volumes of cells were calculated from their diameters. The values of permeability constants were calculated in such a way that it indicated the number of moles of the substance in question that would theoretically enter the cell through 1 μm^2 of surface in one min with a concentration difference between the cell and its surroundings of 1 mol per liter (1 M). In other words the constant provided and approximate measure of the amount of substance that would have theoretically passed though the unit area of cell membrane in unit time with unit difference in concentration on the two sides of the membrane (Jacobs and Stewart 1932). These historical observations serve even today as a basis to develop assays for the measurement of permeability.

2.16.1 Cytotoxicity Assays

Those cytotoxic assays that do not distinguish between apoptosis and necrosis can be divided in two types:

- Plasma membrane permeability (radioactive and non-radioactive) tests measuring the leakiness of dying cells
- Colorimetric assays that measure the reduction in metabolic activity of mitochondria.

As far as the detection of apoptosis is concerned, different methods have been devised, among them:

- Staining of apoptotic cells
- TdT-mediated dUTP Nick-End Labeling (TUNEL assay)
- *In situ* end labeling (ISEL)
- DNA laddering analysis
- Annexin-V analysis
- Measurement of apoptosis related proteins (Western Blotting)
- Comet assay
- p53 protein analysis
- Caspase staining kits

Apoptotic protocols have been reviewed earlier (Banfalvi 2009). As this book deals with the permeability of cells, only the tests related to permeability will be detailed.

Assessment of Cell Viability – Dye Exclusion Test The most simple way to judge morphological and permeability changes of biological membranes is by exclusion of certain dyes or uptake and retention of others. The dye exclusion test is based on the principle that live cells have intact cell membranes that exclude certain dyes (e.g. trypan blue, eosin, propidium iodide, etc.), whereas dead cells can take up these dyes. One of the traditional methods is trypan blue staining where dead cells stain blue, while live cells exclude trypan blue. The trypan blue test cannot distinguish between necrotic and apoptotic cells. Due to the microscopic visualization of dead cells and to the simplicity and rapidity, this technique remained a standard methodology used in microscopy for cell counting and assessment of cell and tissue viability. General methods for the detection of cell death include trypan blue, propidium iodide and Hoechst staining. Cells are routinely counted manually with a hemocytometer. Cell viability should be at least 95 % for healthy log-phase cultures. In recent years, modern automated instrumentation has been introduced to supplement the traditional trypan blue technique with the efficiency and reproducibility of computer control, advanced imaging, and automated sample handling (Louis and Siegel 2011).

Beside dye exclusion, esterase activity, mitochondrial membrane potential, protocols for determining the viability of fixed cells either before or after fixation with amine-reactive dyes suitable for a range of excitation wavelengths have been used. Membrane-impermeable dead cell and live cell dyes as well as dye-exclusion procedures for microscopy are among the preferentially applied methods (Johnson et al. 2013). High performance chromatography (HPC) measurement of viable staining with acridine orange and propidium iodide turned out to be superior to the conventional viability measurement by trypan blue staining (Mascotti et al. 2000).

Membrane Permeability Test Using Fluorescent Dyes A reliable and rapid test to detect toxic chemicals affecting cell membranes uses fluorescent dyes. Fluorescein diacetate enters intact cells freely where it is hydrolyzed to the green fluorochrome

fluorescein. To the contrary, ethidium bromide is excluded from healthy cells and is staining only the nucleic acids of those cells the membrane of which is damaged. The combination of these two dyes results in green cytoplasmic fluorescence of intact cells and red fluorescence of nucleus in membrane-damaged cells. Concentration-dependent effects of various detergents and solvents were quantified by flow cytometry measuring the amount of dye retention (Aeschbacher et al. 1986). A similarly rapid and simultaneous double-staining procedure using fluorescein diacetate and propidium iodide was described to discriminate between live and non-viable cells (Jones and Senft 1985; Yu et al. 1995).

Bioluminescence Permeable Cell Assay Healthy cells are impermeable to ATP but upon permeabilization the glucose used to synthesize and accumulate ATP without further growth can be measured by the intensity of bioluminescence. Cellular ATP biosynthetic activity was calculated from the slope of linearly increasing bioluminescence. This permeable cell assay was then applied as a Permeable Cell Assay to high-throughput measuring the dynamic cellular activity of glycolytic ATP synthesis (Hara and Mori 2006). To measure cellular ATP synthetic activity, combination of osmotic shock and detergent, Triton X-100 treatment was used to make bacterial cells permeable. ATP discharged from permeable cells reacted with external luciferase. Cellular ATP biosynthetic activity was calculated from the slope of linearly increasing bioluminescence (Hara 2009).

Measuring the Relative Cell Permeability of Synthetic Compounds by Conjugation to Dexamethasone Derivative To measure the relative cell permeability of different chemical compounds these molecules are conjugated to a dexamethasone derivative. The entry of the conjugate into the living cells triggers the nuclear transport through the Gal4 DNS binding domain of the glucocorticoid receptor fusion protein and subsequently the activation of Gal4-responsive luciferase reporter gene. The relative cell permeability is thus quantitatively traced back to the level of luciferase expression (Yu et al. 2007).

Cell Permeability Assays to Measure Drug Absorption Caco-2 cell monolayers were cultured on semipermeable plastic supports fitted into the wells of multi-well culture plates. Test compounds were added and after incubation for various lengths of time, aliquots were removed for the determination of the concentration of test compounds and rates of permeability for each compound, called the apparent permeability coefficients. The osmotic reflection coefficient (σ) accounts for restrictions of solute permeability based on the molecular size of a solute relative to the mean pore size for a permeability pathway. Larger molecules encounter greater resistance and are more frequently "reflected" in the membrane, having a higher value for σ, whereas small molecules are less frequently reflected, and therefore have a smaller value for σ. In most cases, estimates of σ are used based on the diffusion behavior of tracer-labeled solutes.

Originally radiolabelled compounds were used in Caco-2 cells monolayer assays that have been replaced by liquid chromatography-mass spectrometry and liquid

chromatography-tandem mass spectrometry (LC-MS-MS). Mass spectrometry eliminated the need for radiolabelled compounds, and permitted the simultaneous measurement of multiple compounds. The measurement of multiple compounds per assay reduced the number of incubations, thereby increasing the throughput of the experiments (van Breemen and Li 2005).

Plasma membrane water permeability method was developed to measure the osmotic permeability and applied to cells and epithelia expressing molecular water channels. This method is based on the integrated intensity of monochromatic light in a phase contrast or dark field microscope that is dependent on relative cell volume. For cells of different size and shape, the increased cell volume was associated with decreased signal intensity; generally the signal decreased 10–20 % for a two-fold increase in cell volume. The signal-to-noise ratio of the transmitted light detection permitted measurement of cell volume changes of <1 %. The method was applied to characterize transfected cells and tissues that express water channels (Farinas et al. 1997; Sehy et al. 2002).

Water channel diffusional permeability for the *Xenopus* oocyte, an important model system for water channel investigation, was typically calculated from intracellular water pre-exchange lifetime, cell volume, and cell surface area. It was debated whether or not intracellular motion affects water lifetime, thus the measured permeability was referred to as the apparent diffusional permeability. Magnetic resonance (MR) spectroscopy was used to measure oocyte water exchange with greater precision and higher signal-to-noise ratio than other methods. MR imaging served to assess both oocyte geometry and intracellular water diffusion for the same single cell. MR imaging confirmed the dependence of intracellular water lifetime on intracellular diffusion.

Hemolysis Indicator Hemolysis is the breakage of the membrane of red blood cells, causing the release of the hemoglobin and other components from the cells to the surrounding plasma (Lippi et al. 2008). Hemolysis can be induced by more than 50 causes, among them the most important ones: hemolytic anemia, hemoglobinopaties, drugs, infections, disseminated intravascular coagulation, transfusion reactions, etc. Hemolysis is an indicator of specimen condition and a source of error for a number of different serum and plasma assays. A growing number of clinical manifestations attributed to hemoglobin release in a variety of acquired and iatrogenic hemolytic disorders suggesting that hemolysis and hemoglobinemia should be considered a novel mechanism of generating human disease (Rother et al. 2005). Post collection hemolysis occurs when red cells are pressed through a small needle, contact with the clot for a longer period of time, when red cells are subjected to heating, freezing, or cell death. Several studies have shown that when blood is drawn from a peripheral catheter, a higher incidence of hemolysis occurs (Kennedy et al. 1996). The best solution against hemolysis is its prevention by the establishment of protective phlebotomy techniques, by education, training, validated procedures, minimal interference.

The upper reference limit for free hemoglobin is 2 mg/dL for plasma and 5 mg/dL for serum. Hemolysis is defined as the free hemoglobin concentration >30–50 mg/dL, conferring detectable pink/red color to serum or plasma. Serum free hemoglobin concentrations range between 0.0 g/L and 2.0 mg/L.

Hemolysis Index Cell-free hemoglobin was quantified by absorbance measurements on serum or plasma at different wavelengths, and the concentration of cell-free hemoglobin was finally accepted as the "hemolysis index" (Dolci and Panteghini 2014). Three types of indexes of hemolysis are generally accepted:

- traditional index of hemolysis (defined as grams of plasma free hemoglobin released per 100 L of blood pumped)
- normalized index of hemolysis (normalized by hematocrit) and
- modified index of hemolysis (taking into account not only hematocrit but also hemoglobin) (Naito et al. 1994).

The tested blood conditions are often not indicated in the reports. Even more disturbing is the fact that different systems and different assay parameters exist that make the measurements of hemolysis one of the biggest challenges in clinical laboratories. Efforts led to standardize the mean of hemolysis index. The standard hemoglobin assay utilizes the reference cyanmethemoglobin method using a spectrophotometer (Lippi et al. 2009). Other non-cyanide methods have also been developed for hemoglobin estimation (van Assendelft and Zijlstra 1989; Shah et al. 2011). In the quantitative copper sulphate test, capillary blood samples were used to fill the microcuvettes and the hemoglobin content was determined by a hemoglobinometer or automatic hematology analyzer calibrated according to the manufacturer's recommendations (Gómez-Simón et al. 2007). Due to the lack of standardization of the index of hemolysis more efforts should be invested into the standardization of the hemolysis index (Simundic et al. 2010).

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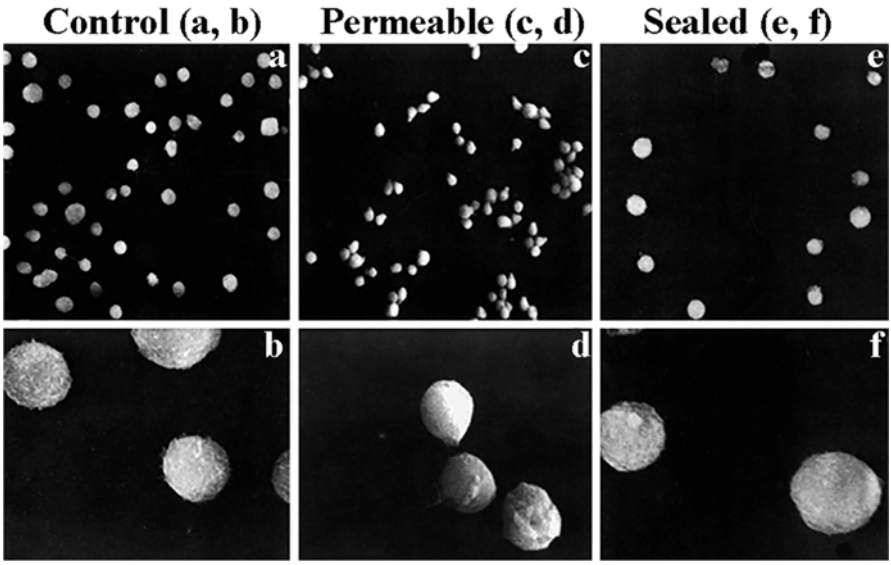
Chapter 3
Methods of Permeabilization

Contents

G. Banfalvi, *Permeability of Biological Membranes*,
DOI 10.1007/978-3-319-28098-1_3



Abstract Viable cells are capable of living, developing and reproducing themselves. Under experimental conditions the parameters of viability are diverse and measured by different viability tests, redox potential, integrity of cell membranes, cellular activities, and cellular enzymes. Each assay shows a different angle and may provide the basis of assays related to viability and cytotoxicity. Non-invasive treatment of cells to maintain viability have received wide attention and remained one of the major challenges facing reparative medicine. Biopreservation is one of the most important processes in maintaining the integrity and functionality of cells held outside the native environment for extended storage times. The importance of preservation is exemplified by the long-term storage of red blood cells (RBCs), critical to ensure safe blood supply for transfusion medicine (Scott et al, Transfus Med Rev 19:127–142, 2005). This chapter describes different cell manipulations that all aim to make cells permeable while maintaining not only the structural but also the functional integrity of cells. Unfortunately there is no unique, fast, reliable solution to permeabilization that could be of general use. The discussion of permeabilization methods will include the advantages and disadvantages of these methods.

Keywords Brij detergents • Channel formation • Channelopathies • Coelenterazine analogues • Cold shock • Cryoprotectants • Cytolysin • Detergents • Digitonin • Dimethylsulfoxide (DMSO) • Electrofusion • Electroinjection • Electroporation • Flip-flop movement • Fluidifying agents • Fusion cell ghosts • Glass beads • Glycols • Granule exocytosis • Granzyme • Hypertonic treatment • Hypotonic treatment • Isotonic solution • Lateral diffusion • Laser pulse • Liposomes • Lysolecthin • Lytic activity • Membrane fluidity • Microinjection • Organic solvents • Osmotic lysis • Penetrating peptides • Perforin • Picnocytic vesicles • Polyethyleneglycols • Polysorbates • Polyvinylpyrrolidone • Pore formation • Pore-forming toxins • Proteinase K • Repeats in toxin • Saponin • Stomatal opening • Streptolysin O • Transfection • Triton X100 • Tween 20

3.1 Physical Methods

3.1.1 Electroporation

The introduction of impermeant probes into living cells without losing physiological function remained an important goal for studying mechanisms of cellular regulation. The frequently used applications to introduce macromolecules into cells involve electroporation, electrofusion, electroinjection, microinjection, transfection, liposome mediated delivery, fusion of red cell ghosts, cell penetrating peptides, osmotic lysis of picnocytic vesicles, laser pulses, glass beads, cold shock, hypotonic treatment. Since among these applications electroporation and hypotonic treatment are the procedures that are most closely related to permeabilization, and are the most frequently used techniques, more attention will paid to them.

Electroporation is the application of controlled electrical pulses to living cells for the permeabilization of their membrane. Electrical pulses delivered by a pulse generator induce transmembrane potential causing reversible breakdown of the cellular membrane. As a result pores are formed that allow macromolecules (DNA, RNA, proteins, antibodies) to enter the cell. Variables of electroporation (field strength, pulse strength, pulse shape) help to maximize the efficiency of permeabilization. Due to the reproducibility, efficiency and low toxicity, electroporation has been applied for the introduction of different molecules into bacterial, yeast, plant, insect and mammalian cells. For electroporation different invasive and non-invasive electrodes have been devised. Non-invasive electrodes:

– Caliper Electrodes are re-usable electrodes for *in vivo* use consisting of a caliper and a pair of electrode plates,
– Gene-paddles, reusable electrodes designed for *in vivo* gene delivery and *in vitro* embryo gene delivery,
– Petri dish (100 mm) Tissue Electrode for electroporation of exogenous molecules into adherent cells or tissues.

– Platinum Electrode for Tissue Slices for *ex vivo* electroporation to introduce genes, drugs into ex-plant tissues.

3.1.2 Electrofusion

These applications include hybridoma production for antibody expression, stem cell development, genetically modified plant protoplast, tetraploid fusion and nuclear transfer for transgenic development.

3.1.3 Electroinjection

This voltage-discharge technique is by now generally accepted. At higher than the threshold voltage, the electrical breakdown of the plasma membrane is accompanied by the sudden decrease of its electrical resistance and the formation of pores in it. The diameter of pores increases with increasing the nanosecond electric pulse intensity (Nesin et al. 2011). The voltage-discharge technique employs glass capillaries with ~1 mm outer and 0.5 mm inner diameter pulled with a micropipette puller to create an inner opening diameter of about 0.4–0.5 μm. After back-filling with a few microliters of proper loading solution, a silver wire (0.25 μm) is passed into the micropipette and connected to the voltage stimulator. The opposite terminal is connected to a second silver wire fixed to the cell containing medium on a microscope slide. Electroinjection turned out to be an efficient method for loading of exogenous proteins into cells while maintaining the integrity of the physiological properties of the cell (Wilson et al. 1991). Although, the technical merit of the voltage-discharge methods to deliver macromolecules into the cytosol is not doubted, they require considerable methodological expertise, are restricted to a small cell number and not adaptable to adherent cells (Walev et al. 2001).

3.1.4 Microinjection

Microinjection is considered a common laboratory technique, along with vesicle fusion, chemical transfection, and viral transduction, for introducing a small amount of substance into a cell. For the microinjection into the target cell under the microscope two micromanipulators are being used, one is holding the pipette and the other holding a microcapillary (0.5–5 μm in diameter) to penetrate the cell membrane or the nuclear envelope (Diacumakos 1978; Burr and Allen 2014). The combination of microinjection and electroporation for the transfection is routinely used for gene therapy applications in mammalian cells. Microinjection as already referred to can also be regarded as a loading process of impermeant molecules into the

cytoplasm. Microinjection is relatively inefficient in the sense that only a small number of cells can be loaded in a tedious process that requires expertise and expensive instruments.

3.1.5 Transfection

During transfection high-intensity pulses open pores in cell membranes, followed by long, low-intensity pulses that drive the material into cells *via* electrophoresis. Parameters are optimized to maximize efficiency and cell viability. Transfection serves to introduce proteins that are missing, to produce proteins in bioreactors, deliver genes for gene correction and small interfering RNA (siRNA) to suppress the expression of certain genes, drug delivery, cancer therapy, fight against viral diseases by preventing their replication.

3.1.6 Liposome-Mediated Delivery

This method has been used preferentially for the introduction of siRNAs into cells and to activate immunity response genes (Sioud and Sørensen 2003). The delivery of functional therapeutic proteins by lipid vesicles into targeted living cells is an equally promising strategy for the treatment of different diseases and cancer (Liguori et al. 2008). In liposome-mediated drug delivery of methothrexate negatively-charged liposomes were more efficient for the delivery than neutral liposomes. Cholesterol was also an essential component of the liposome membrane for optimal drug delivery (Heath et al. 1985). These observations confirm data related to the efficiency of DNA delivery, namely that acidic phospholipids were more effective in both binding and delivery, with phosphatidylserine being the best in both aspects. The inclusion of cholesterol in liposomes reduced the cell-induced leakage and enhanced substantially (two to tenfold) the delivery of DNA to cells (Fraley et al. 1981). Liposome-mediated drug delivery can be efficiently measured by capillary electrophoresis with laser-induced fluorescence (Malek and Khaledi 2003).

3.1.7 Fusion of Red Cell Ghost

When red cells were subjected to hemolysis in a hypotonic medium they became ghosts, but retained a considerable amount of haemoglobin, assumingly by adsorption. If this hemolytic system was rendered isotonic by adding salts, the cells assumed their original volume and shape. However, after hemolysis by water the red cells were unable to retain their spherical shape. These “watery ghosts” formed biconcave disks and have undergone considerable surface changes (Ponder 1942).

3.1.8 Cell-Penetrating Peptides

These peptides are also known as protein transduction domains, membrane translocating sequences, or Trojan peptides. The identification of these peptides at the end of the 1980s came as a denial of the dogma that the plasma membrane is impermeable to hydrophilic molecules. The first cell-penetrating peptides were discovered in 1988 (Frankel and Pabo 1988). The trans-activating transcriptional activator (TAT) from HIV-1 virus could be taken up from the cell culture by different cell types (Green and Loewenstein 1988; Wagstaff and Jans 2006). Cell-penetrating peptides are short (10–40 residues) peptides facilitating the uptake of small molecules and large DNA fragments through endocytosis and are delivered to the endosomes in almost any mammalian cell. The "cargo" is linked to peptides *via* covalent bonds or non-covalent interactions. The cationic nature of cell-penetrating peptides is due to their high arginine and lysine amino acid content. When the effects of peptide length, guanidine content of arginine, and sequence of non-cationic residues were assessed, the arginine content in the penetrating analogs was found to influence eukaryotic cell uptake efficiency, as well as eukaryotic cell viability. All penetration analogs retained the ability to cross eukaryotic membranes giving rise to a distribution within the vacuolar apparatus (Bahnsen et al. 2013). Recently a new class of cell-penetrating peptides designated Xentry has been described. Xentry is represented by the short peptide derived from the N-terminal region of the X-protein of the hepatitis B virus (Montrose et al. 2013). Cell-penetrating peptides are attractive candidates with the potential to deliver drugs into cells. Antimicrobial peptides are believed to act solely through membrane disruption, but do not form equilibrium pores in vesicles, rather cause short-lived, transient leakage events (Wimley 2010; Wimley and Hristova 2011). The pore forming antibiotics are small bacteriocins containing the nonproteogenic lanthionine amino acid produced by lactic acid bacteria. Salivaricin 9 is a newly discovered antibiotic produced by *Streptococcus salivarius*. Salivaricin 9 is a bactericidal molecule targeting the cytoplasmic membrane of sensitive cells penetrating the cytoplasmic membrane and inducing pore formation, which results in cell death. When treated with proteinase K or peptidase, salivaricin 9 has lost all antimicrobial activity, while it remained active when treated with lyticase (zymolase), catalase and certain detergents (Barbour et al. 2013).

3.1.9 Osmotic Lysis of Picnocytic Vesicles

In this procedure, also named influx cell-loading technique, cells are first incubated in culture medium containing hypertonic 0.5 M sucrose, 10 % polyethylene glycol 1000 and the macromolecule to be transferred. The medium containing cells is then diluted with two-third parts of water. Most picnocytic vesicles formed in the presence of sucrose burst in hypotonic medium and release the enclosed macromolecule. Cells remain fully viable after the hypertonic sucrose treatment, and the

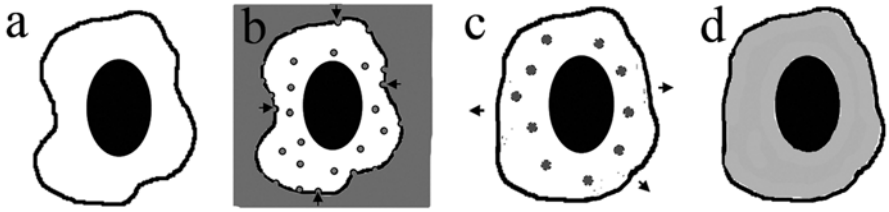


Fig. 3.1 Schematic view of osmotic changes in picnotic vesicles. (**a**) Normal cell. (**b**) Cell placed into hyperosmotic medium containing the material that is carried into the cell by round picnotic vesicles while cell is shrinking. *Arrows* indicate osmotic shrinkage. (**c**) Swelling of cell containing the picnotic vesicles in hypotonic medium. Hypotony may cause the picnotic vesicles to disrupt. Cell swelling is indicated by *arrows*. (**d**) Even distribution of material carried into the cell. Viability of cell is maintained

majority of them survive more successive rounds of osmotic lysis. This procedure, termed osmotic lysis of picnosomes, has been used to transfer substantial amounts of horseradish peroxidase, antiricin antibodies and dextran 70,000 into the cytosol of L929 murine adipocyte cells (Okada and Rechsteiner 1982). It was shown that endosomal compartments containing the hypertonic loading medium do not fuse with lysosomes (Park et al. 1988). Osmotic changes in picnotic vesicles are demonstrated in Fig. 3.1.

3.1.10 Laser Pulses

Cell membrane permeabilization is explained by the creation of fluid plasma within the laser focal spot. Laser intensity for near-infrared optoinjection allows to control the molecules delivered into cells through the pores generated (Peng et al. 2007). Reversible permeabilization for biopreservation using high-intensity femtosecond laser pulses was described (Kohli et al. 2005). When mammalian cells were suspended in impermeable cryoprotectant hyperosmotic sucrose solution, femtosecond laser pulses were used to transiently permeabilize cells for cryoplasmic solute uptake. The kinetics of cells exposed to 0.2, 0.3, 0.4 and 0.5 M sucrose, following permeabilization, were measured using video microscopy, and the post-permeabilization survival was determined by dual fluorescence membrane integrity assay. Using appropriate laser parameters, it was observed that highest survival was achieved with 0.2 M sucrose solution (>90 %), and a progressive decline in cell survival towards higher concentrations (Kohli et al. 2005). Although, the cryoprotective effect of sucrose is known and similarly, the reversible permeabilization in the presence of 0.2 M sucrose with femtosecond laser pulses is not doubted, some explanation to these observations are needed:

(i) The physiological osmolarity of sucrose corresponding to that of mammalian cells is 0.3 M (sucrose is a non-dissociable solute).

(ii) 0.2 M sucrose is hypotonic not hypertonic, but this is advantageous for permeabilization as the sucrose solution will rapidly enter the cell upon pore formation by laser pulses.
(iii) At the isotonic 0.3 M sucrose concentration there will be little or no movement between the intracellular and extracellular solutions.
(iv) At higher than 0.3 M sucrose concentration the cells will shrink as the osmosis will be higher outside than inside.
(v) Hypotonic solution in itself would cause leakage in plasma membrane (Banfalvi 2014). The leakage can be compensated by the cryoprotectant effect of sucrose and prevents cells from disruption. High-intensity femtosecond laser pulses can be applied for membrane surgery and nanosurgical cell isolation and nanosurgical ablation of focal adhesion adjoining epithelial cells.

Highly focused pulsed laser microbeams have been used to dissect, inactivate and perturb cells and subcellular targets. Optoporation, the permeabilization of a cell membrane by laser pulses, has emerged as a powerful non-invasive and highly efficient technique to induce transfection in cells. This technique employs pulsed microbeams to transiently permeabilize the plasma cell membrane and affect the delivery of molecules from the extracellular environment into the permeable cells. Optoporation can be applied using nonspecific or specific modalities. In the nonspecific modality, the pulsed microbeam is focused onto the glass coverslip on which the cells are plated. In the specific modality, termed "optoinjection", the microbeam is focused directly onto the plasma cell membrane and achieves molecular delivery into that cell alone (Krasieva et al. 1998).

Applications of laser beam permeabilization:

(i) The major field of applications of permeabilization with laser irradiation remained the immobilization of human sperms. The reason for application laser beams was that immobilization and permeabilization improved fertilization rates (Palermo et al. 1993; Fishel et al. 1995; Gerris et al. 1995), by facilitating sperm nuclear decondensation after injection of the spermatozoon into the oocyte (Dozortsev et al. 1995). Although, others disagreed (Lacham-Kaplan and Trounson 1994; Hoshi et al. 1995), it was shown that sperm immobilization, leading to the permanent permeabilization of the sperm membrane, improved fertilization rates and led to higher pregnancy rates (Palermo et al. 1996). Laser-induced immobilization of spermatozoa and permeabilization of the sperm tail membrane was applied prior to intracytoplasmic sperm injection (Montag et al. 2000; Ebner et al. 2001; Viziello et al. 2005).
(ii) Reliable membrane permeabilization was achieved by nano- and picosecond irradiation, allowing the penetration of fluorescent labeled antibodies into living cells. These results confirmed that the laser-induced permeabilization approach constituted a promising tool for targeted delivery of larger exogenous molecules into living cells (Yao et al. 2009).
(iii) The permeabilization of cell membranes and the introduction of fluorescein isothiocyanate-dextran and siRNA were enhanced using an optical field gen-

erated in the presence of polystyrene microspheres of 1000 nm diameter excited by a femtosecond laser pulse (Terakawa and Tanaka 2011). Gold nanoparticle mediated laser transfection served efficient siRNA mediated gene knock down (Heinemann et al. 2013). Optotransfection of mammalian cells based on femtosecond laser treatment was facilitated by gold nanorods (Ma et al. 2013).

(iv) Delivery of proteins to mammalian cells *via* gold nanoparticle was mediated by laser transfection (Heinemann et al. 2014).

(v) Femtosecond-laser excitation of cell-adhered gold nanoparticles evoked localized membrane permeabilization, enabled the inflow of extracellular molecules into cells and was recommended for prospective *in vivo* applications (Schomaker et al. 2015).

(vi) Despite the relatively small opening created in the plasma membrane, targeted laser cell perforation allowed specific 3D immunolocalization of cytoplasmic antigens in cultured cells (Jimenez and Post 2014).

(vii) Recently the usual tedious manual targeting of individual cells that significantly limited the addressable cell number was overcome by an experimental setup with custom-made software control, for computer-automated cell optoporation (Breunig et al. 2015).

3.1.11 Glass Beads

A number of methods to introduce macromolecules into cells have been tested. Among the techniques for loading impermeant molecules into the cytoplasm is precipitation with calcium phosphate, membrane breakdown by electric pulses, fusion of cells or liposomes, osmotic lysis of pinosomes, centrifugation loading, retroviral vectors, plant vectors, scrape loading, sonication loading, direct microinjection, glass beads. Loading methods have been described by several authors (Diacumakos 1978; Fechheimer et al. 1986; Graessman et al. 1974; Klein et al. 1987; McNeil et al. 1984). In this subtitle only to those loading methods will be payed attention that allow the healing of wounds mechanically introduced through the plasma membrane with particular emphasis on glass beads. Such a method is scrape loading.

Scrape Loading Cells in loading solution containing KH_2PO_4, glucose, EDTA, NaCl (pH 7.0) were loaded with DNA by scraping them from the dish by using a rubber policeman (McNeil et al. 1984). Scrape loading and sonication loading were used to transfect mammalian cells with plasmid DNA (Fechheimer et al. 1987). Scrape loading and probably sonication loading should be restricted to screening assays for qualitative assessment (McKarns and Doolittle 1992).

Pinosomes (drinking bodies) are tiny fluid-filled vesicles permitting the active transport of fluid through the membrane into the interior of the cell.

The rapid and simple new technique for loading macromolecules into living cells with glass beads was originally described and performed by McNeil and Warder (1987). The cell culture medium of the monolayer was replaced by the macromolecule to be loaded, glass beads (55–500 μm) were sprinkled onto cells, and then washed free of beads while exogenous loading of macromolecules took place. Bead loaded cells remained adherent, homogeneously spread, with short recovery time and applicable for immediate microscopic examination. Cells loaded with macromolecules returned to normal culturing conditions and were immediately available for experiments (McNeil and Warder 1987).

3.1.12 Cold Shock

One of the oldest methods to permeabilize cells applied cold shock. Mouse L cells were rendered permeable to nucleoside triphosphates by a cold shock in a near isotonic buffer. These cells retained their morphologic integrity and used exogenously supplied ribonucleotides and deoxyribonucleotides to synthesize RNA and DNA (Berger and Johnson 1976). For unknown reason in eukaryotic cells this method did not gain broad scale application.

In spite of the complexity of the bacterial cell wall cold shock permeabilization in bacterial cells remained in use. The difficulty of vital staining of permeable bacteria is due not only to the complexity of the bacterial cell wall, but also to the efficient efflux pumps exhibited by many bacteria (Midgley 1986). The Gram-negative bacterial wall consists of four layers, with the outer membrane representing the major permeability barrier. Consequently, Gram negative bacteria are less permeable than the Gram-positive ones. Treatment of bacteria with EDTA or Tris alters the permeability of the outer membrane (Leive 1965; Irvin et al. 1981). The permeabilizing effect of EDTA is based on the chelation of divalent cations in the outer membrane, while Tris is replacing these ions. It was observed that *Bacillus subtilis* cells became permeable to small molecules upon exposure to cold shock that was used for the selective release of ribonuclease inhibitor from cells (Smeaton and Elliott 1967). However, after cold shock treatment to *E. coli*, RNA synthesis was reduced to 70 % and the ability of protein synthesis in these permeable cells was eliminated (Lazzarini and Johnson 1973). When *Escherichia coli* cells were exposed to cold shock (0 °C for 30 min) in the presence of Tris or EDTA, the net uptake of dye was similar to that of fully permeabilized cells (Jearnes and Steen 1994). The effect of rapid and slow chilling was compared in *Escherichia coli* cells. Rapid chilling strongly damaged the cell membrane by disrupting the outer membrane barrier (Cao-Hoang et al. 2008). While rapid chilling was more detrimental than slow chilling, the permeabilization itself by cold shock eliminated the washing of cells and reduced the preparation time to less than 5 min. The results obtained with cold-shock permeabilization were comparable to those seen with ethanol fixation (Walberg et al. 1997). Permeabilization with ethanol will be discussed in the subchapter devoted to permeabilization with organic solvents.

3.1.13 Hypotonic Treatment

Hypertonic fluids have a higher than the isoosmotic concentration (>0.3 Osm) present in normal cells of the body and the blood. Hypertonic agents draw fluid into the intravascular space from cells. Hypertonic saline (3 % NaCl) is a common hypertonic fluid. The salinity of hypotonic solutions is less than the normal saline (0.9 %) and is used as a 0.33 % NaCl solution. Hypotonic solution composed mostly of water will enter the cells rather than remain in the intravascular space. The importance of hypotony during permeabilization is demonstrated by several examples.

Upon hypotonic treatment of rat hippocampal CA1 region (a portion of the hippocampal formation) three types of responses were distinguished:

(i) the volume of "yielding cells" began to increase immediately;
(ii) "delayed response cells" swelled after a latent period of 2 min or more; and
(iii) "resistant cells" whose volume did not change during exposure to hypoosmotic solution.

When the external osmolarity was raised, most cells started to shrank slowly, reaching minimal volume in 15–20 min. Although, the water permeability of the membrane of hippocampal CA1 pyramidal neurons is low compared to that of other cell types (Aitken et al. 1998), one would assume that other cell types could be categorized in the same way, but the delay in response would be different. The composition and the cytoskeletal mechanical support of the plasma membrane are likely to contribute to the resistance to swelling and protect neurons against swelling-induced damage (Aitken et al. 1998). Hypotonic swelling in "yielding" cells activates ion channels and the cell volume regulatory processes increase transiently K^+ and Cl^- conductances (Häusinger 1996; O'Neil 1999; Jakab et al. 2002). After K^+ and Cl^- ions leave the cell, the cell volume is rapidly restored. In addition to ion channel activation, osmotic swelling is known to promote the release of ATP (van der Wijk et al. 2003).

To study plasma membrane permeabilization under different conditions red blood cells are preferentially used since these cells contain only cellular membrane (Segal et al. 1966; Helmerhost et al. 1999; Chapman and Buxser 2002). The damage of erythrocytes is conveniently traced by the detection of lactate dehydrogenase (Li et al. 2005) or by screening the release of hemoglobin by the Drabkin method (Drabkin 1949; Lenfant et al. 2005). Turbidity measurement could be also useful for the rapid detection of hemolytic activity (Young et al. 1986). In red blood cells permeabilized in slightly hypotonic treatment the swelling of erythrocytes was accompanied by a moderate (20 %) decrease of light dispersion, when the screening of hemolysis was not yet possible (Arias et al. 2010). This observation could mean that the pore size at the beginning of permeabilization was not large enough to allow the exit of large molecules such as hemoglobin. The estimated molecular weight of hemoglobin is 64,500 D (Gutter et al. 1956). Encapsulation by hypotonic dialysis was used as carrier of rat and human red blood cells (RBCs). Hypotonic-dialysis encapsulation took place under 80 mOsm/kg for 60 min. The encapsulation was studied using radiolabelled carbonic anhydrase and fluorescently labelled dextran. Both markers were incorporated to a slightly greater extents by human than by rat RBCs during the hypotonic

treatment. Cell recovery of rat and human RBCs loaded with either carbonic anhydrase or fluorescent dextran was 49 % and 80 %, respectively (Alvarez et al. 1996). The administration of hypertonic saline dextran caused a sustained increase in blood pressure and bleeding of uncontrolled hemorrhage (Wade et al. 2003).

Hypotonic permeabilization in combination with other permeabilizing agents such as saponin (50 μg/ml) treatment of live cells for intracellular delivery of quantum dots was achieved (Medepalli et al. 2013). It was shown that hypotonic treatment of cells before the application of electroporation increased significantly the efficiency of electrofusion of murine melanoma (B16-F1) and Chinese hamster ovary (CHO) cells (Usai et al. 2010).

Hypotonic salt solution with Triton X-100 and propidium iodide proved to be a convenient method in studies on bone marrow cells for assessing nuclear DNA content. Other permeabilizing agents such as Tween-20 and saponin were shown to interfere with membrane protein integrity and stainability (Lamvik et al. 2001).

The relatively mild treatment of soybean protoplasts exposing them to hypotonic buffer containing Mes 3 mM, $CaCl_2$ 5 mM and 0.15 M sorbitol is indicated by the observation that electroporation (with two 50 msec 400 V/cm pulses) generated ten times more uptake of calcein fluorescent dye than hypotonic treatment alone (Cutler and Saleem 1987).

Hypotonic buffer containing 10 mM Tris CI, 4 mM $MgCl_2$, 1 mM EDTA, and 6 mM 2-mercaptoethanol (pH 8.0) was applied to permeabilize mammalian cells and measure DNA synthesis. The DNA-synthetic capacity in permeable HeLa cells (Seki et al. 1975) and murine ascites sarcoma cells was highly dependent on the presence of four deoxyribonucleoside triphosphates, adenosine triphosphates, $Mg^{2+,}$ and ionic environment and correlated closely with the DNA replicating activity assayed by [^{3}H] deoxythymidine incorporation in intact cells (Seki and Oda 1977).

Isotonic solution (288 mOsmol) versus hypotonic solutions with mannitol reduced or absent (60, 30, and 0 mM, respectively) osmolality of 259, 228 and 197 mOsmol were monitored to follow iodide anion permeability in rat brain endothelial cells. The isotonic solution contained (in mM): NaCl 87, KCl 4, $MgSO_4$ 0.6, $CaCl_2$ 0.3, KH_2PO_4 0.6, Na_2HPO_4 1.1, Hepes 10, mannitol 90 and glucose 6 (pH adjusted to 7.4). The rapid increase in permeability depended on the degree of hypotonicity. Anion substitution experiments suggested that iodide efflux took place *via* a chloride channel rather than an exchanger (von Weikerstahl et al. 1997).

After hypotonic treatment to permeabilize the protozoan *Toxoplasma gondii* most organelles and the endoplasmic reticulum remained intact (Kimmel et al. 2006). However, mouse liver mitochondria initially incubated in hypotonic HEPES buffer (15 min, 22 °C) released preapoptotic proteins (Uren et al. 2005) suggesting that mitochondria might be more sensitive to hypotony than the plasma membrane. Indeed, the permeabilization of the mitochondrial outer membrane constitutes a major checkpoint of apoptotic and necrotic cell death (Boya and Kroemer 2008), that determines the point of no return of the lethal process (Taylor et al. 2008; Youle and Strasser 2008).

Escherichia coli cells were also made permeable with a hypotonic Tris(hydroxymethyl)aminomethane (TRIS) buffer to utilize exogenous deoxyribonucleoside triphosphates and perform semiconservative replication. The rate of rep-

lication was the same as in cells made permeable with toluene or sucrose (Boye et al. 1981). Treatment with toluene (Moses and Richardson 1970), sucrose (Wickner and Hurwitz 1972), or ether (Vosberg and Hoffmann-Berling 1971) killed the vast majority of exposed cells and is no longer recommended for permeabilization.

Hypotonic Buffer in the Presence of Dextran Poly(ADP-ribose)polymerase activity that requires intact DNA, was measured in permeable murine lymphoma L5178Y cells. These cells were rendered permeable to nucleotides by treatment with hypotonic treatment. The hypotonic buffer contained 9 mM hepes pH 7.8, 5 mM dithiothreitol, 4.5 % dextran (w/v) ~ mol. wt. 110,000 Da, 1 mM EGTA, 4.5 mM $MgCl_2$. Permeabilization at 0 °C lasted for 30 min and was terminated by the addition of ninefold volume of isotonic buffer (Halldorsson et al. 1978). Exponentially growing mouse L cells were selectively permeabilized to small molecules by hypotonic treatment and the presence of dextran sulfate in the permeabilizing buffer was emphasized (Kucera and Paulus 1982). Characterization of ribonucleoside diphosphate reductase activity has been extended to cells in specific phases of the cell cycle and in transition between cell cycle phases, with activity studied both in situ in permeabilized cells and in cell extracts (Kucera et al. 1983). It was demonstrated that mouse thymocytes continued to synthesize DNA under hypotonic conditions in the presence of dextran T-150, the four deoxyribonucleoside triphosphates and ATP. Permeable murine thymocytes could seal their plasma membrane in a serum-enriched medium within a few hours. The reversal of permeabilization of murine thymocytes by hypotonic treatment in the presence of dextran as a molecular coat to prevent the disruption of cells was demonstrated by scanning electron microscopy (Banfalvi et al. 1984) (Fig. 3.2).

3.2 Pore Formation in Membranes

3.2.1 Major Intrinsic Proteins (MIPs)

The Major Intrinsic Protein of the MIP family of the human lens of eye represents about 60 % of the protein in the lens cells. MIP is an aquaporin (Aquaporin 0) that undergoes proteolytic cleavage during the development of the lens (Gonen et al. 2004). Aquaporins are present in different cells (Yool and Campbell 2012; Pareek et al. 2013; Klein et al. 2015; Hill and Shackar-Hill 2015; Verma et al.2015). Members of the diverse and large family of MIP form transmembrane channels involved in the transport of water, small carbohydrates (e.g. glycerol), urea, NH_3, CO_2, H_2O_2 and ion transport in an energy-dependent manner. Based on their permeability properties they are now further subdivided into aquaporins, with real water-selective pores, and aqua-glyceroporins with slightly less selective pores (van Os et al. 2000).

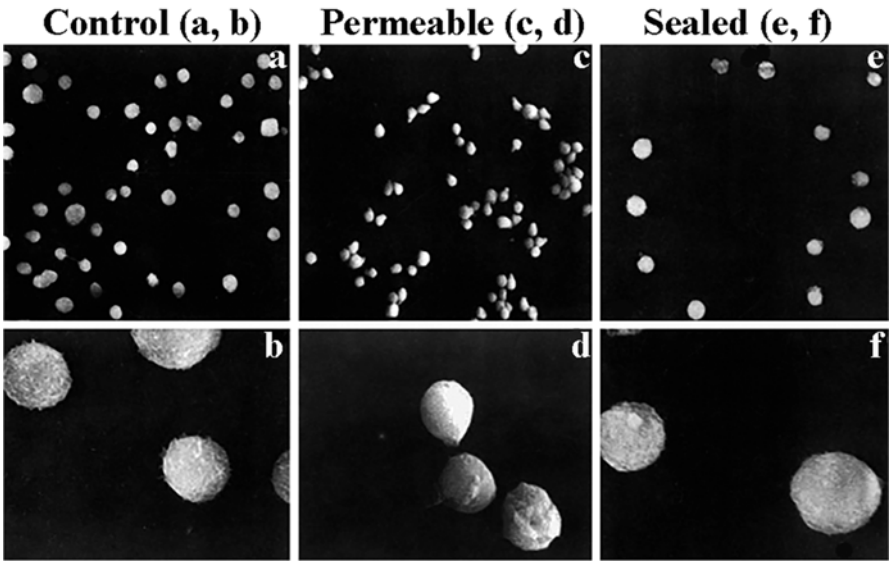


Fig. 3.2 Scanning electron microscopy of intact (**a**, **b**), permeabilized (**c**, **d**) and sealed (**e**, **f**) murine thymocytes. Cells were permeabilized in hypotonic permeabilization buffer at 0 °C for 15 min. Hypotonic buffer for permeabilization (Halldorsson et al. 1978) was slightly modified, containing 9 mM Hepes, pH 7.8, 5.8 mM dithiothreitol, 4.5 % dextran T-150, 1 mM EGTA and 4.5 mM $MgCl_2$. Intact, permeable and sealed cells were fixed on cover slides for 30 min at 0 °C with 2.5 % glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. After washing with the same buffer, cells were fixed further for 30 min at 0 °C with 2.5 % osmium tetroxide in the same buffer. Cell were dehydrated stepwise, using increasing concentrations of ethanol in the range of 50–100 %. Cells were finally washed and stored in amyl acetate. Small pieces of cover slides were glued to copper stubs, and coated with gold for viewing by scanning microscopy at lower (upper row) and higher magnification (lower panels) (Modified with permission Banfalvi et al. 1984, Fig. 2)

Family members of MIP include:

- MIP Aquaporine 0, the major component of lens fiber gap junctions (Gonen et al. 2004).
- Mammalian aquaporins provide the plasma membranes of red cells, as well as kidney proximal and collecting tubules with high permeability to water (Chrispeels and Agre 1994).
- Soybean nodulin-26 – peribacteroid membrane is induced during nodulation in legume roots after *Rhizobium* infection. Phosphorylation of nodulin 26 enhances water permeability and is regulated developmentally and by osmotic signals (Guenther et al. 2003).
- Plant tonoplast intrinsic proteins. Tonoplast is the membrane that separates the vacuole from the surrounding cytoplasm in plant cells. Tonoplast intrinsic proteins allow the diffusion of water, amino acids and/or peptides from the tonoplast interior to the cytoplasm. These proteins have been used as markers for vacuolar identity in plants (Gattolin et al. 2010).
- Bacterial glycerol facilitator protein – facilitates the movement of glycerol across the cytoplasmic membrane. The glycerol facilitator of *Escherichia coli*, GlpF, is

a transport channel in the inner membrane of this Gram-negative bacterium (Maurel et al. 1994).

- Yeast FPS1. Under high osmolarity stress, many fungal species, including *S. cerevisiae*, maintain osmotic equilibrium by producing and retaining high concentrations of glycerol as a compatible solute (Nevoigt and Stahl 1997). Intracellular glycerol concentration is regulated in *S. cerevisiae* in part by the Fps1 plasma membrane glycerol channel (Luyten et al. 1995; Sutherland et al. 1997; Tamas et al. 2003). The yeast glycerol uptake/efflux facilitator protein functions in osmoregulatory pathways to transport glycerol passively out of the cell (Beese-Sims et al. 2011).
- *Salmonella typhimurium* propanediol diffusion facilitator. The propanediol utilization gene (pduF) of *Salmonella enterica* encodes a hydrophobic protein with a strong similarity to the GlpF protein of *Escherichia coli*, which facilitates the diffusion of glycerol. The N-terminal end of the PduF protein includes a motif for a membrane lipoprotein-lipid attachment site as well as a motif characteristic to the MIP (major intrinsic protein) family of transmembrane channel proteins (Chen et al. 1994).
- *Drosophila* neurogenic protein 'big brain' (bib) – encodes a membrane-associated protein that allows the transport of certain molecules and is responsible for signaling for exodermal cells to become epidermoblasts rather than neuroblasts (Doherty et al. 1997).

3.2.2 ATP-Induced Pore Formation

Beside its well established intracellular functions ATP has several extracellular functions (Burnstock 1972, 1976; Fedan et al. 1981; Needham et al. 1987; Gordon 1986) not to be detailed. The extracellular ability of ATP to create permeability lesions in plasma membrane has been described in different cell types such as mast cells (Cockroft and Gomperts 1979; Benett et al. 1981), epithelial cell lines (Heppel et al. 1985), macrophages (Steinberg et al. 1987), lymphocytes (Mustelin 1987), hemopoietic stem cells (Whetton et al. 1988). While these excitatory and inhibitory effects are generated by MgATP, the agonist for permeabilization is free ATP as ATP^{4-}. The pattern of selectivity displayed by the rat mast cell ATP^{4-} receptor is distinct from P2 purinoceptors (Tatham et al. 1990). In conformity with this observation, when divalent cations were omitted from the medium, leakage was effective at lower ATP concentrations. At higher ATP concentration the leakage of larger molecules from the cells became evident (Tatham and Lindau 1990; Cutaia et al. 1996). This observation confirmed earlier evidence that increase in pore size was obtained with increasing ATP concentration in mast cells (Cockcroft and Gomperts 1979). High conductance pathways induced by extracellular ATP with similar properties were found in patch-clamp experiments on macrophages (Buisman et al. 1988; Coutinho-Silva and Persechini 1997). However, high ATP concentration may also lead to pore formation induced cell death. The hypothesis that this nucleotide can kill cells was tested on microvascular cells of the retina. As

predicted ATP-induced cell death required $P2X_7$ purinergic receptor activation that caused pores to form and microvascular cells to die, and as surmised pore formation was accounted for by ATP's lethality (Sugiyama et al. 2005). $P2X_7$ receptors are ATP-gated cation channels, the activation of which in macrophages leads to the opening of a membrane pore permeable to dyes (e.g. ethidium bromide) and to the release of interleukin-1β. Pannexin-1 functions as a hemichannel mediates pore formation and interleukin-1β release by the ATP-gated $P2X_7$ receptor (Pelegrin and Surprenant 2006).

3.2.3 Pore-Forming Proteins

Intracellular pathogens are able to invade the host organism, to bypass its immune response and to survive in its intracellular environment. Pore-forming proteins are among the several tools used by intracellular microorganisms to enter into host cells, inhibit phagocytosis, escape phagosomes or promote pathogen dissemination. However, pore-forming proteins can also behave as double-edged swords that may serve the benefit of the host organism by eliminating infected cells or induce inflammatory processes (Almeida-Campos et al. 2002). Animals and plants defend themselves against these pathogenic invasions, by the rapid mobilization of polycationic helical amphipatic peptides. Interactions provide not only defense but are also attractive approaches for drug design (Duclohier 2002).

Pore and channel forming proteins and peptides comprise a diverse class of molecules ranging from short peptides that are unregulated and large ion channel proteins that are highly regulated and exhibit exquisite selectivity for particular ions. The diversity of regulation and selectivity, together with recent advances in protein "reengineering" technology, is expected to result in wide-ranging biotechnological applications (Panchal et al. 2002). Multiple fluorescence techniques have been applied for the analysis of pore-forming protein structures in membranes to reveal the identity, location, conformation and topography of polypeptide sequences that interact with the plasma membrane (Heuck and Johnson 2002).

Corresponding to their major groups, pore-forming proteins will be distinguished as pore-forming toxins, pore-forming channels and disorders of voltage gated ion channels known as channelopathies.

Pore-Forming Toxins Gram negative bacteria use a type I secretion system to translocate proteins, including pore-forming toxins, proteases, lipases and S-layer proteins, across both the inner and outer membranes into the extracellular surroundings. The *Escherichia coli* alpha hemolysin (HlyA) secretion system is the prototypical and best characterized type I secretion system (Gentschev et al. 2002). Aerolysin is the pore-forming toxin in *Aeromonas* pathogenicity. The pore-forming aerolysin binds to its target cell and then hijacks cellular devices to promote its own polymerization and pore formation (Abrami et al. 2000).

Other Gram-negative bacteria use the more sophisticated type II secretion system to translocate a wide range of proteins from the periplasm across the outer membrane. The inner-membrane platform of the type II secretion system is the nexus of the system and orchestrates the secretion process through its interactions with the periplasmic filamentous pseudopilus, the dodecameric outer-membrane complex and a cytoplasmic secretion ATPase (Korotkov et al. 2012).

A broad range of pathogenic Gram negative bacteria produce RTX toxins (repeats in toxin), exhibit cytotoxic and often hemolytic activity. They are widespread in *Pasteurellaceae* species causing infectious diseases both in animals and in humans (Frey and Kuhnert 2002). The first described group of RTX proteins consisted of toxins, exhibiting cytotoxic pore-forming activity, detected as a hemolytic halo surrounding bacterial colonies grown on blood agar plates (Goebel and Hedgpeth 1982; Muller et al. 1983; Felmlee et al. 1985; Welch 1991). The RTX family of proteins was originally characterized by the presence of arrays of glycine- and aspartate-rich nonapetide repeats (Welch 2001), then by their common feature of export across the bacterial envelop *via* the type I secretion system. RTX protein translocation occurs through an oligomeric secretion channel spanning across the entire Gram-negative bacterial cell envelope including the cytoplasmic membrane, periplasmic space and outer membrane (Linhartová et al. 2010). Pore-forming protein toxins can exist either in a stable water-soluble state or as an integral membrane pore. To convert from the water-soluble to the membrane state, toxins undergo large conformational changes (Parker and Feil 2005). Pore-forming proteins are produced by cells from all kingdoms, with best characterized ones being of bacterial origin and known as pore-forming toxins. The responses to these toxins are also extremely diverse due to multiple downstream effects of pore-induced changes in ion balance (Bischofberger et al. 2012). Crystallographic studies revealed that the construction of many outer membrane proteins and bacterial toxins is based on the beta barrel motif. Two structural classes have been identified. One class is represented by the porins including monomeric or multimeric proteins where each beta barrel is formed from a single polypeptide. Porins are present in the outer membrane of Gram-negative bacteria, in some Gram-positive bacteria, in mitochondria, and in the chloroplast. The second class features proteins where the beta barrel is a multimeric assembly, to which each subunit contributes beta strands (Delcour 2002). The trafficking of metabolites across the outer mitochondrial membrane is thought to be mediated by the pore-forming voltage-dependent anion channel known as the mitochondrial porin (Blachly-Dyson and Forte 2001).

Data indicate that the plasma membrane of hematopoietic cells contains porin, located mostly in caveolae or caveolae-like domains. Extra mitochondrial porin was purified from the plasma membrane of intact cells by a procedure utilizing the labeling reagent NH-SS-biotin and streptavidin affinity chromatography (Bathori et al. 2000).

Staphylococcal Pore-Forming Toxins Some of the transmembrane pore-forming toxins (Prévost et al. 2001) have been described as beta-barrel forming staphylococcal pore-forming proteins. Mutational analysis indicated that the glycine residue is

located as a 'joint' between the two halves of the β-barrel, suggesting a two-step transmembrane β-barrel pore formation mechanism in which the upper extramembrane and bottom transmembrane regions are formed independently (Yamashita et al. 2014). These beta-barrel pore-forming toxins belong to the staphylococcal alpha-hemolysin family and represent the prototype of bacterial exotoxins with membrane-damaging function, which share sequence and structure homology. These toxins are secreted in a soluble form which finally converts into a transmembrane pore by assembling an oligomeric beta-barrel, with hydrophobic residues facing the lipids and hydrophilic residues facing the lumen of the channel (Menestrina et al. 2001). Alpha hemolysin is a cytotoxin secreted by Staphylococcus aureus that assembles from a water soluble monomer to form a membrane bound heptameric beta barrel on the surface of susceptible cells, perforating the cell membranes, leading to cell death and lysis (Montoya and Gouaux 2003).

***Treponema denticola*,** a Gram negative anaerobic oral spirochete pathogen, has been shown to adhere to fibroblasts and epithelial cells, as well as to extracellular matrix components present in periodontal tissues, and to produce several deleterious factors that may contribute to the virulence of this bacterium. These bacterial components include outer-sheath-associated peptidases, chymotrypsin-like and trypsin-like proteinases, hemolytic and hemagglutinating activities, adhesins that bind to matrix proteins and cells, and an outersheath protein with pore-forming properties (Sela 2001).

Granule Exocytosis The contents of secretory vesicles of the cytotoxic lymphocytes ($CD8^+$, natural killer cells) are secreted toward the target cell, and some of the toxins penetrate into the target cell. These toxins invoke caspase activation, the so called "granule exocytosis mechanism" that provides a variety of additional strategies to overcome the inhibitors of the caspase cascade that may be produced by viruses. Further molecular players are the pore-forming protein perforin and granule-bound serine proteases or granzymes (Smyth et al. 2001).

Oligomerization into a Pore for Cytochrome C Efflux The proapoptotic cascade activates BID protein by its truncation, which in turn oligomerizes BAK or BAX into pores that result in the release of cytochrome c (Korsmeyer et al. 2000).

Lytic Activity of Perforin and Granzyme This mechanism of killing involves exocytosis of lymphocyte granules which cause pores to form in the membranes of the attacked cells, fragments nuclear DNA and lead to cell death. The cytotoxic granules contain perforin, a pore-forming protein, and a family of serine proteases termed granzymes. Both perforin and granzymes are involved in the lytic activity of caspases leading to apoptosis (Kam et al. 2000).

Acanthaporin Human pathogens are known to generate soluble protein toxins resulting in pores in membranes, resulting in the death of target cells and causing tissue damages and severe, often fatal diseases. The pathogenic parasite *Entamoeba histolitica* amoeba is a bacteria-feeding enteric protozoan that produces a pore-forming

toxin named acanthaporin (Michalek et al. 2013). Acanthaporin was cytotoxic not only for human neuronal cells but also exerted antimicrobial activity against a variety of bacterial strains by permeabilizing their membranes (Sönnichsen et al. 2013).

Lysteriolysin O is a non-enzymatic, cytolytic, thiol-activated, cholesterol-dependent, pore-forming toxin protein. This pH-sensitive endosomolytic bacterial hemolysin is produced by the Gram positive bacterium *Listeria monocytogenes* (Provoda and Lee 2000). *Listeria monocytogenes* has evolved mechanisms to exploit host processes to grow and spread from cell to cell without damaging the host cell. The pore-forming protein listeriolysin O mediates escape from host vacuoles and utilizes multiple mechanisms to avoid causing toxicity to infected cells (Portnoy et al. 2002; Cossart 2002).

Pore-Forming Colicins Colicin is a water-soluble pore-forming protein. This bacteriocin is produced by and toxic to some strains of *Escherichia coli* and evolved to interact simultaneously with several components of the complex membrane of the target cell that transforms itself into a membrane protein, and becomes an ion channel (Lakey and Statin 2001).

Cholesterol-dependent cytolysins are a large family of pore-forming toxins that are produced by many species from the genera *Clostridium*, *Streptococcus*, *Listeria*, *Bacillus*, and from *Arcanobacterium haemolyticum* (formerly known as *Corynebacterium hæmolyticum*). These toxins are well known as beta-hemolytic proteins and exhibit two hallmark characteristics, the absolute dependence on the presence of membrane cholesterol and the formation of extraordinarily large pores (Tweten 2005). These pore-forming proteins include the human perforin proteins and the complement membrane attack complex (Gilbert 2010).

Pore-Forming Channels

These channels exhibit extreme molecular diversity and mechanisms to buffer cell excitation and counteract vasoconstrictive influences. The molecular diversity is exemplified by their vascular potassium channel isoforms. The interaction of several molecular mechanisms is regarded to fine tune the potassium channel activity in response to various normal and pathological challenges (Korovkina and England 2002).

Type A Potassium Channels Rapidly inactivating A type potassium channels are important determinants of firing frequency in many excitable cells (Herson and Adelman 2003).

Calcium Activated Potassium Channels Prominent representatives of potassium channels are calcium activated potassium channels, which are highly selective for potassium ions requiring intracellular calcium for channel gating. The families of the large conductance calcium activated and voltage dependent potassium channels, have been widely studied (Weiger et al. 2002).

HERG K^+ Channels The *ether-a-go-go related gene* (hERG) encodes the pore-forming subunit of a delayed rectifier voltage gated K^+ channel (Abott et al. 1999). The strange name "ether-á-go-go" was coined by Kaplan and Trout (1969) as a humorous reference to how the legs of mutant flies shake under ether anesthesia like the go-go dancers. HERG K^+ ion channels are crucial for normal action potential repolarization in cardiac myocytes (Vandenberg et al. 2001; Tseng 2001).

ATP Sensitive Potassium Channels Associated missense polymorphisms (KCNJ11, E23K and *ABCC8* A1369S) may progress to type 2 diabetes (Florez et al. 2012). E23K polymorphism of pancreatic beta cells generates changes in the pore-forming ATP sensitive K^+ channels. E23K significantly enhances the spontaneous open conformation of these channels, and modulates their sensitivity toward inhibitory and activatory adenine nucleotides. Evidence was provided that in Caucasians, E23K could be an important genetic risk factor for type 2 diabetes (Schwanstecher and Schwanstecher 2002). ATP sensitive potassium channels are widely expressed in many cell types including neurons. ATP sensitive K^+ channels, are heteromeric membrane proteins that consist of two different subunits: the pore-forming, two transmembrane spanning potassium channel subunit and the regulatory, transmembrane spanning sulphonylurea receptor (Liss and Roeper 2001). Glucose sensing neurons function much like pancreatic beta cells where glycolysis regulates the activity of an ATP sensitive K^+ channel (Levin 2001).

Epithelial $Ca2^+$ Channel This channel constitutes the rate-limiting apical entry step in the process of active transcellular Ca^{2+} transport. It belongs to a superfamily of Ca^{2+} channels that includes the vanilloid receptor and transient receptor potential channels. The "gatekeeper" Ca^{2+} channel consists of six transmembrane spanning domains, including the pore-forming hydrophobic stretch between domain 5 and 6. These channels are the prime targets for hormonal control of active Ca^{2+} flux from the urine space or intestinal lumen to the blood compartment (Hoenderop et al. 2002a, b).

Polyphosphate and Polyhydroxybutyrate Complexes Complexes of the two polymers, isolated from bacterial plasma membranes form voltage-dependent, Ca^{2+}-selective channels in planar lipid bilayers that are selective for divalent over monovalent cations, permeant to Ca^{2+}, Sr^{2+}, and Ba^{2+}, and blocked by transition metal cations in a concentration-dependent manner (Reusch 2000).

Voltage Dependent K^+, Ca^{2+}, and Na^+ Channels The principal pore-forming (alpha) subunits of each channel show either an exact or an approximate fourfold symmetry. These channels share similar transmembrane topology, and all are gated by changes in membrane potential. Furthermore, these channels all possess an auxiliary polypeptide, designated the beta subunit, which plays an important role in their regulation. Despite considerable functional similarities and abilities to interact with structurally similar alpha subunits, however, there is considerable structural diversity among the beta subunits (Hanlon and Wallace 2002). The pore-forming subunits (alpha) of many voltage-dependent K^+ channels and the modulatory beta-subunits

exist in the membrane as components of macromolecular complexes, able to integrate a myriad of cellular signals that regulate ion channel behavior (Torres et al. 2007).

Light-Dependent Stomatal Opening Plants lose water through open stomata. Volume control of the pore-forming guard cells represents a key step in the regulation of plant water status. These sensory cells are able to integrate various signals such as light, auxin, abscisic acid, and CO_2 (Dietrich et al. 2001).

3.2.4 Channelopathies

Channels are macromolecular protein complexes within the lipid membrane. Studies of human inherited channelopathies have clarified the functions of many ion channels. Regularly more than one gene regulate the function of a channel, consequently different genetic mutations may manifest with the same disorder (Celesia 2001). Over the past quarter of century numerous defects in channels, irrespective of their types, became known referred to as channelopathies. Channelopathies have been defined as diseases of disrupted channel functions caused by mutations in genes encoding channel proteins.

Hyperekplexia ("exaggerated surprise") is a rare hereditary, neurological disorder that may affect newborns (neonatal) or prior to birth (*in utero*), as well as children and adults. Hereditary hyperekplexia is characterized by generalized stiffness immediately after birth; excessive startle reflex (eye blinking and a flexor spasm of the trunk) to unexpected (particularly auditory) stimuli; and a short generalized stiffness following the startle response during which voluntary movements are impossible.

The importance of transport of anion transport is demonstrated by the multitude of channelopathies caused by mutations impairing chloride ion transport that can lead to diverse symptoms such as cystic fibrosis, myotonia, epilepsy, startle disease, hyperekplexia (startle disease), lysosomal storage disease, deafness, blindness, renal salt loss, lung infection and fibrosis, kidney stones, renal salt loss, neurodegeneration, osteopetrosis, male infertility, and probably many others, yet to be discovered.

An interesting feature of channelopathic disorders is that they are caused by mutations in genes belonging to non-related gene families of chloride channels and transporters, ABC transporters, gamma amino butyric acid (GABA) and glycine receptors (Plannels-Cases and Jentsch 2009). Indeed, a heterogeneous group of disorders belong to the channelopathies caused by the dysfunction of ion channels that can be located in the membranes of any cell and in many cellular organelles. Major

groups of channelopathies have been categorized based on their organ system of occurrence (Kim 2014):

- *Nervous system* (e.g. generalized epilepsy with febrile seizures are full-body convulsion, familial hemiplegic migraine, episodic ataxia, hyperkalemic and hypokalemic periodic paralysis). That channelopathies could affect nerves as well as muscles was demonstrated in 1995, by the discovery that episodic ataxia type 1, a rare autosomal dominant disease, was the consequence of mutations in one of the potassium channel genes (Browne et al. 1995; Rose 1998).
- C*ardiovascular system*: long QT syndrome, short QT syndrome, Brugada syndrome, and catecholaminergic polymorphic ventricular tachycardia. LQT2 is one form of the congenital long QT syndrome. Long QT syndrome (LQTS) is an inherited or acquired heart condition in which delayed repolarization of the heart following a heartbeat increases the risk of episodes of irregular heartbeat that originates from the ventricles. Long QT syndrome results from mutations in the human ether-a-go-go-related gene (HERG), and more than 80 mutations, usually causing single amino acid substitutions in the HERG protein. HERG encodes the ion channel pore-forming subunit protein for the rapidly activating delayed rectifier K^+ channel in the heart (January et al. 2000).
- *Respiratory system*. Mutations in cystic fibrosis transmembrane conductance regulator gene (CFTR), are responsible for cystic fibrosis, a relatively well known channelopathy. CFTR protein is a multifunctional protein with a main function of Cl^- channel. CFTR is expressed in epithelia (upper airways, intestine, pancreas, etc.) (Edelman and Saussereau 2012). *Polycystins* are membrane-associated proteins involved in renal cystic disease. Mutations in polycystin1, polycystin 2 and polycystin L genes are responsible for almost all cases of autosomal dominant polycystic kidney disease (Sandford et al. 1999).
- *Endocrine system*. In endocrine cells, ion channels play an important role in hormonal secretion, Ca^{2+}-mediated cell signaling, transepithelial transport, cell motility and growth, volume regulation and cellular ionic content and acidification of lysosomes. Channelopathy can cause endocrine disorders or endocrine-related manifestations, such as pseudohypoaldosteronism type 1, Liddle syndrome, Bartter syndrome, persistent hyperinsulinemic hypoglycemia of infancy, neonatal diabetes mellitus, diabetes insipidus, cystic fibrosis, Dent's disease, hypomagnesemia with secondary hypocalcemia, nephrogenic diabetes insipidus, thyrotoxic hypokalemic periodic paralysis, etc. (Rolim et al. 2010).
- *Ion channel dysfunction* plays an important role in hormonal secretion (neonatal *diabetes mellitus*, familial hyperinsulinemic hypoglycemia, thyrotoxic hypokalemic periodic paralysis, familial hyperaldosteronism, etc.).

  Inherited disorders of voltage gated Na+ ion channels causing epilepsy. Two distinct pathways are known for generating epileptic phenotypes based on inherited disorders of voltage gated Na^+ ion channels. The first pathway is the direct involvement of mutations in genes encoding the pore-forming subunits of the channel and favor repetitive firing and network hyperexcitability. The

second pathway involves mutation of other genes that lead to downstream modifications in Na^+ channel expression (Noebels 2003).

Cardiac K^+ channels. Heterogeneous expression of voltage gated potassium channels was found in the heart. In the mammalian myocardium, potassium (K^+) channels control resting potentials, amplitude, duration, refractory periods and automaticity of action potentials. K^+ channels are composed of four pore-forming α-subunits assembled as tetramers or dimers forming K^+ selective pores and modulated by accessory subunits. Molecular cloning has revealed the presence of a large number of K^+ channel pore forming (alpha) and accessory (beta) subunits in the heart (Nerbonne et al. 2001) and voltage-gated K^+ channel diversity in the mammalian myocardium (Nerbonne 2000). In the diseased myocardium remodeling of voltage gated K^+ currents occurs, influencing propagation and rhythmicity leading to increased dispersion of ventricular repolarization and reentrant arrhythmias (Nerbonne and Guo 2002).

Mutations in voltage gated Na^+ channels. Voltage gated Na^+ channels are glycoprotein complexes responsible for initiation and propagation of action potentials in excitable cells. Mammalian Na^+ channels are heterotrimers, composed of a central, pore-forming subunit and two auxiliary beta subunits. Multifunctional Na^+ channel beta subunits modulate channel gating and regulate the level of channel expression at the plasma membrane. Three genes encode Na^+ channel beta subunits with at least one alternative splice product. Mutation in the beta1 subunit gene has been linked to generalized epilepsy (Isom 2001).

Voltage-gated calcium channels belong to the group of voltage-gated ion channels found in the membranes of excitable cells (muscle, glial cells, neurons, etc.) with an about 1000-fold greater permeability to the calcium ion Ca^{2+} than to Na^+ (Catterral et al. 2005; Yamakage and Namiki 2002). Voltage-gated Ca^{2+} channels are involved in a large variety of cellular functions such as excitation-contraction coupling, hormone secretion, firing and pacemaker activity, gene activation and proliferation (Lory et al. 2000). Voltage-gated Ca^{2+} channels mediate Ca^{2+} entry into cells in response to membrane depolarization. Electrophysiological studies revealed different Ca^{2+} currents designated L, N, P, Q, R, and T-type. The high-voltage-activated Ca^{2+} channels that have been characterized biochemically are complexes of a pore-forming alpha1 subunit of approximately 190–250 kDa; a transmembrane, disulfide-linked complex of alpha 2 and delta subunits; an intracellular beta subunit; and occasionally a transmembrane gamma subunit (Catterral 2000). Inactivation of channels controls the amount of Ca^{2+} entry during an action potential and is believed to play an important role in tissue-specific Ca^{2+} signaling. Ca^{2+} channels of a given subtype may be inactivated by three different conformational changes: fast and a slow voltage-dependent inactivation processes and in some channel types by an additional Ca^{2+}-dependent inactivation mechanism. Inactivation kinetics of Ca^{2+} channels are determined by the

intrinsic properties of their pore forming alpha1-subunits and by interactions with other channel subunits (Hering et al. 2000).

- *Urinary system* (Bartter syndrome, Dent's disease, nephrogenic diabetes insipidus, autosomal-dominant polycystic kidney disease, and hypomagnesemia with secondary hypocalcemia). Bartter syndrome an inherited abnormal thickening of the ascending limb of the loop of Henle, is characterized by low potassium levels (hypokalemia), increased blood pH (alkalosis), and normal to low blood pressure
- *Immune system* (myasthenia gravis, neuromyelitis optica, Isaac syndrome, and anti-NMDA [N-methyl-D-aspartate] receptor encephalitis) (Kim 2014). A range of antibody-mediated disorders have been associated with the neuromuscular junction, with autoantibodies to specific ligand-gated receptors, voltage-gated ion channels or related proteins. Antibodies have been detected in autoimmune forms of autonomic neuropathy and in disorders of the central nervous system (Buckley and Vincent 2005). The pathogenicity of autoantibodies to ion channels has been demonstrated in most of the autoimmune conditions, and patients responded well to immunotherapies that reduced the levels of the pathogenic autoantibodies. Autoimmune channelopathies are supposed to have a good prognosis, especially if diagnosed and treated early, if they do not belong to the paraneoplastic syndromes defined as disorders caused by cancer, and are not a direct result of cancer invasion of the affected organ or tissue (Kleopa 2011)
- *Signal transduction cascades regulating apoptosis*. Several ion channels in the plasma membrane, in particular the N-type K^+ channel, the chloride channel, cystic fibrosis conductance regulator, and an outward rectifying chloride channel, as well as the mitochondrial permeability transition pore, are involved in signal transduction cascades regulating apoptosis (Gulbins et al. 2000). Dent's disease is a rare X-linked recessive inherited condition that affects the proximal renal tubules. Patients who have mutations in the *CLCN5* gene (Dent 1), which encodes a kidney-specific chloride/proton antiporter, and patients with mutations in the *OCRL1* gene (Dent 2) develop this channelopathy.
- *Mitochondrial channelopathies*. Mitochondria, the powerhouses of the cells, are the most important cellular organelles to contribute to aging. Aging is related to the production of reactive oxygen species affecting apoptosis and ATP synthesis. Due to the multiple ion channels and transporters present in mitochondria, their dysfunctions (mitochondrial channelopathies) are likely to accelerate the aging process (Pi et al. 2007).
- *Ion channels in cancer*. The ubiquitous presence of ion channels in virtually all cells and their critical involvement in diverse biological functions suggests that cancer can also be ascribed, at least in part, to ion channel malfunction. Although, cancer is not cataloged as a channelopathy, the non-traditional roles of ion pumps and channels are recognized and suggested that ion channels and ion pumps contribute to cancer progression (Litan and Langhans 2015). An example could be the colonization of cancer cells in other tissues that is promoted by channelopathies. Ion channels could be the next frontier in cancer research (http://www.

alomone.com/upload/newsletters/modulator%2017%20papers/ion%20channels%20in%20cancer.pdf). This is demonstrated by the well-known chlorotoxin, a non-identified ion channel as a potent anti-cancer drug (Bronstein-Sitton 2003). Chlorotoxin, a 36-amino acid peptide was isolated from the venom of the *Leiurus quinquestriatus* scorpion as a putative Cl^- channel inhibitor (DeBin and Strichartz. 1991). It was found that chlorotoxin could inhibit invasiveness of glioma cells *in vitro* (Soroceanu et al. 1999).

3.2.5 Streptolysin O Treatment

Among the various cell poration methods we find glass bead-loading (McNeil and Warder 1987), digitonin (Adam et al. 1990) and more importantly streptolysin O permeabilization (Bakhdi et al. 1985; Koffer and Gomperts 1989; Cattaneo and Vicentini 1989; Graves et al. 1990; Narashimhan et al. 1990; Tan et al. 1992; Liu et al. 1993; Bryant 1992). Streptolysin O is a 69 kD bacterial protein forming an alpha helix, toxic to eukaryotic cells. It belongs to the cholesterol-binding bacterial exotoxins, forming large pores in the plasma membrane of mammalian cells (Bhakdi et al. 1996). Since the plasma membrane contains more cholesterol, internal membranes are less exposed to streptolysin and will not be permeabilized. For example mitochondria contain less than 3 % and the endoplasmic reticulum 6 % cholesterol by mass. As already mentioned, by number cholesterol can make up almost half of the cell membrane, but it has a smaller molecular weight, thus the proportion of cholesterol by mass is only about 20 % in the plasma membrane (Alberts et al. 2002). Cholesterol plays a key role in the initial binding of streptolysin to the plasma membrane. It is not involved in the formation of the membrane-penetrating toxin channels. Membrane damage by streptolysin resembles other channel formers, e.g. staphylococcal alpha-toxin (Bhakdi et al. 1985).

The two major types are streptolysin O and streptolysin S. Streptolysin O is the prototype oxygen-labile, cytotoxic 69-kDa α-helical protein produced by strains of *Streptococcus pyogenes* and other hemolytic streptococci (Bhakdi et al. 1985; Sierig et al. 2003). Streptolysin O is hemolytically active only in its reversibly reduced state. Streptolysin S is stable in the presence of oxygen. Another difference is that streptolysin O is antigenic, the smaller streptolysin S has no antigenic character. The transfer of exogenous proteins into culture animal cells was achieved by streptolysin O permeabilization of plasma membranes (Liu et al. 1993). The pore-forming toxin streptolysin O can be used to reversibly permeabilize adherent and nonadherent cells, allowing the delivery of molecules with up to 100 kDa mass through the plasma membrane to the cytosol. Resealed cells were viable for days and retained the capacity to endocytose and to proliferate (Walev et al. 2001). The pores produced by streptolysin O exceed 13 nm in diameter (Buckingham and Duncan 1983). Antibodies were introduced into cultured myocytes using a developed streptolysin-O permeabilization technique that allowed the targeted cells to

accumulate large biomolecules without perturbing their normal physiological state (Boyle and Lieberman 1999).

The native chromatin conformation was expected to be preserved under mild conditions during streptolysin permeabilization. Results demonstrated that such a permeabilized cell *in vitro* transcription system could be efficient and used to test crude nuclear fractions as well as purified proteins expressed in bacteria. Permeabilization could be a useful tool for the reproduction of transcriptional regulation on chromatin templates *in vitro* as well as the investigation of the biochemical functions of specific transcription factors or signal transduction effectors (Liu et al. 1993). Streptolysisn O- and S-mediated pore formation in the eukaryotic membrane could be accounted for by the trypan blue dye uptake in injured cells and could contribute to keratinocyte damage. Compared to streptolysin O, streptolysin S has a modest influence on streptococcal pathogenicity (Sierig et al. 2003). Direct membrane damage attributable to streptolysin O was reported by others (Madden et al. 2001; Bricker et al. 2002). The cytotoxic effect of streptolysin O can be ascribed to its ability to bind cholesterol and to form holes in the plasma membrane of cells, while the membranes of the endoplasmic reticulum and nucleus remain unaffected (Fig. 3.3).

Perfringolysin, a Cholesterol Dependent Cytolysin belonging to the streptolysin family, has been revealed (Rossjohn 1997), as pore forming, and studied by the same research group (Palmer et al. 1998; Shatursky et al. 1999). The monomers of this toxin diffuse in the membrane bilayer, oligomerize and form homotypic aggregates that create large transmembrane pores. These pores allow large proteins (e.g. antibodies) to enter, but are small for organelles to pass (Campbell et al. 1992). However, as these pores are generally lethal, the application of streptolysin in cell permeabilization was restricted to the investigation of fast cellular processes for only a short period of time (Bakhdi et al. 1993).

Streptolysin O permeabilization turned out to be a simple and effective method to introduce relatively large molecules into rat cardiac myocytes. Moreover, these

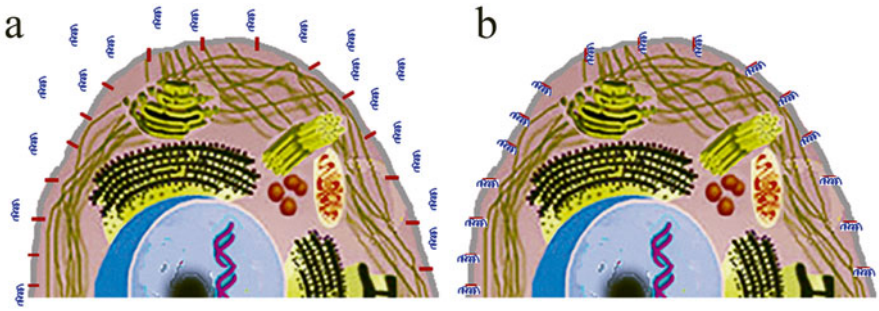


Fig. 3.3 Pore formation in the presence of streptolysin O. (**a**) Eukaryotic cell with cholesterol (*red bars*) embedded in the plasma membrane (*blue line*). (**b**) Incubation in the presence of streptolysin O (*blue* α-helical spirals) resulting in preferential binding to cholesterol and pore formation exclusively in the plasma membrane. The pore size allows to introduce proteins (e.g. antibodies), but prevents the loss of cellular organelles

ventricular myocytes could be resealed after permeabilization and maintained their viability after subsequent incubation with 5 % fetal bovine serum (Fawcett et al. 1998). The permeabilization procedure of myocytes was performed as previously described (Sutherland et al. 1980; Morgan and Morgan 1982). The work of other investigators led to the speculation that solution I rendered cells "hyperpermeable" (McClellan and Winegrad 1978). Solution I consisted of 10 mM EGTA, 5 mM Na_2ATP, 120 mM KCl, 2 mM $MgCl_2$ and N-Tris(hydroxymethyl)methyl-2-aminoethane sulphonic acid (TES) (Morgan and Morgan 1984). The reason of "hyperpermeabilization" has not been explained, but could be related to the advantages of the slightly hypotonic concentration of the permeabilizing solution. Hypotonic driven permeabilization and the delivery of quantum dots into cells as well as reversible permeabilization was achieved by using low concentrations of cell permeabilization agents like saponin (Medepalli et al. 2013). Cells reversibly permeabilized by Streptolysin O could be reprogrammed and partially dedifferentiated in the cell-free system from egg extracts. However, the permeabilizing activity of Streptolysin O was not stable, and therefore difficult to control its activity (Miyamoto et al. 2008). These authors suggested to replace the reversible permeabilization of streptolysin O by digitonin to induce nuclear reprogramming and to activate pluripotent genes in their cell-free system. It was assumed that the reversal of permeabilization was related to the inherent structure of plasma membrane allowing to insert small holes or fine needles or else to remove a lipid mosaic to penetrate the membrane without causing the cell to burst and permits the self-sealing when the permeabilizing agent has been removed or the permeabilizing effect was over.

Cholesterol-dependent cytolysins as pore-forming proteins were expressed by several genera of pathogenic, primarily Gram-positive bacteria causing different diseases. These bacteria include *Streptococcus pyogenes*, responsible for upper respiratory infections, *Bacillus anthracis*, which mostly generates cutaneous infections, and *Listeria monocytogenes*, which causes gastroenteritis (Cassidi and O'Riordan 2013). While the secretion of streptolysin O may spare the extracellular pathogen *S. pyogenes*, from phagocytic killing by the lysis of the target cells (Sierig et al. 2003), in contrast, the phagosome-specific lysine listeriolysin O is necessary to induce autophagy during *Listeria monocytogenes* infection within the cytosol of intact macrophages (Meyer-Morse et al. 2010).

Serratia marcescens hemolysin represents another type of hemolysin that was studied with regard to structure, activation and secretion. This hemolysin has nothing in common with the pore forming toxins of *E. coli* type (RTX toxins), the *Staphylococcus aureus* alphatoxin or the thiol activated toxin of group A beta-hemolytic streptococci (Streptolysin O). The uniqueness of the secretory mechanism of this hemolysin is that its activation requires phosphatidylethanolamine as a cofactor (Hertle 2000).

3.2.6 *Coelenterazine Analogues*

Coelenterazine is a light-emitting luciferin molecule present in many aquatic organisms (Fig. 3.4), the substrate for a group of bioluminescent enzymes obtained from marine organisms (Shimomura 1997, 2006). The commercially available coelenterazine analogues suitable for measurements in live cells use the *Renilla luciferase* reporter enzyme (Zhao et al. 2004).

The photoprotein aequorin has been widely used as an intracellular probe for Ca^{2+} for decades by emitting light in the presence of Ca^{2+} while decomposing into apoaequorin, coelenteramide and CO_2. The reverse reaction in the presence of apoaequorin, coelenteramide and O_2 produces aequorin (Shimomura and Johnson 1975, 1978). Several kinds of semi-synthetic aeqorins were prepared from synthetic analogues of coelenterazin (Shimomura et al. 1988, 1989, 1990). Coelenterazine serves to permeate the plasma membrane and aequorin to study intracellular Ca^{2+} levels. The advantage of avoiding microinjection to introduce aequorin into cells is that microinjection is difficult to perform and can be impractical when cells are small (Shimomura et al. 1993). Recently the analytical use of Ca^{2+}-regulated photoproteins isolated from luminous coelenterates shifted to real-time non-invasive visualization of intracellular calcium concentration in cells and whole organisms. A limitation of this approach for *in vivo* deep tissue imaging is that the damaging blue light emitted by the photoprotein is highly absorbed by tissues. Novel coelenterazine analogues were synthesized and their effects on the bioluminescent properties of recombinant obelin from *Obelia longissima* and aequorin from *Aequorea victoria* have been utilized (Gealageas et al. 2014).

3.3 Organic Solvents

To introduce large molecules (e.g. antibodies) into cells, they have to be fixed with cross-linking agents such as formaldehyde, paraformaldehyde or glutaraldehyde then permeabilized. Two major types of chemicals are used for permeabilization, organic solvents and detergents. This subchapter provides a brief review of organic solvents. The detergents will be discussed in the next subchapter. The organic

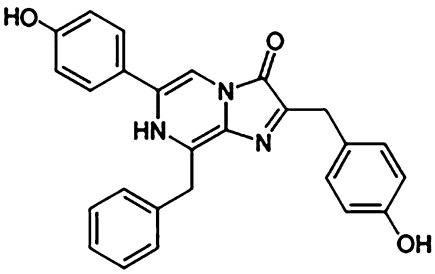


Fig. 3.4 Chemical 2D structure of coelenterazine. Molecular formula: $C_{26}H_{21}N_3O_3$. IUPAC: 6-(4-hydroxyphenyl)-2-[(4-hydroxyphenyl) methyl]-8-(phenylmethyl)-7H-imidazol[3,2-a] pyrazin-3-one

solvents dissolve lipids from cell membranes making them permeable to antibodies. Because the organic solvents also coagulate proteins, they can be used to fix and permeabilize cells at the same time (Jamur and Oliver 2010). The time of fixation depends on cell type, furthermore the fixation strength and time are shorter for cells than for tissue sections. For immunocytochemistry, the target cells or tissues are fixed to a slide. There are different methods to attach cells to the surface of the slides by:

– linkage to the solid support by chemical linkers,
– adherent cells can be grown on microscope slides,
– suspension cells are centrifuged onto slides.

Fixation immobilizes antigens, retains cellular and subcellular architecture and permits access to antibodies. There are two major groups of fixatives, organic solvents and cross-linking agents. For fixation it is better to use cross-linking agents such as paraformaldehyde or formaldehyde as they preserve cell structure, but may reduce antigenicity. Organic solvents (e.g. alcohol, ether, aceton) remove lipids (permeabilize), dehydrate and precipitate (fix) cells while maintaining the cellular and subcellular structures.

Classification of Fixatives The most simple classification distinguishes between protein coagulative and crosslinking (noncoagulative) agents. A more refined way of distinction listed below is based on the classification of Bancroft and Stevens (1986). According to this classification most popular fixatives for immunochemical staining are aldehydes, protein-denaturing agents, and acetone.

Major groups of fixatives include:

- Aldehydes: formaldehyde (formalin = 40 % solution of formaldehyde, paraformaldehyde), glutaraldehyde, acrolein, glyoxal; formaldehyde mixtures containing mercuric chloride, acetic acid, zinc, periodate lysine.
- Protein-denaturing agents (precipitants): acetic acid, methanol, ethanol, industrial methylated spirits.
- Oxidizing agents: osmium tetroxide, potassium permanganate, chromic acid, potassium dichromate. These compounds have found certain applications in specific histological preparations.
- Mercurials (B-5 and Zenker's fixative) that despite of being rapid, penetrate poorly and produce tissue shrinkage. B-5 fixative (containing the corrosive mercuric chloride and sodium acetate) is routinely used for bone marrow biopsies, and lymph nodes. Zenker's fixative (named after Konrad Zenker, German histologist) is employed to animal or vegetable tissues for microscopic study. Zenker' fixative provides excellent fixation of nuclear chromatin, connective tissue fibers and cytoplasmic organelles, primarily mitochondria. Zenker's fixative contains mercuric chloride, potassium dichromate, sodium sulfate, water, and acetic acid. The modified Henker's solution known as Helly's fixative or "formol Henker" is preferred for the staining of mitochondria. The additional formalde-

hyde component in the "formol-Henkel" fixative reacts with dichromate and produces formic acid and chromic ions that change the orange solution to greenish.
- HOPE. The fixative abbreviated HOPE (hepes-glutamic acid buffer-mediated organic solvent protection effect) is a formalin-like fixative with excellent preservation capability used in immunohistochemistry and enzyme histochemistry.
- Carbodiimide cross-linking agents. These peptide coupling compounds are frequently used because carbodiimide binding does not need the prior activation of the carboxylic acid (http://www.sigmaaldrich.com/technical-documents/articles/chemfiles/carbodiimides.html)
- Physical fixation: heat; microwaves previously mentioned, but not to be discussed.
- Miscellaneous fixatives: nonaldehyde-containing fixatives, picric acid, acetone. Picrates (salts or esters of picric acid *alias* 2,4,6-trinitrophenol) react with histones and amino acids and precipitate proteins. Acetone will be discussed separately.

Of the different types of chemical fixatives crosslinking agents (e.g. aldehydes) have already been mentioned. More attention will be given to other chemical fixatives primarily to precipitating agents (alcohols), and less consideration will be given to other fixatives. In the process of chemical fixation the cellular structures are preserved in a state close to the living cells and tissues. Chemical fixatives stabilize the proteins and nucleic acids of the samples by making them insoluble. Physical fixation, e.g. heat, microwave drying to remove the water component of the sample, paraffin embedding of delicate tissues or embedding in cryoprotective medium then snap-freezing and storage in liquid nitrogen and sectioning will not be detailed.

3.3.1 Alcohols (Methanol, Ethanol)

Alcohol permeabilization has been preferentially used for DNA analysis by flow cytometry (Ormerod 2002) and phospho-epitop staining of proteins (Krutzik and Nolan 2003). Alcohols fix and permeabilize cells by dehydrating them and solubilize molecules out of the plasma membrane. Proteins become more accessible to antibodies during the process and cells are permeabilized to a greater extent than with detergents such as saponin, allowing efficient access to nuclear antigens. Flow cytometry analysis of intracellular proteins use commercially available kits (e.g. Leukoperm, IntraStain, Fix&Perm) (Koutna et al. 2012). The flow cytometric application of ethanol as permeabilizing agent for DNA analysis of synchronized fractions of human erythroleukemia K562 cells is exemplified by Fig. 3.5. Applications of reversible permeabilization will be detailed in Chap. 4.

Methanol Alcohols, such as methanol or ethanol are commonly used to permeabilize cells. Several protocols are available for intracellular staining with methanol permeabilization. For immunostaining the methanol permeabilization step involves the covering of formaldehyde or paraformaldehyde fixed cells or tissue sections.

a

Fraction-number	Flow rate ml/min	Non-irradiated cells: Cell number ($x10^6$)	Non-irradiated cells: C-value	Gamma-irradiated cells: Cell number ($x10^6$)	Gamma-irradiated cells: C-value
1	21.5	19.2	2.07	17.6	2.06
2	23.5	15.8	2.18	13.8	2.11
3	25.5	16.7	2.46	19.9	2.38
4	27.6	18.1	2.73	15.8	2.37
5	29.7	17.9	2.95	18.2	2.52
6	31.9	18.4	3.21	19.8	2.85
7	34.0	17.9	3.47	19.1	3.13
8	36.1	13.2	3.69	12.3	3.42
9	38.3	8.9	3.95	9.0	3.70

Recovered by elutriation: 1.48×10^8 cells (98.6%) | 1.45×10^8 (96.6.0%)

Loaded to elutriation chamber: 1.5×10^8 cells (100%) | 1.5×10^8 (100%)

Loss of cells during elutriation: 2×10^6 cells (1.3%)

Further loss of cells due to radiation: 3×10^6 cells (2.0%)

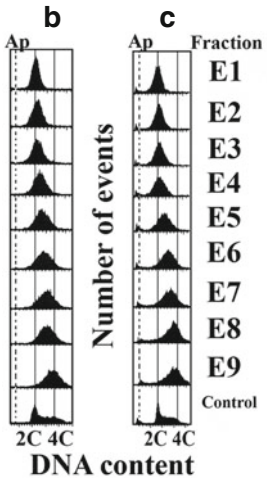


Fig. 3.5 Characterization of human erytroleukemia K562 cell populations synchronized by centrifugal elutriation and subjected to flow cytometry after ethanol permeabilization. K562 cells were exposed to 4 Gy γ-irradiation, incubated for 2 h. (**a**) Fractions of control and gamma-irradiated cells were obtained by elutriation (With permission, Banfalvi, p. 336). Cell cycle patterns of various elutriated fractions (1–9) in untreated (**b**) and irradiated K562 cells (**c**) were analyzed by FACS after ethanol permeabilization and propidium iodide staining. For staining the nuclear material, cells were placed for 15 min in ice cold 0.1 M ammonium citrate containing 50 μg/ml propidium iodide followed by the addition of an equal volume of cold 70 % ethanol. The control population was not elutriated. DNA content is expressed in C-values. *2C*, diploid genome content; *4C*, tetraploid DNA content; *Ap*, small apoptotic cells generated by γ-irradiation appearing in the sub-Go/G1 window (Reproduced with permission (Banfalvi et al. 2007)

Formaldehyde is normally used only to fix cells, paraformaldehyde may itself transiently permeabilize the plasma membrane to some extent, but unlikely to allow access to the nucleus. To permeabilize the nuclear membrane paraformaldehyde is used in combination with a detergent, preferentially with Triton X-100. The fixation and permeabilization methods depend on the sensitivity of the epitope and antibody and may require some optimization. Different periods of permeabilization ranging between 10 min and 1 month at storage temperatures (from +4 °C to −80 °C) are followed by rinsing in physiological buffer (normally PBS) and staining with antibody.

Flow cytometry protocols for intracellular staining typically use cold methanol as a permeabilization agent to detect phosphorylated proteins, transcription factors under increased reactivity of antibodies to specific nuclear antigens (https://www.rndsystems.com/resources/protocols/flow-cytometry-protocol-staining-intracellular-molecules-using-alcohol). However, it was demonstrated by reflection contrast microscopy and transmission electron microscopy that acetone or methanol fixation resulted in complete loss of integrity of intracellular structures in contrast to paraformaldehyde or glutaraldehyde fixation. Poor preservation of plasma membrane integrity after fixation in acetone or methanol was seen by scanning electron microscopy. Fixation with paraformaldehyde before methanol treatment reduced

damage to intracellular and plasma membranes (Hoetelmans et al. 2001). Less attention has been paid to the introduction of immunoreagents into cells that can cause protein extraction and relocation not representing the *in vivo* situation. To avoid pitfalls originating from experiments in dead, permeabilized cells other authors suggested the complementation of fixation with live-cell imaging when it comes to the scrutinization of protein localization (Schnell et al. 2012).

Methanol fixation methods (https://www.usbio.net/misc/Immunocytochemistry):

- Cells are fixed in −20 °C methanol for 5–10 min. No permeabilization step are needed following methanol fixation.
- Methanol-Acetone Fixation. Cells are fixed in cooled methanol for 10 min at −20 °C. Excess methanol is removed. Permeabilization with cooled acetone takes place for 1 min at –20 °C.
- Methanol-Acetone Fixation in 1:1 methanol and acetone mixture at −20 °C for 5–10 min.
- Methanol-Ethanol Fixation in 1:1 methanol and ethanol mixture at −20 °C for 5–10 min.
- Paraformaldehyde-Methanol Fixation in 4 % paraformaldehyde for 10–20 min. Rinsing briefly with PBS. Permeabilization with cooled methanol for 5–10 min at −20 °C.
- Methanol-Acetic acid 3:1 is generally used for the for the isolation of mitotic chromosomes from cell cultures and chilled on ice before use.

Methanol was introduced as a fixative in hematology (Romeis 1948; Roulet 1948) and remained the most common fixative for blood and bone marrow aspirate smears. The fixative effect of methanol is based on protein dehydration and precipitation. As fixative methanol was traditionally applied in hematology before the Romanowsky staining (Romanowsky 1891). This staining was the forerunner of several other staining methods (Giemsa, Jenner, Wright, Field, and Leishman), to differentiate cells in pathologic conditions. Methanol is rarely used alone as a fixative beside blood and bone marrow aspirate smears. Fixative mixtures containing methanol are usually water free. As commercially available ethanol (95 %) is more expensive and not freely available in some institutions, it was replaced by methanol and was reported to be as effective as ethanol for fixation of smears (Kumarasinghe et al. 1997). In Carnoy's fluid ethanol is substituted with methanol and the fluid is named methacarn. Methacarn consists of 60 % absolute methanol, 30 % chloroform and 10 % glacial acetic acid. Methacarn fixation of tissues takes only 4 h. Specimens can be stored in fixative for several weeks without apparent harm to tissue morphology or antigenicity (Puchtler et al. 1970; http://www.cpl.colostate.edu/pathology/protocols/methacarn.htm).

Ethanol There are serious reservations against the use of ethanol for cell fixation. Although, both methanol and ethanol remove the lipids from the membrane, dehydrate cells, and precipitate proteins, ethanol has a stronger tendency to disrupt hydrophobic and hydrogen bonds in cellular structures. Early attempts to remove lipids from tissues revealed that no single pure solvent appeared to be suitable as a general-purpose lipid extractant, but evidence was presented that ethanol (20 ml/g

tissue) extraction for 5 min extracted essentially all the lipids from liver homogenates (Lucas and Ridout 1970). Other observations indicate that the stimulation of plasma membrane calcium pump is the result of a direct interaction of ethanol with the C-terminal regulatory domain of the Ca^{2+} pump (Cervino et al. 1998). When ethanol and calmodulin were present simultaneously, the stimulatory effects of ethanol and calmodulin have been found to be additive suggesting that ethanol and calmodulin stimulate the Ca^{2+}-ATPase through different mechanisms. The stimulatory effect of ethanol was significant pointing to its potential implications of toxicological relevance (Benaim et al. 1994). Due to the fact that ethanol affects the structure of some proteins, it is usually not the preferred agent to fix cells. Although, ethanol is not a preferential agent for fixing and permeabilizing cells, the toxicity of 70 % ethanol has been found to be the most effective in killing microbes (bacteria, fungi) and some viruses, but ineffective against bacterial spores. The killing effect of 70 % ethanol is related to the destruction of bacterial cell membrane and by denaturation of proteins and lipids in solutions. Ethanol in 70 % solution is generally used for wiping microbiological working areas. Some institutions and hospitals use somewhat higher ethanol concentration, but ethanol is an effective disinfectant at concentration between 70 and 90 %. The percentage of ethanol is a product of experience with 70 % being the best percentage for killing pathogens and other microbes. Interestingly, no experimental evidence has been presented why diluted ethanol does kill more efficiently than undiluted ethanol, but the reason is likely to be related to denaturation (Ali et al. 2001). Higher than 90 % ethanol concentration may immobilize and "seal" the bacteria leading to their survival.

Davidson's fixative is an ethanol containing mixture particularly useful for preparing tumor and bone marrow specimens, gynecologic material, fatty breast, and medical biopsies. This fixative consists of two parts of 37 % formalin, three parts of ethanol, one part of glacial acetic acid, three parts of tap water and sufficient eosin to color. Acetic acid alone is associated with tissue swelling; combining with alcohol may result in better preservation of tissue morphology. Acetic acid is present in Davidson's fixative as a denaturant that is applied in combination with other precipitating fixatives (http://microvet.arizona.edu/research/aquapath/davidson.htm).

To get the right ethanol dilution the following instructions should be kept in mind:

(i) The concentration of ethanol as an azeotrop is around 95.6 %. Diluted ethanol is no longer an azeotrope and its evaporative loss will result in a gradual decrease of ethanol concentration. It is not advised to keep diluted (70 %) ethanol in unsealed beakers on the bench or elsewhere as it may lose its disinfectant effect.

(ii) Due to the formation of hydrogen bonds between ethanol and water, the mixing of 700 ml ethanol (100 %) and 300 ml water will result in less than 1 L 70 % ethanol. To get the correct concentration 700 ml absolute alcohol is brought to 1000 ml with distilled water. Water in chemistry and biology means distilled water unless otherwise noted (e.g. tap water, sea water, spring water, demineralized, bi-distilled water, etc.).

(iii) Alcohol is to be stored in glass bottles.

3.3.2 Acetone

Organic solvents including acetone remove lipids and dehydrate cells while precipitating their proteins. The most popular fixatives used for immunochemical staining techniques are aldehyde fixatives, protein-denaturing agents, and acetone. Acetone similarly to alcohol penetrates tissues poorly and is regularly used on tissue sections or cytological preparations. For cell-surface markers acetone appears to be one of the best and most commonly used fixatives. The only restriction to be imposed is the avoidance of extended buffer washes during immunocytochemical staining that can create morphological changes in acetone-fixed tissue such as damages of cell membranes and chromatolysis. Fixing frozen sections at room temperature in acetone followed by drying at room temperature could improve morphology, but may increase background staining. Extended fixation in acetone should be avoided to prevent the fragility of tissues (Renshaw 2007). Acetone is a noncoagulative fixative that removes lipids from cell membranes and aids antibody penetration. The combination of acetone with nonionic detergents (Triton X- 100, Tween 20) or saponin with fixative solutions can be more efficient (Laurila et al. 1978), but is not recommended for electron microscopy as it can deteriorate subcellular morphology (Renshaw 2007). The sensitivity of cells to acetone depends on cell type. The human skin cells are more sensitive to acetone than fibroblasts (Pace and Elliot 1962).

3.3.3 Ether

Chemical methods have been employed to extract intra cellular components from microorganisms by permeabilizing the outer-wall barriers. This can be achieved with organic solvents that act by creating pores through the cell membrane. Among these solvents are toluene, ether, phenylethyl alcohol, dimethyl sulfoxide (DMSO), benzene, methanol, chloroform. Other chemical permeabilizations were achieved with antibiotics, thionins, surfactants (e.g. Triton, Brij, Duponal) chaotropic agents, and chelates that will be discussed under Mild Detergents.

Ether treated bacteria, such as *E. coli* and other Gram-negative bacteria, contained in a cell-free system were used to study murein biosynthesis (Maass and Pelzer 1981). Cells of *Saccharomyces cerevisiae* were permeabilized by ether for the isolation of coenzyme NADH, resulting in the recovery of 80 % NADH present in the cell (Goyal and Chand 1996). This method is utilized as ether shake applied by clinical microbiologists to clean up samples heavily contaminated with Gram-positive organisms. Permeabilized cells were prepared by treatment with diethyl ether (Vosberg and Hoffmann-Berling 1971; Dupont and Clarke 1991). Whole-cell permeabilization for in situ assays has been achieved with organic solvents including ether or a solution containing toluene and ethanol (Oertel and Goulian 1979).

3.3.4 Toluene Treatment

Interest in whole-cell permeabilization to develop enzyme assays has arisen when it became evident that *in vitro* results cannot be extrapolated to enzyme concentrations found *in vivo* (Serrano et al. 1973). It turned out that the comparison of different permeabilization methods was difficult because each procedure produced cells with different permeability alterations and the degree of permeabilization varied depending on experimental conditions. As there is no uniform method of permeabilization that could be generally used, several chemicals and combinations of permeabilizing agents have been tested, compared and recommended.

Toluene treatment of bacteria has been originally used for sterilization purposes. Toluene treatment to permeabilize bacterial cells has been used for 50 years to make enzymes within a cell accessible to exogenous substrates (Jackson and DeMoss 1965). During the early attempts of whole cell permeabilization toluene was one of the most frequently applied permeant to introduce various compounds into cells (Moses and Richardson1970; Burger and Glaser 1973; Deutsch et al. 1976; Peterson et al. 1971; Reeves and Sols 1973; Henderson and Snell 1971; Hilderman and Deutscher 1974; Matlib et al. 1977; Basabe et al. 1979). The cell membranes of *Salmonella typhimurium* and *Escherichia coli* have been made permeable with toluene to introduce S-adenosylmethionine into the cell for study of the course of methylation (Paoni and Koshland 1979). In the presence of Mg^{2+}, toluene removed very little protein, phospholipid, or lipopolysaccharide from *E. coli*. In the absence of Mg^{2+}, or in the presence of EDTA, toluene removed considerably more cell material, including several specific cytoplasmic proteins such as malate dehydrogenase (de Smet et al. 1978). Electron microscopic and biochemical examinations of toluene-treated liver cells confirmed that they were relatively intact and lost only small amounts of cellular constituents to the suspension medium (Hilderman et al. 1975). Similarly to whole cells, mitochondria made permeable with toluene lost only small amounts of their proteins and retained a major fraction of the nucleotides and coenzymes and allowed the study of mitochondrial enzymes at approximate *in vivo* concentrations (Matlib et al. 1977). The transfer of ribonucleic acid (tRNA) nucleotidyltransferase was studied after making cells permeable to macromolecules by treatment with toluene (Deutscher 1974).

It became known at the beginning of the 1970s that toluene made bacterial cells permeable to molecules such as nucleoside triphosphates. Toluene treatment provided an important and easily obtainable *in vitro* system, to study macromolecular biosynthesis including DNA synthesis (Moses and Richardson 1970), RNA synthesis (Peterson et al. 1971), and peptidoglycan synthesis (Schrader and Fan 1974). Toluene-treated cells permeable to macromolecules have also been used in enzyme purifications (Deutscher 1974). Nascent DNA synthesized in toluene permeabilized *Bacillus subtilis* cells in the presence of 5′ mercurydeoxycytidine triphosphate and 2′ 3′ dideoxyATP has been isolated and subjected for the analysis of Okazaki fragments (Banfalvi and Sarkar 1983). The origin and degradation of RNA primers at the 5′ termini of nascent DNA chains was followed in *B. subtilis* cells permeabilized

with toluene (Banfalvi and Sarkar 1985). When tetracycline susceptible and resistant *E. coli* cells were permeabilized with toluene, it was found that protein synthesis in the two strains became equally sensitive to tetracycline, suggesting that an intact membrane was required for resistance (McMurry et al. 1986).

Combined toluene-ethanol treatment was applied for young hyphae of *Neurospora crassa* that were made permeable to UDP-glucose and trypan blue (Quigley et al. 1987). Permeabilization has been accomplished by toluene-ethanol combined treatment in *Neurospora crassa* and nuclear, mitochondrial and cytosolic enzyme activities have been measured in permeable cells (Basabe et al. 1979).

3.4 Mild Detergents

Detergents are useful reagents to mimic lipid bilayers due to their self-assembling properties, but with complex properties in solution. Mild detergents are widely used for intramembrane protein manipulation. Many membrane proteins solubilized with these agents tend to denature and/or aggregate (Privé 2007). Based on their chemical structure different types of detergents have been distinguished (Table 3.1):

Ionic detergents have a hydrophilic head group that can be either negatively (anionic) or positively (cationic) charged. Ionic detergents serve the disruption of cellular structures and denaturation and separation of proteins. Ionic detergents bind to protein molecules and mask their native charge.

Non-ionic detergents. The hydrophilic head group of non-ionic detergents is uncharged. They are preferred to break lipid-lipid and lipid-protein interactions, but have a limited potential to disturb protein-protein interactions. Non-ionic detergents are known as non-denaturing agents and are used for permeabilization and to isolate biologically active (membrane) proteins.

Zwitterionic detergents protect the native state of proteins without altering the native charge of the protein molecules. Zwitterionic detergents are used for isoelectric focusing and 2D electrophoresis. Synthetic zwitterionic detergents are known as sulfobetaines. Sulfobetaines retain their zwitterionic characteristics over a wide range of pH. Zwitterionic detergents are most efficiently and widely used for 2D gel electrophoresis. The structural details of these molecules (CHAPS, CHAPSO, sulfobetaine 3-10 (SB 3-10), etc. are given in the websites of http://www.sigmaaldrich.com/life-science/biochemicals/biochemical-products.html?TablePage=14572925; http://www.gbiosciences.com/research-products/protein-research/detergents-and-accessories/zwitterionic-detergents.aspx.

Chaotropic agents are molecules dissolved in water that are able to disrupt the hydrogen bonding network and increase the hydrophobic effects within the solution. Chaotropic agents reduce the hydration shells around hydrophobic amino acids, and nucleic acids and may cause denaturation. Chaotropic agents are: butanol, ethanol, guanidinium chloride, lithium perchlorate, lithium acetate,

Table 3.1 Type of detergents

Type	Chemicals
Ionic	Cholate, deoxycholate, hexadecyltrimethylammonium bromide, sodium dodecyl sulfate, sodium dodecylbenzenesulfonate
Non-ionic	Benzalkonium chloride, Brij compounds, digitonin, maltosides, Triton X compounds, Tween compounds
Zwitterions	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)
	3-[(3-Cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO)
	N-Decyl-N,N-dimethyl-3-ammonio-1-propanesulfonate (Sulfobetaine 3–10)
Chaotrops	Guanidinium chloride, sodium dodecyl sulfate, thiourea, urea

magnesium chloride, phenol, propanol, sodium dodecyl sulfate, thiourea, urea, etc. More attention will be paid only to those mild detergents that are able to permeabilize biological membranes.

3.4.1 Digitonin

Digitonin is the glycoside of the plant *Digitalis purpurea* (foxglove). It is used as a nonionic detergent to water-solubilize effectively lipids. Beside solubilizing membrane proteins, other membrane-related applications include the precipitation of cholesterol, and most importantly from our point of view the permeabilization of cell membranes. Digitonin, is commonly employed for pore formation in various cell membranes (Kholodkov et al. 2015). In contrast to other detergents (e.g. Triton X-100) digitonin has the ability to permeabilize preferentially the plasma membrane of mammalian culture cells while leaving the nuclear envelope (Griffis et al. 2003) and other intracellular organelles intact (White et al. 2015). Similarly, by the optimization of the concentration of digitonin, the plasma membrane was permeabilized, but not the endoplasmic reticulum of mammalian cells (Tissera et al. 2010).

Digitonin permeabilization did not affect mitochondrial function, and allowed the determination of mitochondrial membrane potential of *Trypanosoma cruxi* in situ (Vercesi et al. 1991). The preferential permeabilization of digitonin was developed into a simple and effective method for assessing the cytosol vs. mitochondrial localization of the apoptosis regulator Bax protein by digitonin fractionation, which uses a low concentration of detergent to permeabilize the plasma membrane without damaging intracellular membranes. This method allowed the separation of the cytosolic light membranes from the heavy membranes (mitochondria from nuclei) by centrifugation (Dewson 2015). Digitonin extraction served also the measurement of enzymatic activity of released lysosomal hydrolases (Aits et al. 2015). To the contrary, other authors found that the tuning of digitonin concentration allowed the selective permeabilization of not only the plasma but also the mitochondrial membranes (Niklas et al. 2011). Digitonin permeabilization leaved the nuclear membrane

intact and competent for faithful, signal-dependent translocation through the nuclear pore complex (Liu et al. 1999). Nucleocytoplasmic transport of fluorescently labeled DNA using a digitonin-permeabilized cell system showed that as a result of nuclear import DNA accumulated in the nucleus in over 80 % of the permeabilized HeLa cells (Hangstrom et al. 1997).

Another major advantage of digitonin induced permeabilization is its reversibility tested in bovine, mouse, and porcine somatic cells. When permeabilized cells were treated with *Xenopus laevis* egg extracts, cells showed exchange of nuclear proteins from extracts and after resealing of the membrane, the cells showed up regulation of gene expression. These results suggested that reversible permeabilization with digitonin could be used to induce nuclear reprogramming and to activate pluripotent genes by a cell-free system (Miyamoto et al. 2008).

Digitonin was used to permeabilize cells for antibody penetration into the nucleoplasm but was less efficient than NP-40 (David and Yewdell 2015). The Tergitol-type NP-40 (nonyl phenoxypolyethoxylethanol) detergent is related but not identical with Nonidet P-40 (octyl phenoxypolyethoxylethanol). NP-40 is a powerful detergent used to open all membranes within a cell including the nuclear membrane. Partial permeabilization of plasma membrane of canine myocardium and ventricular myocytes with digitonin and cell disruption, followed by differential and sucrose density centrifugation provided rapid separation of non-nuclear membranes and cytosol from nuclei (Tadevosyan et al. 2015).

Digitonin is not the only compound derived from plant that is able to permeabilize plasma membrane. Similarly to digitonin betulinic acid isolated from the bark of white birch (*Betula pubescens*) elicits eryptosis/erythroptosis in human erythrocytes through Ca^{2+} loading and membrane permeabilization. In addition betulinic acid was reported to induce apoptosis through mitochondrial dysfunction making it a potential anti-cancer agent (Gao et al. 2014). Digitonin is not to be confused with the cardiac drug digoxin (digitalis or digitoxin), althouh both digitonin and digoxin have been derived from the same plant.

3.4.2 Saponin

Saponins are amphipatic glycosides with vast structural and functional diversity causing soap-like foaming in aqueous solutions isolated from various plant species (Hostettman and Marston 1995), especially from the green parts of potato (Fig. 3.6) and tomato (Ahmed and Müller 1981). Commercial saponins are extracted mainly from Yucca schidigera and Quillaja saponaria. Saponin is a mixture of triterpenoid molecules and glycosides that permeabilize cells by interacting with cholesterol present in the cell membrane (Melan 1999). It is assumed that the widely used detergent saponin could extract cholesterol from membranes (Mercanti and Cosson 2010), but less prone than non-ionic detergents like Triton X-100 or NP-40 to extract membrane proteins from cellular membranes (Goldenthal et al. 1985). The interactions of saponins with lipid membranes are largely unknown, as are the roles of cholesterol or the branched sugar moieties on the aglycone backbone. A

curvature-driven permeabilization mechanism dependent on the interaction between saponin and sterols and on the molecular shape of the saponin and induction of local spontaneous curvature was proposed (Lorent et al. 2013). It is likely that saponin as a relatively mild detergent solvates cholesterol present in the plasma membrane. At low concentrations (<0.5 mg/ml) of saponin internal membranes remain intact. Saponin is typically used at 0.5–1 mg/ml. It is useful for labeling smaller molecules that exist in a soluble state within the cytoplasm.

One of the saponins, solanine contains the trisaccharide solatriose consisting of glucose, galactose and rhamnose (Fig. 3.6). It is assumed that solanine causes the depolarization of mitochondrial membrane and increases the calcium content of the cytoplasm. At least some of the elevated calcium level originates from the damaged mitochondria relative to intact cells where the mitochondrial calcium concentration is significantly higher than in the cytoplasm. The elevated calcium concentration in the cytoplasm is likely to lead to apoptosis (Gao et al. 2006). α-Hederin (kalopanax-saponin A was isolated from the leaves of *Hedera helix* (ivy) (van de Haar 1912; Choi et al. 2008). This saponin molecule possesses strong hemolytic activity (Chwalek et al. 2006; Gauthier et al. 2009), and cytotoxicity against *in vivo* tumors; which could be utilized against various cancer cell lines, and similarly to other saponins could result in apoptosis (Choi et al. 2008; Kumara and Huat 2001; Swamy and Huat 2003). From an ecological perspective, saponins are synthesized by a variety of plants to function as antimicrobial phytoprotectants (Papadopoulou et al. 1999). It was expected that the antibacterial effect of the detergent-like saponins could be utilized against multidrug-resistant *E. coli* strains, although cytotoxicity against these bacterial strains was not observed by others (Arabski et al. 2012).

Permeabilization of mammalian plasma membranes by incubation of cells with saponin has been used with smooth muscle cells (Guthrie et al. 1991) and cells or tissue preparations from liver (Fabbrizzio et al. 1994), pancreas (Schultz 1990), skeletal muscle (Huchet and Leoty 1994), cardiac muscle (Zhu and Nosek 1991), and other cells (Fabbrizio et al. 1994). Despite their utility for other purposes, these methods were limited because they either allowed only a small percentage of cells to incorporate membrane-impermeable molecules (Shubeita et al. 1992) or, severely compromised myocyte functions (Johnson et al. 1996).

The usefulness of saponin (from *Gypsophila plants* in the carnation family) as a tool for investigating the interior of different intracellular compartments of rat hepatocytes has been demonstrated by differential permeabilization. At low saponin concentration (40 μg/ml) the plasma membrane, at higher concentration (200 μg/ml) the endoplasmic reticulum was permeabilized. The Golgi complex became permeable at intermediate saponin concentration (Wassler et al. 1987). Saponin permeabilization allowed the cytometric analysis of nuclear and other intracellular antigens (Jacob et al. 1991).

Saponin is known to interact with membrane cholesterol, selectively removing it and leaving holes in the membrane. The disadvantage of other detergents such as Triton X-100 and Tween-20 is that they are non-selective in nature and may extract proteins along with the lipids (Jamur and Oliver 2010). Pores created by saponin in the plasma membrane are large enough for the entry of antibodies. To prevent the leaking out of proteins, saponin-treated cells are exposed to crosslinking reagents,

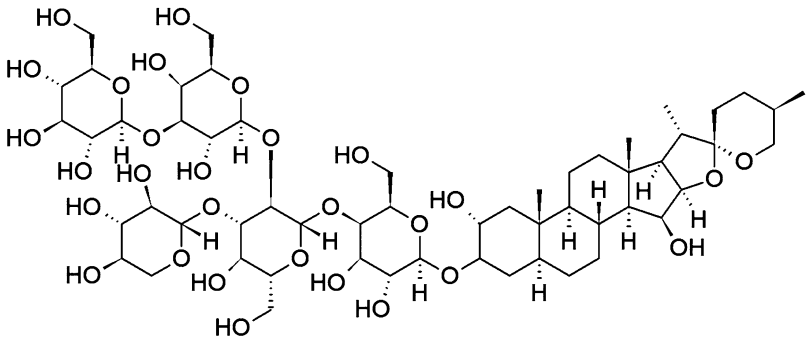


Fig. 3.6 Chemical structure of the saponin α-solanine

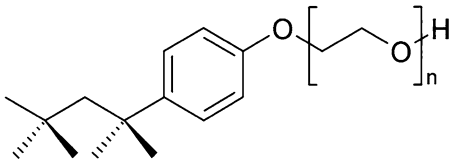


Fig. 3.7 Chemical structure of Triton X-100 (polyethylene glycol p-(1,1,3,3-tetramethylbuthyl)-phenyl ether). Other names: octylphenol ethylene oxide condensate; Octoxynol-9; toctylphenoxy-polyethoxyethanol. Average length of ethylene oxide units, n = ~8.5

such as formaldehyde. Saponin permeabilization has become the detergent of choice in phosphoepitope staining protocols (Pala et al. 2000).

The combination of paraformaldehyde with saponin was less efficient, it caused an increase in non-specific binding of antibodies (Koutna et al. 2012). These authors have also found that proteins were more accessible to antibodies when cells were permeabilized with alcohol than with saponin, allowing efficient access to the nuclear antigens. Reversible permeabilization was obtained when osmosis driven fluid transport achieved by hypotonic environment was combined with low concentrations of permeabilizing agents like saponin (Medepalli et al. 2013).

Despite their usefulness the methods of saponin permeabilization were limited only to a small percentage of cells to incorporate membrane-impermeable molecules (Shubeita et al. 1992). Saponin severely compromised cellular functions unless permeabilization was improved in the presence of ATP (Johnson et al. 1996). Another limitation was that detergent permeabilization was performed at relatively high room temperature (Schultz 1990; Guthrie et al. 1991; Hutchet and Leoty 1994; Zhu and Nosek 1991), leading to proteolysis and cell-degradative processes. Permeabilization lasted for the duration of the experiments (Schultz 1990; Guthrie et al. 1991; van Heugten et al. 1994) or allowed the release of intracellular proteins (Fabbrizio et al. 1994). Finally the effects of agonists such as cAMP or 1,4,5-inositol tris-phosphate, were either abolished (van Heugten et al. 1994) or could not be evaluated (Guthrie et al. 1991; Hutchet and Leoty 1994; Zhu and Nosek 1991).

3.4.3 Triton X100

Triton X100 (Fig. 3.7) is a non-ionic surfactant for the recovery of membrane components under non-denaturing conditions used primarily for immunofluorescent staining. Triton X-100 solvates efficiently cellular membranes without disturbing protein-protein interactions. Efficient Triton X-100 concentrations for permeabilization range from 0.1 to 1 %. Triton X-100 has no antimicrobial potential. When Triton-X100 was administered at or near the minimum effective concentrations, cell permeabilization could be reversed within 24 h, and cells continued to proliferate and showed metabolic activity during the restoration of membrane integrity (van de Ven et al. 2009).

Triton X-100 is closely related to IGEPAL® CA-630 or Nonidet P-40, which has somewhat shorter ethylene oxide units. Nonidet P-40 will not be discussed as it is no longer commercially available. Triton X-100 is more hydrophilic than IGEPAL® CA-630 and is not considered functionally interchangeable for most applications (http://www.snowpure.com/docs/triton-x-100-sigma.pdf). Sodium dodecyl sulfate (SDS) is another non-ionic detergent commonly used to denature proteins for electrophoresis. Although, SDS could be useful as a permeabilizing agent, but it induces slight denaturation of fixed cells in order to reveal epitopes, thus no further consideration will be given to SDS.

Triton X-100 is among the most widely used nonionic surfactants for either lysing cells and extract cytoplasmic protein and other cellular organelles or to permeabilize the living cell membrane for transfection (Genusso et al. 2004; Rajagopal et al. 2002; Hipfner et al. 1994). Similarly to the improved permeabilization of cardiac myocytes with saponin in the presence of ATP (Johnson et al. 1996), the motility of equine sperm permeabilized with 0.02 % Triton X-100 was maximized by the presence of ATP but not with cAMP (Loux et al. 2014).

3.4.4 Tween 20 and Tween 80

Tween 20 is polysorbate 20. Its synonyms are polyethylene glycol sorbitan monolaurate, polyoxyethylenesorbitan monolaurate. Other commercial names: Scattics emulsifier, Alkest TW 20. The chemical structure of Tween 20 contains a polar ethylene tail, a polar polyethylene head group and a lauric acid ester with an apolar tail (Fig. 3.8a). The structure of polysorbate 80 is similar but contains oleic acid instead of lauric acid (Fig. 3.8b). The brand names of Tween 80 are Alkest TW 80, Scattics, Canarcel, Poegasorb 80. Polysorbates are relatively stable to electrolytes, saponify with strong acids and bases. Polysorbates are known to be incompatible with alkalis, heavy metal salts, phenols, and tannic acid. The oleic acid ester makes Tween 20 more sensitive to oxidation than Tween 20. Prolonged storage can lead to their oxidation to peroxides (Rowe et al. 2009).

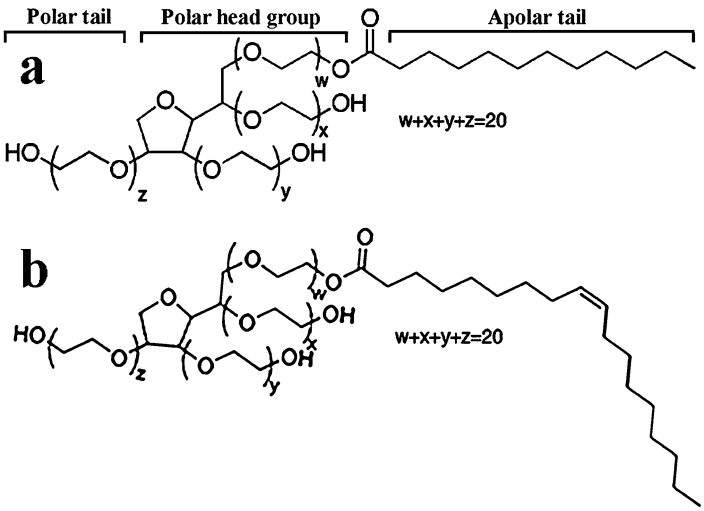


Fig. 3.8 Structures of polysorbate 20 (Tween 20) (**a**), and polysorbate 80 (Tween 80) (**b**). Organic solvents among them Tween 20 dissolve lipids from cell membranes and make them permeable to antibodies. Organic solvents also coagulate proteins, fix and permeabilize cells at the same time

The advantage of Tween 20 is that it acts very gently on cell lipids (Hjerten and Johansson 1972) for permeabilization of cells that were fixed in 0.25 % paraformaldehyde so that the immunofluorescent staining of surface antigens and the antigenic reactivity of internal antigens could be preserved. Although, Tween 20 reduced the size of individual cells, it permitted excellent staining of intracellular proteins, with very low coefficients of variation on the DNA distributions, and preservation of the integrity of cell surface antigens (Schmid et al. 1991). The disadvantage of Triton X-100 and Tween-20 is that they are non-selective in nature and may extract proteins along with lipids (Jamur and Oliver 2010). The solubilization and permeabilization of plasma membrane with Tween 20 is viewed schematically in Fig. 3.9. For flow cytometric analysis six different permeabilizing agents (NP-40, proteinase K, saponin, streptolysin, Triton X-100 and Tween 20) were compared to detect 18S rRNA in HeLa cells. It was found that highest level of mean fluorescence was obtained with the Tween-20 permeabilization method, and caused a dramatic improvement in quality of cell morphology and stability in environment. Maximum frequency of permeabilization and fluorescent intensity were obtained when the cells were treated with 0.2 % Tween-20 and incubated for 30 min (Amidzadeh et al. 2014). The reason why the mean fluorescence intensity using Tween-20 was significantly higher than other permeabilization methods is unclear, but could be related to the chemical structure of Tween 20, to the impact of incubation time for the better performance (Amidzadeh et al. 2014), or to the stoichiometric relationship of binding of Tween.20 to membrane lipids. Mild solubilizers (Tween 20, saponin, digitonin, and leucoperm) generate larger pores for antibodies to pass without dissolving plasma membranes and are suitable for antigens in the

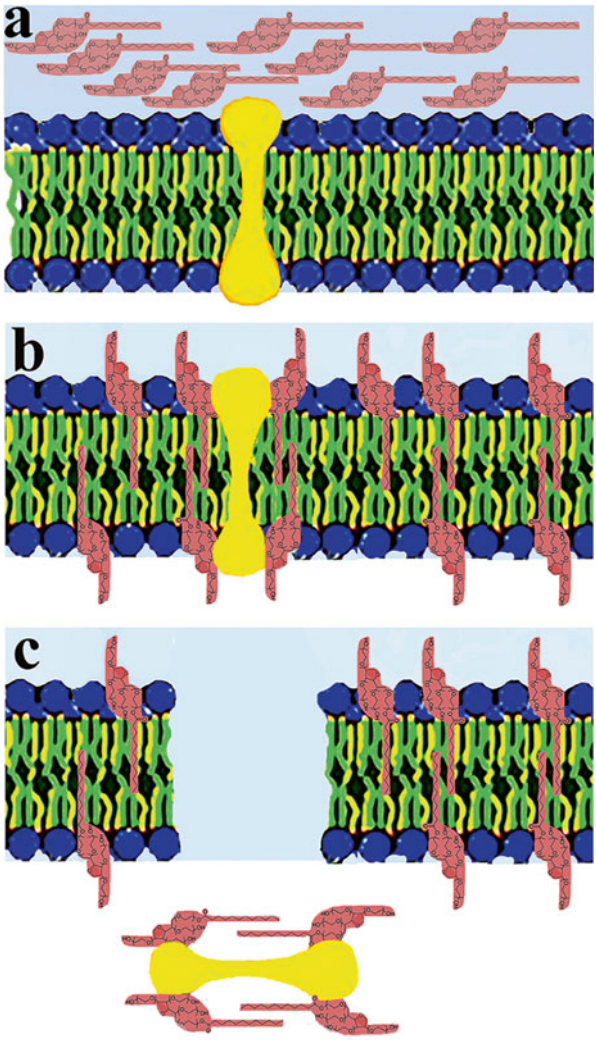


Fig. 3.9 Solubilization of membrane protein and permeabilization of plasma membrane by Tween 20. (**a**) Plasma membrane in the presence of Tween 20. (**b**) Binding of Tween 20 to membrane lipids and intramembrane proteins. (**c**) Permeable membrane with a solubilized protein at the bottom removed from the membrane

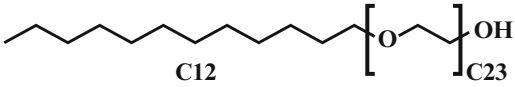


Fig. 3.10 The chemical structure of Brij 35 detergent

cytoplasm or the cytoplasmic face of the plasma membrane and for soluble nuclear antigens (https://stemcell.soe.ucsc.edu/sites/default/files/intracellular_staining.pdf). Tween 80 as a nonionic surfactant and emulsifier has other applications often used in food and cosmetic industry.

3.4.5 Brij Detergents

The Brij™ detergents (Brij 28, Brij 35, Brij 58, Brij 98) nonionic detergents are most frequently used as solubilizing agents and surfactants. Unlike viscous mild detergents, Brij solutions are easy to pipette and dispense. To prevent their oxidation and the formation of peroxides and other degradation products, Brij surfactant solutions are prepared and packaged under nitrogen atmosphere. The structure of Brij 35 is given in Fig. 3.10.

In the past five decades polyoxyethylene-detergents including polyoxyethylene-octylphenols (NP-40, Triton X compounds), -sorbitans (Tween detergents), -alcohols (Lubrol series), and particularly polyoxyethylene-fatty acid ethers (Brij series) became popular agents for the purification of intramembrane proteins (Miseta et al. 1995). Brij detergents (Brij 58 and 98) have been used to reveal new aspects of membrane microdomains in erythrocytes. Membrane microdomains enriched in cholesterol, sphingolipids known as lipid rafts, and specific proteins are involved in important physiological functions. Studies suggested that detergent-resistant membranes are in liquid-ordered state and are rich in raft-like components. Brij and Triton X-100-detergent resistance of membranes is reflected by different degrees in the lateral order of membrane microdomains (Casadei et al. 2014). Among the detergents compared (Brij 58, CAPS, Triton X-100) Brij 58 was the most powerful substance with regard to conferring solubility (Krause et al. 2002). Brij detergents are among the most powerful detergents to solubilize membrane proteins. Since they are less efficient as permeabilizing agents, their further discussion would be superfluous.

3.4.6 Lysolecithin

Although, lysolecithin (lysophosphatidylcholine) in itself would not be sufficient to permeabilize a membrane, it contributes to a marked decrease in its glycoprotein viscosity. Permeabilization measurements showed that lysolecithin increased the retardation ability of glycoproteins to hydrogen, reducing the ability to resist acid penetration (Slomiany et al. 1986).

Permeability studies demonstrated a dramatic increase in the permeability of liposomes at high lysolecithin concentration. These observations were assumed to have implications with respect to the hemolytic process. The extrapolation of these model experiments to the erythrocyte membrane, supported the hypothesis that an important stage in the lysis by lysolecithin was a change in cation permeability, inducing swelling of the cell and leading to a progressive osmotic breakdown of the membrane (Mandersloot et al. 1975). The membrane damaging effect of lysolecithin was seen as inflammation when 6–20 mg/ml lysolecithin was introduced into the rabbit gallbladder. The permeability increase in gallbladder resulted in enzyme liberation (Sjödahl et al. 1975).

The treatment of Chinese hamster ovary cells with lysolecithin made cells permeable to proteins and small molecules. Lysolecithin treatment yielded cell preparations that were capable of normal replicative DNA synthesis at *in vivo* rates, when supplied with deoxyribonucleoside triphosphates (dNTPs) (Miller et al. 1978, 1979; Castelot et al. 1979). Lysolecithin-permeabilized infected cell extracts were used to investigate the *in vitro* biochemical phenotypes of poxvirus temperature sensitive mutations (Condit et al. 1996). Although, it is not known why lysolecithin permeabilization has lost its attraction in the last two decades, it is only suspected that the relatively high concentration of lysolecithin (25–250 μg/ml) could be ascribed to its low selectivity and accounted for by the termination of its use. At the end of the 1980s we have studied the temporal order of replication of 39 genes in human erythroleukemia (K562) cells. Optimal lysolecithin concentration for the permeabilization of K562 cells was found to be 50 μg/ml. The nuclear content at the time a specific gene was replicated during the S phase was given in C-value. One of the reasons why the exact replication time could not be determined was probably due to the lack of proper permeabilization with lysolecithin. Details of the application of permeabilization for the determination of the temporal order of gene replication will be given in Table 4.3 of Chap. 4.

3.5 Enzymatic Digestion

3.5.1 Proteinase K

Proteinase K is one of the most active endopeptidases, working over a wide pH range, stable and not easily degradable. Proteinase K rapidly inactivates endogenous nucleases including RNases and DNases (Brdiczka and Krebs 1973). Optimal concentration may vary depending on cell type, time of incubation. Digestion normally starts at around 1 μg/ml proteinase K concentration. Incubation has to be monitored to sea the creation of pores in the cell membrane and the facilitated accession to DNA or RNA for labeling with in situ hybridization technique (Dingley et al. 2005). The digestion process should not go too far to avoid the damage of antigens and the destruction of tissue and cellular integrity.

For immunofluorescence and mRNA in situ hybridization of vertebrate and invertebrate embryos in the majority of cases, permeabilization was achieved by treating the fixed embryos with detergents, cold organic solvents or a mixture of both (Hammati-Brivanlou and Harland 1989; Harland 1991; Schulte-Merker et al. 1992; Stachel et al. 1993; Leung et al. 2000; Thisse and Thisse 2008). For mRNA in situ hybridization, proteinase K or pronase E have been used for continued permeabilization (Allende et al. 1996; Kang et al. 2002) indicating that proteinase K in itself is not suitable for complete permeabilization. These observations are in conformity with earlier results indicating that proteinase K alone without additives such

as SDS (0.1–0.5 %) could not permeabilize the plasma membrane of the HeLa cells (Sambrook 1982).

In addition to phospholipids, the outer membrane of Gram-negative bacteria contains proteins and large amounts of lipopolysaccharides. The fungal cell wall is composed of a chitin layer that sits adjacent to the plasma membrane topped by a (1–3)-β-glucan network that also contains (1–6)-β-glucosidic linkages (van der Weerden et al. 2010). The outermost layer of the cell wall is composed of highly glycosylated proteins that are anchored *via* glycopeptide linkages to the (1–3)-β-glucan layer. The outer proteinaceous layer of *Fusarium oxysporum sp. vasinfectum* hyphae could be removed, through direct digestion with proteinase K, but was not directly involved in the permeabilization of plasma membrane (van der Werden et al. 2010). When the pore forming peptide salivaricin 9 was treated with proteinase K or peptidase, it has lost its antimicrobial activity, while remained active when treated with lyticase, catalase and certain detergents (Barbour et al. 2013). The permeabilization of cells by proteinase K to create pores in the cell membrane is probably related to its major proteolytic activity by the digestion and removal of contaminating, unwanted enzymes (e.g. nucleases) and other proteins including transmembrane proteins. The proteinase K based solutions that are breaking the protein cross-links, unmask antigens and epitopes in fixed and paraffin embedded tissue sections and enhance the staining intensity of antibodies.

3.6 Agents Affecting Membrane Fluidity

The Davson-Danielli membrane model assumed that all cell membranes are identical. However, this turned out not to be the case. A further false assumption of this model was that membrane proteins would be exposed on all sides to a hydrophilic environment providing a stable configuration. These shortcomings of the Davson-Denielli model led to the fluid mosaic model of Singer and Nicolson that divided the membrane proteins into two categories, integral and peripheral proteins and differentiated among membrane regions based on their protein configuration and concentration. The Singer and Nicolson model distinguished among outside and inside peripheral proteins: (*i*) proteins that are exposed to one surface, (*ii*) proteins extending through the membrane and exposed to both surfaces, and (*iii*) proteins that are moving laterally along the membrane.

Membrane fluidity is the viscosity of the lipid bilayer of a cell or a synthetic lipid membrane. Fluidity refers primarily to the ability of molecules within the membrane (i.e. phospholipids, cholesterol, proteins, etc.) to traverse horizontally through the membrane. Membrane fluidity is the reverse of of membrane microviscosity, which in turn is inversely proportional to rotational and lateral diffusion rates of membrane components (Hollan 1996). The membrane fluidity is affected mainly by the rapid lateral movements of lipids known as lateral diffusion (Fig. 3.11), less frequently by transverse diffusion known as flip-flop transbilayer lipid motion (Fig. 3.12) and by the asymmetrical distribution of lipids in each side of the bilayer.

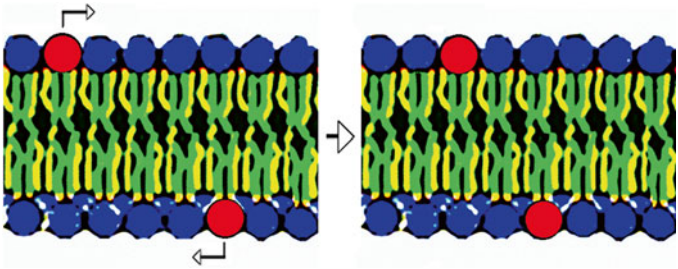


Fig. 3.11 Lateral diffusion of membrane lipid or protein molecules in the lipid bilayer

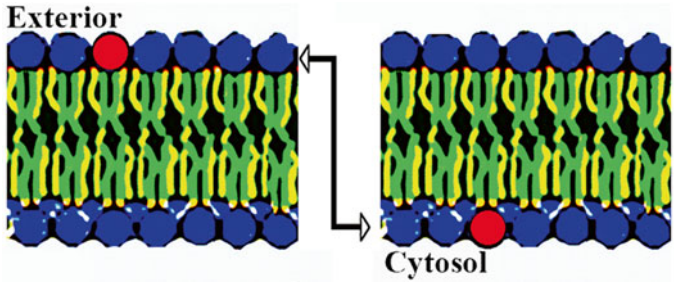


Fig. 3.12 Flip-flop movement or transverse diffusion of a lipid or protein molecule from one membrane surface to the other

Verification of Lateral Diffusion That proteins in the fluid mosaic model are distributed randomly throughout the plane of the membrane due to their mobility known as lateral diffusion was verified by electron microscopy to view the lipid bilayers cleaved by freeze fracture. Another process to track lateral diffusion is called fluorescence recovery after photobleaching (FRAP). This process uses fluorescence labeling to tracking lipid molecules. The cell surface is labeled first with a chromophore, then analyzed under a fluorescence microscope on one section of the illuminated area. On this site, the fluoresced molecules are then destroyed by bleaching them (e.g. by laser) and visualizing their exit or entrance in the illuminated area. Mobile molecules have two different states, bleached or unbleached. The fluidity of the membrane depends on:

(i) The lipid composition including:

- *Saturated fatty acids* that contain only single bonds, form straight chains, are solid at room temperature, make the membrane less fluid and in the organism as animal fats may clog the arteries.
- *Unsaturated fatty acids* contain one or more double bonds, form bent chains (*cis* configuration), prevent the formation of week apolar interactions among fatty acid chains, are liquid at room temperature and make membranes more fluid, and as vegetable oils do not clog the arteries.

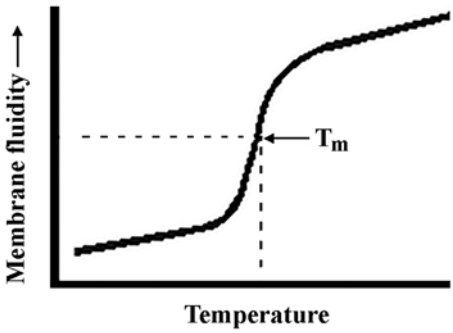


Fig. 3.13 Relationship between membrane fluidity and temperature

– *Cholesterol* stabilizes fluidity and acts as a bidirectional regulator of membrane fluidity. It fills the gaps between the phospholipids to prevent over fluidity or under fluidity. At high temperatures cholesterol stabilizes the membrane and raises its melting point, at low temperatures intercalates between the phospholipids and prevents clustering together and stiffening.

(ii) *Temperature*. The fluidity of the membrane depends on the temperature. The fluidity of membrane shows a sharp transition at the melting temperature (Tm) (Fig. 3.13). At the melting point a sharp transition takes place between the more rigid gel-like and the fluid-like state of the membrane. The melting point is a reflection of the lipid composition in the membrane. Low melting point indicates reduced fluidity and the presence of saturated fatty acids and/or higher proportion of cholesterol. High melting point reflects elevated unsaturated fatty acid and/or lower cholesterol content.

(iii) *Pressure*. The fluidity of the membrane can be increased by lowering the external pressure. The presence of more unsaturated fatty acids, desaturation of saturated fatty acids, shortening the chain length of fatty acids, reduced cholesterol and sphingomyelin content will lower the melting point of membranes and less thermal energy will be required to achieve the same level of fluidity as membranes made with lipids with saturated chains (Gennis 1989). The composition of the phospholipid bilayers is affected by the saturated and unsaturated fatty acid content of the diet that should avoid or be low in trans fats and cholesterol.

The fluidity of lipid bilayers and monolayers in artificial membranes can also be controlled by changing the temperature or the lateral pressure. Higher external pressure generated by hyperosmosis causes the shrinkage of cells primarily through the lateral pressure, reducing its permeability. In hypotonic environment the internal lateral pressure causes the cells to swell, reducing the lateral connections and may lead to permeabilization, leakage or cell disruption.

Diseases Related to Membrane Fluidity Changes Membrane fluidity changes have been described in thrombocythaemia, hyperlipidaemia, hypercholesterolaemia,

hypertension, diabetes mellitus, obesity, septic conditions, in allergic and burnt patients, in alcoholics, in Alzheimer's disease and in schizophrenia (Hollan 1996).

3.6.1 Protective Agents on Membrane Fluidity

Protein crystals during cryoprotection are mechanically fragile. They contain and can be surrounded by large amounts of water, formation and growth of water ice crystals during cooling, that degrade protein crystal order and diffraction properties (Garman and Schneider 1997). Best cryoprotection is attained when water is cooled into an amorphous, vitreous or glassy state (Berejnov et al. 2006). Cryoprotectants are essentially anti-freeze solutions that decrease freeze-induced cellular damages. In the process of cryopreservation (cryoconservation) cells or tissues are cooled to sub-zero temperatures. Cryopreservation should be performed without additional damage caused by the formation of ice during freezing. This can be avoided by coating the material to be frozen with a class of molecules termed cryoprotectants. Cryoprotectants are essential ingredients for cell freezing; protecting cells during their cooling from room temperature to −70 in deep freezer or to −196 °C in liquid nitrogen. The general requirements for cryoprotectants are: (*i*) low molecular weight, (*ii*) nontoxic nature, (*iii*) inexpensive, (*iv*) providing high level of cell viability post thaw, and (*v*) no influence on the cells.

The mechanism by which the cryoprotectors exert their action on the cells remains elusive. It was suggested that most of them play a role as plasma membrane protectors attributed to protection of protein functionality and/or to solute binding to proteins acting as a water substitute (Crowe et al. 1988; Leslie et al. 1995). Among the cryoprotectants are amino acids. Monosodium glutamate was also successfully used to protect different kinds of micro-organisms, including lactic acid bacteria, during freezing and freeze-drying (Font de Valdez et al. 1985; Hubalék 1996; Carvalho et al. 2003). L-proline amino acid enhances the stability of proteins and membranes in low-water activity and high-temperature environments (Csonka 1989) and inhibits aggregation during protein refolding. L-proline is regarded as a potential protective agent for industrial microorganisms and enzymes.

The two major classes of cryoprotectants are:

– Penetrating intracellular agents enter cells and prevent the formation of ice crystals that would cause membrane damage and cell leakage such as dimethylsulfoxide (DMSO), glycerol, polyethyleneglycol (PEG).
– Impermeable extracellular agents improve the osmotic imbalance that would occur during freezing (e.g., sucrose, trehalose, and dextrose).

Detailed description of cryoprotectant will follow the fluidifying effects of plasma membrane.

3.6.2 Fluidifying Effect on Plasma Membrane

Leptin improves the membrane fluidity of human erythrocytes in a nitric oxide-dependent manner (Tsuda et al. 2002).

Tamoxifen is a non-steroidal antiestrogen drug, which is used to prevent and treat breast, liver, pancreas and brain cancers. Tamoxifen has a fluidifying effect on plasma membrane (Kazanci and Severcan 2007). Increased lipiodol (poppyseed oil) uptake in hepatocellular carcinoma is possibly due to increased membrane fluidity by dexamethasone and tamoxifen (Becker et al. 2010). Tamoxifen and related 17β-estradiol reduced the membrane fluidity of human breast cancer cells (Clarke et al. 1990). Tamoxifen and its related compounds decreased membrane fluidity in ox-brain phospholipid liposomes in the order of their effectiveness: 4-hydroxytamoxifen > 17β-oestradiol > tamoxifen > *cis*-tamoxifen >*N*-desmethyltamoxifen > cholesterol (Wiseman et al. 1993).

3.7 Cryoprotectants

3.7.1 Penetrating Cryoprotectans

Dimethylsulfoxide (Me_2SO, DMSO). This is the most widely used cryoprotectant used as a 10 % DMSO solution. Studies related to cryoprotection have indicated that the known cellular toxicity of DMSO at room temperature is connected to the induction of differentiation in stem cells (Mareschi et al. 2006). This has led to the search for "xeno-free" cryopreservants to avoid the toxicity of DMSO either alone or utilizing reduced concentrations of DMSO and slow cooling (Hunt 2011). As the penetration of intracellular agents may cause other harmful effects, impermeable extracellular cryoprotectants were suggested to be optimal for cryopreservation (Janz et al. 2012). Alternatives for DMSO (Inaba et al. 1996; Monroy et al. 1997; O'Neil et al. 1997; Hardikar et al. 2000; Maruyama et al. 2004) included the use of PEG as a cryoprotectant (Monroy et al. 1997), hydroxyethyl starch (HES/HEAS), a nonionic starch derivative in combination with DMSO as co-cryoprotectants (Maruyama et al. 2004), microencapsulation of islets with alginate-poly(L-lysine)-alginate (APA) before cryopreservation (Inaba et al. 1996), microencapsulation of islets with chitosan-alginate (Hardikar et al. 2000). These methods have been found valid to different extent. The introduction of polymers as cryoprotectants was supposed to increase the viscosity of the medium at low temperature, and inhibit ice crystal formation during cooling or re-warming, to avoid permeation of cells, and to decrease the freezing point of the medium, thus leading to fast freezing (O'Neil et al. 1997). In spite of these efforts to find alternative cryoprotectans, and to avoid its drawbacks, the use of DMSO could not be avoided. DMSO could not be replaced and remained the gold standard in cell freezing. Attempts were made to reduce the

side effects of DMSO on patients and to improve the safety of hematopoietic cell transplantation by the combination of trehalose (30 mmol/L) with DMSO (2.5 % v/v) or sucrose (60 mmol/L) with DMSO (5 % v/v). These combinations produced results similar to those for 10 % (v/v) DMSO (Rodrigues et al. 2008).

Polyvinylpyrrolidone (PVP) The cryoprotectant solution containing 30 % (v/v) ethylene glycol and 1 % (w/v) PVP-40 was originally developed for preserving brain sections (de Olmos et al. 1978). PVP polymer was made from the monomer N-vinylpyrrolidone. It was recommended as a new and potent cryoprotectant for islet cell freezing on the basis of the hypothesis that the low molecular weight compounds, such as DMSO and glycerol, permeate the cell and interact hydrophobically with intracellular proteins. These low molecular weight compounds perturbed the cytoskeletal architecture of frozen cells and diminished islet cell integrity and function (El-Shewy et al. 2004). Human adipose tissue-derived adult stem cells could be effectively cryopreserved and stored in liquid nitrogen using a freezing medium containing the high-molecular-weight polymer, polyvinylpyrrolidone (PVP), as cryoprotective agent rather than dimethylsulfoxide (DMSO) (Thirumala et al. 2010). Another study indicated that the supplementation of the ethylene glycol and sucrose cryoprotectant solution with 7.5 % PVP was optimal for the successful vitrification of the two-cell stage murine embryos (Kim et al. 2008).

Glycols (ethylene glycol, propylene glycol, glycerol) are alcohols containing two or more hydroxy groups. With the exception of ethylene glycol, glycols are noncorrosive, have very low volatility and very low toxicity, Although toxic, ethylene glycol is commonly used at high concentration as automobile antifreeze. Propylene glycol reduces the ice formation in ice cream. Glycerol and DMSO have been used to reduce ice formation in sperm, oocytes, and embryos during cold-preservation in liquid nitrogen (Imrat et al. 2013; Karlsson et al. 2014). X-ray diffraction measurements on aqueous glycerol mixtures confirmed that the polycrystalline-to-vitreous transition occurs within a span of less than 2 % w/v and the form of polycrystalline ice (hexagonal or cubic) depends on the cryoprotectant agent concentration and cooling rate (Berejnov et al. 2006).

Hyperosmotic solutions also protect plants and animals from water stress. Major organic osmolytes include low molecular weight polyhydric alcohols, urea, amino acids and their derivatives. The advantage of low molecular weight organic substances is that they allow the generation of high osmolyte concentration in the internal environment ("milieu interior") that also serves as a cryoprotectant against freezing. Insects, fish and amphibians in polar regions have developed antifreeze compounds and proteins to minimize freezing damage during winter periods. Though, most of the organic solvents are not cryoprotectants per se, they may serve freezing adaptations (Storey 1997; Yancey 2001). Certain osmolytes such as triethylamine N-oxide (TMAO) work better than others in stabilizing proteins in freeze-thaw cycles (Göller and Galinski 1999). TMAO is an osmolyte found in saltwater fish, sharks, rays, molluscs, and crustaceans. Decomposed triethylamine is the main odorant of degrading seafood.

Polyethyleneglycols (PEG 200, PEG 2,000, PEG 20,000) (H-(O-CH_2-CH_2)$_n$-OH) are less efficient cryoprotectants relative to the smaller glycols. Here only the protection of PEG-coated vesicles, such as gene therapy vectors e.g. PEG-coated viruses are mentioned. PEG shields these vectors from inactivation by the immune system and to detarget them from organs where they may build up and have a toxic effect (Kreppel and Kochanek 2007). The size of PEG polymer has been shown to be important, with larger polymers achieving the best immune protection.

Percoll formulated by Pertoft et al. (1978) is used for the isolation of cells, organelles, viruses by density centrifugation. Percoll consists of 23 % (w/w) colloidal silica particles (15–30 nm in diameter) coated with polyvinylpyrrolidone (PVP). Purified percoll possesses low viscosity, low osmolarity and no toxicity towards cells and their constituents. Some batches of percoll with higher endotoxin content can cause inflammation and fever, thus percoll has been replaced with other colloids in assisted reproductive technology to select sperm from semen by density gradient centrifugation (Mortimer 2000).

Ca2$^+$, Zn2$^+$ and H$^+$ protection of cells was provided against membrane damages induced by hemolytic agents (Bashford et al. 1989). Divalent cations and protons act at the extracellular side in conformity with the extracellular protection of plasma membrane (Janz et al. 2012).

3.7.2 Impermeable Cryoprotectants

Sugars are among the natural cryoprotectants in the animal kingdom. Insects preferentially protect themselves by sugars or polyols as cryoprotectants, frogs use glucose, salamanders glycerol.

Sucrose This sugar ($C_{12}H_{22}O_{11,}$ Mw:342.3) is a disaccharide, the combination of α-D-glucose and ß-D-fructose extracted and purified from sugar cane or beet used in huge quantities for human consumption. The word sucrose was coined in 1857 (Miller 1857). This disaccharide is also less frequently referred to as saccharose (Berthelot 1860). Sucrose as a cryoprotectant is a partial dehydrant that prevents the formation of ice crystals in frozen tissue sections. However, due to its relatively high molecular mass, a 30 % (w/v) sucrose solution used as cryoprotectant has only a 0.876 M concentration with a freezing point of −1.63 °C, and is less suitable for deep freezing than other small molecular weight protectants such as glycols. Indeed, the use of sucrose solutions enabled storage of tissue sections at refrigerator temperatures for a few days but longer-term storage was risky and either bacterial/fungal growth or evaporation of the storage solution compromised the integrity of the tissue (Hoffmann and Le 2004). Better results were obtained when 30 % sucrose infiltration and dry-ice freezing was replaced by the combination of glycerol-DMSO infiltration and freezing in isopentane that virtually eliminated freezing artifacts (Rosene et al. 1986). Commercially available cryoprotectant solutions preferentially use the com-

bination of sucrose with other protectants such as sucrose + ethylene glycol, sucrose + PVP + ethylene glycol or other combinations without sucrose, e.g. ethylene glycol + glycerol, etc. In spite of intensive research to improve quick-freezing, a generally applicable, simple and reliable cryopreservation technique is still missing.

Trehalose Unicellular organisms subjected to stress, synthesize huge amounts of trehalose, which helps them in retaining cellular integrity. This is likely to occur by prevention of denaturation of proteins by trehalose. Trehalose is a natural alpha-linked disaccharide formed by two α-glucose units. This disaccharide has also proved useful in the cryopreservation of sperm and stem cells and in the development of organ preservation solutions (Jain and Roy 2009). Trehalose was successfully applied as a cryoprotectant for the freeze preservation of carrot and tobacco cells (Bhandal et al. 1985). As a cryoprotectant agent it was found to enhance the recovery and preservation of human pancreatic islets after long-term storage (Beattie et al. 1997). Trehalose (5 %) significantly ameliorated the cryopreservation of cord blood progenitor cells in a preclinical protocol. The increased recovery of these cells relative to 10 % DMSO improved the engraftment of cord blood transplants (Zhang et al. 2003). Trehalose in combination with sucrose and DMSO reduced the side effects caused by treatment with DMSO alone (Rodrigues et al. 2008). Recently the effectiveness of the solution containing intracellular and extracellular trehalose in the cryopreservation of cord blood cells has been confirmed and demonstrated that trehalose is a useful cryoprotectant (Motta et al. 2014).

Dextrose Dextrose is preferentially extracted from corn. Dextrose is chemically identical with α-D-glucose found in the blood. α-D-glucose is also known as dextrose or grape sugar. The cryoprotectant combination of dextrose with DMSO in a xeno-free procedure was a better alternative than the standard DMSO protocol in primary human hepatocyte cryopreservation. The improved cryopreservation of human hepatocytes uses the polystyrene box freezing and the new Stem-Cellbank (CB) medium. The CB protocol contains 10 % DMSO, glucose and anhydrous dextrose described in the Japanese Pharmacopeia as cryoprotectant (Saliem et al. 2012). It was found that the addition of dextrose sugar to intracellular cryoprotectant solution containing glycerol maintained the viability and integrity of plasma membrane of buffalo's epididymal spermatozoa after thawing (Yulnawati et al. 2009). For myofibrillar proteins effective cryoprotectants were found among carbohydrates, such as sucrose, sorbitol maltodextrins, and polydextrose (Lanier and MacDonald 1991; Tornaniak et al. 1998; Auh et al. 2003; Herrera and Mackie 2004).

Xeno-Free
A major disturbing factor with cell cultures is the presence of serum and animal (xenogenic) products. The use of commercially available serum free, albumin free and xenogen-free (Xeno-free, XF) and cost effective medias is recommended.

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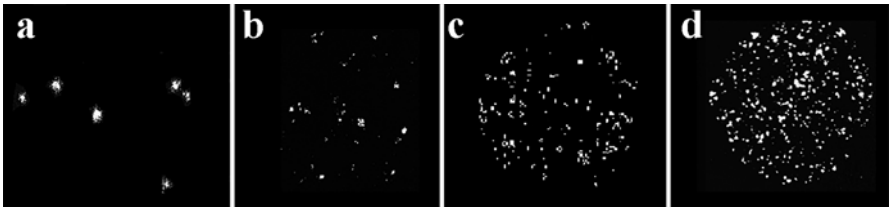
Chapter 4
Applications of Permeabilization

Contents

G. Banfalvi, *Permeability of Biological Membranes*,
DOI 10.1007/978-3-319-28098-1_4



This figure is identical with Fig. 4.13 and explanation is given there.

Abstract The permeabilization of cells has obtained several applications. This chapter deals with these applications and includes cationic cell killing, gene delivery, anticancer effects, delivery of quantum dots, electrofusion, macromolecular biosynthetic processes (poly-ADP ribose, DNA, RNA), temporal order of gene replication and gene expression, visualization of replicons and intermediates of chromosome condensation, linear order and spatial arrangement of chromosomes, genotoxic chromatin changes, among them heavy metal (Cd, Pb, Ni, Hg, Ag) treatment and irradiation (α, γ and UV-B) induced chromatin damages.

Keywords Anticancer permeabilization • Arrangement of chromosomes • Avicins • Chromatin condensation • Chromatin images • Cell cycle synchronization • Contact-killing • DNA synthesis • Early replicative intermediates • Electrofusion • Gene delivery systems • Gene expression • Gene replication • Genotoxic chromatin changes • Heavy metals • Hybridoma • Hypertonic solution • Hypotonic solution • Irradiation • Nascent DNA • Okazaki fragments • Poly-ADP ribosylation • Polyamines • Polyamino acids • Polycationic peptides • Polycations • Propofol • Quantun dots • Replicons • RNA primer • Stimulated lymphocytes • Subunits of chromosomes

4.1 Polycations as Permeabilizing Agents

Electrophoretic measurements have demonstrated that the bacterial cell surface is usually negatively charged. The adsorption of polycations onto the negatively charged bacterial cell surface took place to a greater extent than that of monomeric cations because of their higher charge density. The cytoplasmic membrane permeability turned out to be facilitated by the polycations because of the presence of a large number of negatively charged species such as acidic phospholipids and some membrane proteins in the membrane (Franklin and Snow 1981). Consequently the disruption of the membrane and the subsequent leakage of K^+ ions and other cytoplasmic constituents was enhanced by polycations. These considerations led to the conclusion that cationic disinfectants of polymeric forms exhibited higher antibacterial activity than those of monomeric or dimeric forms (Ikeda et al. 1984).

4.1.1 Cationic Contact-Killing

Cationic contact creates antimicrobial surfaces that prevent the attachment of viable bacteria. Cationic contact-killing is based on highly swollen, compliant surfaces that resist bacterial attachment and at sufficient density of mobile cationic charge can effectively disrupt bacterial cell membranes. These cationic disinfectants of polymeric forms exhibited high antibacterial activity against Gram-positive bacteria, whereas they were less active against Gram-negative bacteria (Lichter and Rubner 2009).

4.1.2 Polycation Gene Delivery Systems

Polycations have gained increasing attention as non-viral gene delivery vectors. Polycation gene delivery systems tried to mimic the mechanisms that viruses use for the endosomal escape. DNA plasmids that formed particulate complexes with a variety of cationic polyamino acids and cationic lipids were used to transfect mammalian cells in culture (Pouton et al. 1998). Polycation gene delivery systems were coupled with synthetic amphipathic peptides mimicking viral fusogenic peptides, histidine-based gene delivery systems for pH-responsive endosomal escape (Cho et al. 2003).

(Lys)16-based reducible polycation/DNA/fusogenic peptide particles provided a gene delivery system which was stable in the extracellular environment and, on reductive depolymerisation, could release DNA plasmids for nuclear translocation (Parker et al. 2007).

Functionalization turned polycations with poor delivery activity into efficient carriers, polylysine that, on its own was lacking nucleic acid transfer activity, displayed

high efficiency in siRNA delivery after modification with polyethylene glycol and pH-responsive endosomolytic peptide (Meyer et al. 2008). Recently polycation liposomes were used to deliver small interfering RNA (siRNA) to tumors with the considerable potential for cancer treatment (Kenjo et al. 2013). A poly-D, L-succinimide (PSI)-based biodegradable cationic polymer mimicked faithfully the effect and the amine composition of the polyethylenimine-mimetic biodegradable polycation gene vector, the gold standard often used for gene delivery (Shen et al. 2013).

4.1.3 Polyamins

Polyamins are naturally occurring constituents and present in plant and animal cells at millimolar concentrations playing regulatory roles (Tabor and Tabor 1984). Polyamines such as spermine and spermidine are polycationic amines that control the function of Ca^2, Na^+/K^+ ATPases and maintain membrane potential, regulate intracellular pH and cell volume. These polyamines inhibit the hypotonic swelling of rat liver mitochondria (Tabor 1960). The inhibition of inner membrane permeability transition of isolated rat heart and liver mitochondria by spermidine (Lapidus and Sokolove 1992, 1994; Rigobello et al. 1993) was stimulated by an increased concentration of matrix calcium (Gunter and Pfeiffer 1990; Zoratti and Szabo 1995). The permeability transition was induced by the passage of small (<1500 Da) molecules through the pore and inhibited by cyclosporin A (Crompton et al. 1988).

4.1.4 Cationic Polyamino Acids

Pro-inflammatory cationic proteins secreted by human granulocytes increased vascular permeability and induced oedema, which are likely to be mediated by the damage of the vascular endothelium (Henson and Johnston 1987). The cationic nature of these molecules is attributed to the high proportion of lysine and/or arginine (Zeya and Spitznagel 1968).

Synthetic polycationic peptides exert similar pro-inflammatory effects *in vivo* (Stein et al. 1956; Sela and Kalchalski 1959; Vehaskari et al. 1984; Needham et al. 1988; Morgan et al. 1989) and also need the presence of basic amino acid residues, including lysine, arginine and ornithine to be able to inhibit the permeability transition induced in mitochondria by calcium ions and inorganic phosphate (Rigobello et al. 1995). *Bacillus thuringiensis subsp. medellin* was reported to produce the Cry11Bb protein of 94 kDa, which is toxic for mosquito larvae due to permeabilization of the plasma membrane of midgut epithelial cells. A mechanism of membrane permeabilization was suggested that included the transmembrane- and surface potential-dependent insertion of this polycation peptide into the lipid bilayer and its

oligomerization leading to the formation of ion channels and to the mitochondrial permeability transition pore opening in a cyclosporin A-insensitive manner (Lemeshko et al. 2005). The action of bactericidal polycationic peptides was compared in *Yersinia* spp. by testing peptide binding to live cells and changes in outer membrane morphology and permeability (Bengoechea et al. 1998). The *Yersinia* genus of bacteria in the family Enterobacteriaceae are Gram-negative, rod-shaped bacteria, and are facultative anaerobes. Some members of *Yersinia* are pathogenic in particular, *Y. pestis* the causative agent of the plague. *Yersinia pseudotuberculosis* causes the Far East scarlet-like fever in humans.

Polycations induce loss of fixed anionic sites in the glomerular capillary wall and epithelial changes similar to those reported in proteinuric conditions indicating that such membrane alterations are accompanied by an increase in glomerular permeability (Kelley and Cavallo 1978).

4.2 Anticancer Effect of Permeabilization

Anticancer drugs are used in different types of cancers in conjunction with surgery, radiotherapy, and immunotherapy as a combined modality approach particularly against metastases. The types of cancers are normally classified based on the affected area that can be anywhere in the body. The classification of antineoplastic agents involves alkylating agents, antimetabolites, natural products (plant and microorganism products), miscellaneous drugs, hormones and antagonists. It is not the aim of this subchapter to review different types of cancers and antineoplastic products, but to focus on those anticancer treatments that are related to the permeability of biological membranes.

4.2.1 Anticancer Effect of Polycationic Peptides

Although, these peptides have already been mentioned, their anticancer effect is discussed here. Poycationic peptides for anticancer treatment were designed on the basis of 16-mer and 14-mer fragments derived from the Cry11Bb protoxin (Lemeshko 2013). Most *Bacillus thuringiensis* strains produce several plasmid-encoded delta-endotoxin genes and package related protoxins (Aronson 1995). These peptides caused mitochondrial, but not red blood cell membrane permeabilization. Conjugation of the cell penetrating hepta-arginine vector through their N- or C-termini resulted in even more active peptides, which permeabilized the red blood cells in the presence of valinomycin. The designed peptides were active against the human leukemia Jurkat cells but not against the normal wild type CHO cells (Lemeshko 2013).

4.2.2 Avicins

Avicins are a class of triterpenoid natural saponins originally isolated from the Australian desert tree *Acacia victoriae*, and have been shown to selectively inhibit the growth of tumor cells from a wide variety of tissues (Haridas et al. 2001, 2004; Hanausek et al. 2001; Mujoo et al. 2001; Jayatilake et al. 2003). Avicin D and G structures have been described (Jayatilake et al. 2003). Avicin G was also shown to affect cell integrity, cytokinesis, and protein ubiquitination in fission yeast. *Saccharomyces pombe* cells treated with a lethal concentration of avicin G (20 μM) exhibited a shrunken morphology, indicating that avicin G adversely affected cell integrity (Gutterman et al. 2005). The apoptotic effect of avicins in transformed cells is probably related to channel formation in membranes. Avicin channels in the mitochondrial outer membrane may favor apoptosis by altering the potential across this membrane and the pH of the intermembrane space (Li et al. 2005; Lemeshko et al. 2006). Avicin G and avicin D were significantly more efficient than saponin in inducing cytotoxicity in PC3 human prostate cancer cells. The size of the pores generated by avicin G and avicin D in the plasma membrane was estimated to be wider than the hydrodynamic radius of PEG-8000. These observations indicated that the anticancer activity of avicin G and avicin D could be related, at least partially, to their high ability to permeabilize biological membranes (Arias et al. 2010).

Accumulating evidence suggests that avicins are likely to induce tumor cell death *via* multiple mechanisms (Wang et al. 2010). Avicin D could induce non-apoptotic cell death mediated by autophagy by decreasing cellular ATP levels, stimulating the activation of AMP-activated protein kinase and inhibiting the mammalian target of rapamycin activity (Xu et al. 2007). Rapamycin and its derivatives are known therapeutic agents with both immunosuppressant and anti-tumor properties. Alternatively, avicins could induce cell apoptosis by the activation of the Fas pathway (Xu et al. 2009). Fas ligand (CD95L) belongs to the tumor necrosis factor family, the binding of which to its receptor induces apoptosis. Avicins were originally identified as potent inhibitors of tumor cell growth, but later found to be pleiotropic compounds that also possess pro-apoptotic, anti-mutagenic and anti-inflammatory activities in mammalian cells, and also mediate antioxidant defense through thiol binding. Increasing evidence has shown that avicins may affect multiple cellular processes to suppress tumor cell growth or trigger cell death (Wang et al. 2010). These contradictory results indicate that the exact mechanism of avicins has not been clarified.

4.3 Delivery and Cellular Imaging of Quantum Dots

Quantum dots are inorganic semiconductor crystals with unique optoelectronic properties offering a great opportunity as a new generation of fluorescent probes for bringing together multidisciplinary research such as cellular imaging, biomolecular delivery and to study cellular processes.

4.3.1 Toxicity of Quantum Dots

Toxicity studies revealed that the toxicity of quantum dots depends on several factors such as size, charge, concentration, protective coating, functional groups, oxidation, mechanical stability, etc. (Hardman 2006).

4.3.2 Functionalization of Quantum Dots

To avoid common problems when using quantum dots, their aqueous solubility has to be improved as well as their surface- and bio-functionalizations are necessary. Surface modifications include the attachment of biomolecules, ligand exchange, improvement of amphiphilic character, inorganic encapsulation, just to mention the most important types of functionalizations.

4.3.3 Bioimaging of Quantum Dots

Biological applications of quantum dots (QDs) include *in vitro* and *in vivo* imaging, detection of pathogens and toxins, gene technology and Fourier resonance energy transfer. Here only the *in vivo* and more importantly the *in vitro* imaging applications will be dealt with. Details of surface functionalization of quantum dots have been recently reviewed by Karakoti et al. (2015).

Live cell monitoring to track cytoplasmic processes remained limited because of the inefficient methods to deliver surface-functionalized quantum dots with excellent optical properties for the specific labeling to subcellular structures. Conventional methods to attain delivery of quantum dots involved endocytosis, lipid based delivery and electroporation. During the assessment of long-term intracellular fate and stability of quantum dot-peptide conjugates it was found that although, quantum dots remained sequestered within acidic endolysosomal vesicles for at least 3 days after initial uptake, but the cell penetrating peptide remained stably associated with the QD throughout this time. Further techniques have been designed to either actively deliver QDs directly to the cytosol or to combine endocytosis with subsequent endosomal escape to the cytosol. Active delivery methods such as electroporation and nucleofection delivered only modest amounts of quantum dots to the cytosol as aggregates. A variety of transfection polymers also resulted in primarily endosomal sequestration of quantum dots, but with low efficiency (Delehanty et al. 2010). These authors demonstrated that an amphiphilic peptide mediated the rapid quantum dot uptake by endocytosis followed by a slower efficient endosomal release. Similar intracellular delivery of quantum dot-protein cargos was mediated by cell penetrating peptides (Medintz et al. 2008). These quantum dot conjugates were distributed primarily in endolysosomal compartments indicating their endo-

cytic driven uptake. Pharmacological agents incorporated into block copolymer micelles, administered or attached to the surface of the quantum dots showed that the final biological outcome (e.g. cell death, proliferation or differentiation) also depended on the physical properties of these nanoparticles (Maysinger et al. 2007). To bypass the endocytic pathway, cellular microinjection of quantum dot-fluorescent protein assemblies was also utilized as an alternate delivery strategy (Medintz et al. 2008). However, the efficiency of delivery of quantum dots by cellular microinjection is known to be very low.

Antibody-coated quantum dots within biodegradable polymeric nanospheres were designed that underwent endolysosomal translocation *via* pH-dependent reversal of nanocomposite surface charge polarity and after entering the cytosol were hydrolyzed and released their quantum dot bioconjugate content. As compared to conventional intracellular delivery techniques this approach allowed the high throughput cytoplasmic delivery of quantum dots with minimal toxicity to the cell and facilitated multiplexed labeling of subcellular structures inside live cells without the requirement of cell fixation or membrane permeabilization (Kim et al. 2008).

4.3.4 Permeabilization to Introduce Quantum Dots

Permeabilization allowed the introduction of quantum dots into cells, but the survival of cells was not secured. Direct internal quantum dot labelling was investigated in *E. coli* cells permeabilized using three methods including chloroform-SDS treatment, lysozyme-EDTA treatment and osmotic shock treatment. No interaction was observed between osmotic shock treated cells and quantum dots (Yang et al. 2013).

In prefixed and permeabilized cells, quantum dots were readily internalized regardless of cell type, and their intracellular location was primarily determined by the properties of the quantum dots themselves (Williams et al. 2008). Reversible permeabilization was necessary to deliver quantum dots into viable cells. Multicolor labeling of individual cells caused further complications since it often necessitated the combinatorial use of various targeting probes, multistep bioconjugation, different delivery strategies, cellular fixation, membrane permeabilization and spectral convolution (Delehanty et al. 2011). Osmosis driven endocytosis-free intracellular transport of quantum dots was achieved by creating a hypotonic environment and reversible permeabilization by using low concentrations of cell permeabilizing agents like saponin in a hypotonic buffer (Medepalli et al. 2013). Reversible permeabilization was introduced some 30 years earlier when hyposmotic driven permeabilization that would have caused disruption of cells was prevented by the presence of dextran as a molecular coat and made possible regeneration in the presence of fetal bovine serum (Kucera and Paulus 1982). This protocol of reversible permeabilization obtained several applications (Banfalvi 2014) to be detailed after the subchapter “Medical applications of hypo- and hypertonic solutions”.

4.4 Cell-Cell Electrofusion

Electropermeabilization or electroporation is the artificial merging of cell membranes by the delivery of appropriate short and intense electrical pulses to living cell cultures or tissues resulting in transient and reversible alterations (Neumann et al. 1989). The application of an electric field across the cell causes the redistribution of ions inside the cell because the cell membranes act as insulators. Although, ions move within the electric field corresponding to their charge, but are trapped inside the cell and accumulate at the two poles in line with the electric field. The polar distribution within the cell creates an electric potential across the plasma membrane and upon exceeding the threshold of about 1 V, the conformational changes within the membrane induce a state of permeability.

Membrane fusion is a naturally occurring process during secretion as a result of merging into a union of plasma membrane and the membrane of internal vesicles, during the formation of secondary lysosomes, in egg fertilization (Ogle et al. 2005), viral infection (Kubo et al. 2003) and tissue repair (Vassilopoulos et al. 2003). It was in 1973 when it was discovered that high electric field pulses in a very narrow range of intensity and duration led to a transient (reversible), experimentally controllable increase in the permeability of the cell membrane (Zimmermann et al. 1973). The effect of electric pulses was regarded as a reversible electrical breakdown to emphasize that these membrane perturbations were reversible (Zimmermann et al. 1974). However, the yield of electrofusion is normally very low when fusion partners differ considerably in their size, since the extent of electroporation and the membrane fusogenic state are proportional to the cell radius (Rems et al. 2013).

The applications of electropermeabilization summarized by Šalomskaitė-Davalgien (2009) include:

- transfection of bacterial, fungal, plant and animal cells
- introduction of various compounds inside cells (metabolites, dyes, drugs, peptides, enzymes and others) (Neumann et al. 1989; Belehradek et al. 1994; Mir et al. 1988; Orlowski and Mir 1993; Shulin 2008)
- electrochemotherapy, allows enhanced delivery of some anticancer drugs into the tumor cells (Cemazar et al. 1999; Mir et al. 1991). This cancer therapy has proven particularly effective with bleomycin and cisplatin (Cemazar et al. 1999; Mir and Orlowski 1999; Marty et al. 2006; Sadadcharam et al. 2008; Satkauskas et al. 2005)
- electro-gene therapy is also based on electroporation of cells in tissues, allowing the uptake of plasmid DNA (Andre and Mir 2004)
- Cell electrofusion *in vitro* (Neumann et al. 1989; Rols and Teissie 1989; Sugar et al. 1987; Teissie and Blangero 1984) and *in vivo* cell electrofusion (Grasso et al. 1989; Heller 1993; Mekid and Mir 2000).

Electrofusion, particularly the production of hybridomas also utilizes the permeabilization process. It was shown that cells of those tumor models that showed a high tendency to electrofuse *in vitro* also maintained this propensity *in vivo* in

tumors following electroporation treatment (Šalomskaitė-Davalgienė et al. 2009). Beside electroporation, *in vitro* cell-cell fusion can be induced by fusogenic agents (e.g. polyethyleneglycol, Sendai virus).

4.4.1 Hybridoma

The most important fusions of cells by electroporation are hybridomas that combine the properties of both parent cells and offer the production of monoclonal antibodies against bacteria, viruses and other naturally occurring substances (Zimmermann 1982). Hybridomas (hybrid cells) produce antibodies in huge amounts and are used for diagnostic and therapeutic purpose. The production of hybridomas starts with the injection of a specific antigen into a mouse, followed by the collection of an antibody-producing cell from the mouse's spleen, then fusing this antigen producing cell with a myeloma tumor cell. After its formation the hybridoma multiplies indefinitely in cell culture and can be used to produce a specific antibody.

After the pioneering work of cell electroporation as an efficient method of permeabilization (Neumann and Rosenheck 1972), hybridoma cells maintained their viability (Kinosita and Tsong 1977; Zimmermann 1982; Hughes and Crawford 1989) and cell-cell fusions have been routinely used to obtain somatic hybrids. It was taken as a further advantage that the strong electrical pulses not only permeabilized the cell membrane, but when cells were in close contact, the transient permeability disappeared and turned to an intracellular process leading to cell fusion (Teissié et al. 1992).

Electroporation and electrofusing approaches have been successfully applied *ex vivo* and *in vivo* during the electrofusion of human cells to rabbit cornea (Grasso et al. 1989; Heller 1993). The occurrence of cell fusion was reported within some type of tumors (B16, LPB) *in vivo* after they were exposed to permeabilizing electroporation (Mekid and Mir 2000).

4.4.2 Inhibition of Electrofusion by Propofol

The fusion can be inhibited by the local anesthetic propofol (2,6-di(propan-2-yl) phenol), which is not identical with Profadol, Propanol, or Propranolol (Fig. 4.1). The membrane permeability of human lymphoma cells decreased by a factor of two with increasing propofol concentrations up to about 0.1 mM concentration. Upon removal of propofol the effects of electroporation remained reversible and more effective than tetracaine during the inhibition of electrofusion (Reitz et al. 1999).

Overdosed and a prolonged treatment of propofol caused cellular cytotoxicity in multiple organs and tissues such as brain, heart, kidney, skeletal muscle, and immune cells; yet, the underlying mechanism remained unknown, particularly in vascular endothelial cells. Intraperitoneal administration of a propofol overdose in

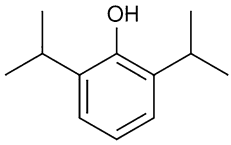


Fig. 4.1 Chemical structure of propofol containing a phenolic hydroxyl group and thus resembles that of α-tocopherol (vitamin E), a natural antioxidant

mice caused increased peritoneal vascular permeability. These results demonstrated the cytotoxic effects of propofol overdose, including necrosis-like cell death and mitochondrial apoptosis on endothelial cells *in vitro* and the endothelial barrier dysfunction by propofol *in vivo* (Lin et al. 2012).

Two mechanisms were suggested to explain the effects of the general anaesthetic propofol:

(i) Ca^{2+}-induced permeabilization of mitochondria, and
(ii) scavenging of free radicals during oxidative stress, inhibition of mitochondrial swelling and release of accumulated Ca^{2+} (Eriksson 1991).

The lysosomal/mitochondrial apoptosis caused by propofol has also been demonstrated in macrophages (Hsing et al. 2012). Both *in vitro* and *in vivo* studies have shown that the antioxidant activity of propofol resulted partly from the phenolic chemical structure (Ansley et al. 1998). Thus, it was logical to assume that propofol could also reduce the cisplatin-induced oxidative stress (Moghadam et al. 2014). Cisplatin has been used as a strong anti-cancer agent, but its use was limited, due to its severe side effects particularly ototoxicity and nephrotoxicity (Boulikas and Vougiouka 2003; Yao et al. 2007; Rybak et al. 2009). Indeed, propofol reversed not only mitochondrial permeability (Javadov 2000), but also reduced ischemia-reperfusion damages (Ko et al. 1997; Xia et al. 2011; Lai et al. 2011) supporting the notion that propofol could have prevented certain steps of the oxidative stress.

4.5 Medical Applications of Hypo- and Hypertonic Solutions

The medical usefulness of hypotonic treatment was demonstrated by the improvement of the ocular surface using hypotonic 0.4 % hyaluronic acid eye drops (Lester et al. 2000). It is assumed by the author of this book that the high molecular weight of hyaluronic acid could have played the same effect as dextran by preventing cells from disruption in the hypotonic medium and contributing significantly to cell proliferation and migration. Hypotonic riboflavin solution was administered to swell the cornea beyond 400 μm, and then the cornea was subjected UV irradiation (Hersh et al. 2011). Hypo-osmotic and low NaCl concentration treatment was used to raise the free intracellular calcium level in rat hippocampal neurons (Borgdorff et al. 2000). Less convincing was the conventional resuscitation of traumatic hemorrhagic shock involving the intravenous administration of isotonic (normal saline), slightly hypotonic (lactated Ringer's) or slightly hypertonic solution beginning in

the prehospital setting. Hypertonic and hypotonic solutions were given to resuscitate severely burned children. When the hypertonic lactated saline group received significantly more sodium than the hypotonic lactated saline group, there was no difference in sodium balance at 48 h postburn (Caldwell and Bowser 1979). The risks of intravenous administration of hypotonic fluids for pediatric patients led to recommendations for eliminating hypotonic fluids as stock items in both prehospital and emergency department settings (Jackson and Bolte 2000). Not surprisingly it was found that isotonic fluids were safer than hypotonic fluids in hospitalized children requiring maintenance by intravenous fluid therapy in terms of plasma sodium levels (Wang et al. 2014).

To the disputed question whether hypo- or hyperosmotic shock should be used, evidence was provided that suggested the benefit of hypertonic solution in early clinical trials (Rizoli et al. 1998; Holcroft et al. 1989; Mattox et al. 1991; Vassar et al. 1991, 1993; Younes et al. 1992; Bulger et al. 2008). Nevertheless, the use of hypertonic solution did not gain general acceptance (Wade et al. 1997a, b, 2004). Hypertonicity has been shown to modulate the innate immune response *in vitro* and in animal models of hemorrhagic shock, but the effect on the inflammatory response in humans remained largely unknown (Bulger et al. 2007, 2008). It is by now generally acknowledged that normal saline and lactated Ringer's are the two balanced salt solutions most commonly used in current fluid resuscitation.

4.6 Poly-ADP Ribosylation in Permeable Cells

Poly(ADP ribosyl)ation is a post-translational modification of nuclear proteins catalyzed by poly (ADP-ribose) polymerase. Poly(ADP ribosyl)ation takes place in most eukaryotic cells immediately after the generation of DNA strand breaks and is implicated in DNA repair and other processes related to DNA strand breakage. To measure poly(ADP-ribose) polymerase activity levels cells have been permeabilized and radioactively labeled with NAD^+ as substrate. Poly(ADP-ribose) polymerase can be activated by introducing DNA strand breaks by prior exposure of living cells to carcinogens or subjection to DNase I. Here only those measurements of poly(ADP-ribose) polymerase synthesis are mentioned that were performed in permeabilized cells:

(a) When the permeable cells were allowed to synthesize both poly(ADPR) and DNA simultaneously, the synthesis of one polymer had no effect on the rate of synthesis to the other macromolecule (Berger et al. 1978). Cells were permeabilized by these authors in a hypotonic buffer consisting of 10 mM Tris-HCl, pH 7.8, 1 mM EDTA, 4 mM $MgCl_2$, 30 mM 2-mercaptoethanol at 0 °C and incubated in an ice bath for 15 min. The suspension of DNA synthesis by inhibitors of topoisomerase II was not paralleled by the decrease of poly(ADP-ribose) synthesis, indicating that topological changes in DNA synthesis are independent of the homopolymer formation and suggested that DNA and

poly(ADP-ribose) synthesis are topologically independent processes. In conformity with this idea the inhibition of poly(ADP-ribose) synthesis by nicotinamide had no significant effect on DNA synthesis in permeable and intact cells (Sooki-Toth et al. 1986).

(b) Cellular regulation of ADP-ribosylation of proteins involved the selective augmentation of ADP-ribosylation of histone H3 in permeable murine thymocytes (Sooki-Toth et al. 1989).

(c) Poly (ADP ribosyl)ation, is an immediate response of most eukaryotic cells to DNA strand breaks and has been implicated in DNA repair and other cellular phenomena. Double-stranded oligonucleotides were conveniently used as chemically and stoichiometrically well-defined poly (ADP ribose) polymerase activators in permeabilized mammalian cells. Hypotonic permeabilization was accomplished using a buffer of 10 mM Tris-HCl (pH 7.8), 4 mM $MgCl_2$, 1 mM EDTA, and 30 mM β-mercaptoethanol (Grube et al. 1991).

(d) The overexpression of poly(ADP-ribose) polymerase did not promote survival after DNA damaging agents and γ-irradiation in cells subjected to complete permeabilization in hypotonic cold shock performed in the presence of 0.05 % (w/v) digitonin (Bernges et al. 1997).

(e) The involvement of poly(ADP-ribosyl)ation in DNA repair and the cellular recovery from DNA damage, led to the speculation that the higher poly(ADP-ribosyl)ation capacity of long-lived species might more efficiently help to slow down the accumulation of unrepaired DNA damage and of genetic alterations, as compared with short-lived species (Bürkle et al. 1994). The study of possible relation between longevity and poly(ADP-ribosyl)ation involved the measurement of maximal oligonucleotide-stimulated poly(ADP-ribose) polymerase activity in permeabilized, Epstein-Barr virus transformed lymphoblastoid cell lines. Poly(ADP-ribose) polymerase activity measured in various mammalian species provided evidence for the notion that longevity is associated with a high poly(ADP-ribosyl)ation capacity (Muiras et al. 1998).

(f) At high resolution of cell synchronization by centrifugal elutriation multiple non-overlapping repair and replication peaks were obtained. The elutriation profile of DNA repair peaks measured in permeable cells corresponded to the DNA fragmentation pattern measured by random oligonucleotide primed synthesis assay. The number and position of poly(ADP-ribose) peaks during S phase resembled those seen in the DNA replication profile. These results indicated that PAR synthesis is temporally coupled to DNA replication serving the purpose of genomic stability (Szepessy et al. 2003).

4.7 DNA Synthesis in Permeable Cells

It was realized in the 1960s that *in vitro* DNA synthesis in the presence of isolated DNA resulted in repair rather than in replicative DNA synthesis due to the fact that DNA isolation generated strand breaks. This recognition led to the development of

in vitro replication systems where the DNA remained intact and the replicative machinery functional. Although, no *in vitro* system can be perfect, individual systems may provide specific advantages with respect to certain aspects of DNA replication. A common requirement towards each *in vitro* replication system is to provide the presence of high concentration of cellular components approaching the *in vivo* demands.

In vitro replication systems were obtained by:

(a) Microinjection. Microinjected DNA (plasmid) initiated cellular DNA synthesis and not DNA repair or plasmid replication (Hyland et al. 1984). The applicability of microinjection is limited by the small population of cells affected.
(b) Isolated of nuclei. The replicative machinery in eukaryotic cells is located in the nucleus. Isolated nuclei act as independent units of replication (Blow and Watson 1987) and can be regarded as analogues of toluene treated permeable bacterial cells. For the optimal replicative function of isolated nuclei the addition of ATP, dNTPs and cytoplasmic fraction were needed. The initial rate of DNA replication in isolated nuclei approached the *in vivo* rate, but was gradually slowed down as the replication capacity was exhausted and only few percentages of DNA were replicated.
(c) Cell free extracts. Lysed cells were placed on a thin, transparent sheet made of regenerated cellulose with low permeability (e.g. cellophane). Small molecules diffused through the membrane, large molecular weight materials were simply layered on the lysate. Similarly to permeable cells this system was unable to initiate a new cycle of replication and only the ongoing one continued. DNA synthesis in lysed cells was semiconservative, discontinuous and could be adapted for the examination of bacterial and phage replication (Olivera and Bonhoeffer 1972).
(d) DNA replication in permeable cells. Permeable cell systems have been used mainly for studying DNA synthesis and has shown to be useful for analytical studies of DNA replication and repair synthesis (Sheinin et al. 1978; Seki and Oda 1986). It was observed that chromatin structure at the sites of DNA replicated in permeable cells was similar to that at the sites of DNA replicated in living cells, and that some structural changes (possibly toward the maturation) of newly replicated chromatin occurred after the DNA replication in permeable cells (Seki et al. 1986). In permeable cells the reassembly of native chromatin structure upon completion of repair was not an active process (Kaufmann et al. 1983).

Examples of applications of prokaryotic and eukaryotic permeable cell systems will be given in accordance with the subject of this book.

4.7.1 DNA Synthesis in Permeable Bacteria

One of the oldest and best known bacterial replicative system is the application of toluene treated cells (Moses and Richardson 1970). Less frequently used method was the permeabilization of cells with ether (Dürwald and Hoffmann-Berling 1971).

Toluene-treated cells were adapted and preferentially used for the permeabilization of *Bacillus subtilis* (Matsushita et al. 1971).

The major advantage of the application of these organic solvents was that after the suspension of *in vivo* replication by permeabilization, the semiconservative replication continued under *in vitro* conditions by the addition of the four deoxyribonucleotide triphosphates (dNTPs) and ATP. Moreover, in the presence of dNTPs, but in the absence of ATP, repair synthesis could be measured. These methods were suitable to investigate discontinuous DNA replication that could be blocked by inhibitors of replicative and repair DNA synthesis. Further advantages of toluene permeabilization were that cells were capable of being passed through not only nucleotides, but also other small molecules and drugs allowing the examination of their effects on DNA replication while retaining functional macromolecules (DNA, RNA, proteins) and preventing the passage of macromolecules in and out of the cells. The disadvantage of permeable cells was that the ongoing cellular processes continued, but a new cycle could not be initiated, thus they could not be regarded as viable cells. Chromosome initiation was absent and toluene treated permeable *B. subtilis* cells allowed only the measurement of ongoing DNA replication (Sueoka et al. 1973).

Synthesis of Nascent DNA in Permeable B. subtilis Cells DNA synthesis in prokaryotic cells is an extremely fast process with the incorporation of ~1600–1700 base pairs per sec. To study the initial steps of DNA replication it was advantageous that the process could be slowed down. From this perspective it was an important observation that in permeable cells the *in vitro* rate of DNA replication represented only a few percentages of the *in vivo* process. Under *in vitro* conditions the synthetic rate was still high (80–100 nucleotide/s, which could be further slowed down by 30 % in the presence of the nucleotide analogue Hg-dCTPn to 70–75 nucleotides/s. Another requirement of *in vitro* DNA synthesis was to achieve high (~80 %) replicative/repair ratio. The ratio of replicative *versus* repair DNA synthesis in *Escherichia coli* strains (D 110, JC 5029, B/r) was less than 4:1 (<80 % replicative, >20 % repair synthesis) and showed significant variation, the reason of which remained unexplained (G Banfalvi and CC Richardson, personal communication). In *B. subtilis* (*B. subtilis* 168 indol$^-$, and its pol A mutants BD 274, polA59, trpC2, thr-5) strains the repair/replication ratio was about 9:1 corresponding to 90 % replicative and 10 % repair synthesis (Table 4.1) and was decisive in choosing *B. subtilis* rather than *E. coli* cells to study DNA replication in permeable cells.

Reactions in 100 µl lasted for 30 min at 37 °C in toluenized cells (5×10^8) (Banfalvi and Sarkar 1983). Complete reaction mixture contained 70 µM potassium phosphate buffer (pH 7.5), 13 mM $MgSO_4$, 1.5 mM ATP, 40 µM each of dATP, dGTP and 5-Hg-dCTP, 100 µM each of CTP, UTP and GTP, 10 mM 2-mercaptoethanol, 6 µM [^{3}H]dTTP (7 Ci/mmol) and permeable cells. Reactions were terminated by the addition of 100 µl of 1 M perchloric acid. Samples were centrifuged (3000 g, 5 min) washed three times with ice cold 0.5 M perchloric acid. Precipitate was dissolved in Soluene (Packard) solution and radioactivity of the dissolved precipitate was determined.

Table 4.1 Mercury-DNA synthesis in permeable *E. coli* and *B. subtilis* cells

Reaction mixture	Relative speed of DNA synthesis expressed in percentages of the complete reaction mixture in	
	E. coli	*B. subtilis*
Complete	100.0	100.0
– dNTP (–dATP, –dGTP, –Hg-dCTP)	9.1	6.0
– ATP (repair synthesis)	21.3	9.3
+ Novobiocin (40 μM)	35.7	28.3

Toluene treated *B. subtilis* cells maintained the replicative character of DNA synthesis and 5-Hg-dCTP substituted effectively dCTP as one of the four substrates of DNA replication. Moreover, the synthesized mercury-substituted DNA was biologically active and could be transformed (Bhattacharya and Sarkar 1981, 1982). Mercury nucleotides have been introduced earlier as potential tools in nucleic acids research (Dale et al. 1973). Mercury labeled nucleic acids have been selectively isolated on thiol-substituted affinity matrices (Dale and Ward 1975). Mercury nucleotides have been also applied for the examination of *in vitro* transcription in eukaryotic and viral systems (Smith and Huang 1976; Zasloff and Felsenfeld 1977; Konkel and Ingram 1977; Nguyen-Hun et al. 1978). Mercurated nucleotides provided us with a means to study the chemical nature of early intermediates of DNA replication in permeable *B. subtilis* cells (Banfalvi and Sarkar 1983). The replicative and repair DNA synthetic potentials in permeable *E. coli* and *B. subtilis* are compared in Table 4.1.

4.7.2 Isolation and Analysis of Nascent Mercury-DNA Synthesized in Permeable Bacteria

To be able to analyze early fragments of nascent DNA not only the synthetic rate of DNA replication was reduced but also the fragment length was limited by the incorporation of dideoxy ATP as chain terminating nucleotide. The impact of dideoxy ATP, and inhibitors on DNA replication in our *in vitro* system of permeable *B. subtilis* cells is demonstrated in Table 4.2.

Synthesis in permeable cells (5×10^8) lasted for 30 min at 37 °C as described under Table 4.1 with the exception that 2′,3′-dideoxyATP (40 μM) and inhibitors HPUra or Novobiocin were added to the incubation mixture.

The incorporation of [^{3}H]-dTTP in the presence of ddATP decreased by about 75 % to 18–22 nucleotides/s, but maintained its replicative character proven by the stimulation of ATP and inhibition with 6(p-hydroxyphenylazo)-uracil (HPUra) or novobiocin (Table 4.2). A further step to reduce the rate of Hg-DNA synthesis was the lowering of temperature of the incubation. In order to get as short DNA fragments as possible not only the presence of ddATP was useful, but also the short pulse of synthesis. DNA was synthesized in toluene permeabilized cells (10^9) in the presence of

Table 4.2 Inhibition of ATP-dependent replicative DNA synthesis in permeable *B. subtilis* cells in the presence and absence of 2′,3′-dideoxyATP

Reaction mixture	Incorporation of [^{3}H]-dTTP (pmol)	
	– ddATP	+ ddATP
– ATP	2.4	1.8
+ ATP (1.5 mM)	22.2	5.9
+ ATP, + 6 (p-Hydroxyphenyl)-azouracil (HPUra) (0.2 mM)	3.5	2.6
+ ATP, + Novobiocin (50 μg/ml)	8.2	3.7

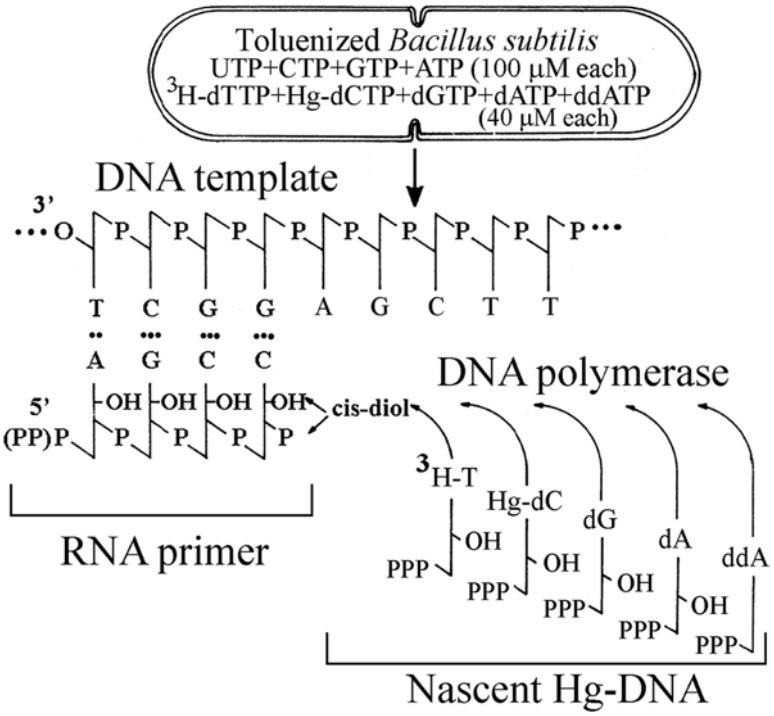


Fig. 4.2 Schematic representation of synthesis of RNA-linked nascent ^{3}H-Hg-DNA in permeable cells of *Bacillus subtilis*

5-Hg-dCTP (40 μM), 2′,3′-dideoxyATP (40 μM) at 25 °C for 1 min. The synthesis of ^{3}H-Hg-DNA in permeable *B. subtilis* cells is schematically viewed in Fig. 4.2.

After Hg-DNA synthesis cells were lysed in the presence of nuclease inhibitors, detergent and proteinase K. Sulfopropyl-Sephadex gel filtration served to separate the synthesized polynucleotides of the cell lysate from the small molecular weight substrates. Polynucleotides were heat-denatured at 100 °C and subjected to thiol-agarose affinity chromatography. The Hg-DNA obtained by thiol-agarose affinity chromatography contained dideoxyAMP nucleotide at its 3′-end. This DNA was labeled at its

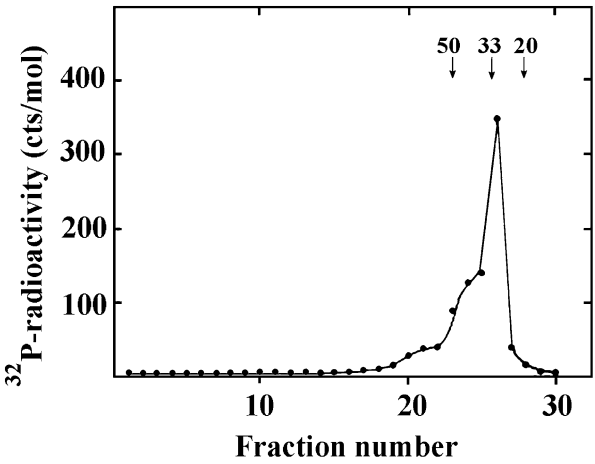


Fig. 4.3 Polyacrylamide gel electrophoresis of 5′-^{32}P-labeled, internally ^{3}H-dTTP-labeled and mercurated nascent DNA. *B. subtilis* ^{3}H-Hg-DNA was terminally labeled with [γ-^{32}P]ATP and polynucleotide kinase. Electrophoresis in 14 % (w/v) polyacrylamide gel was compared to molecular weight standards run parallel in the gel (With permission Banfalvi and Sarkar 1983, Fig. 2).

5′-end with ^{32}P, and its length was determined in 14 % polyacrylamide gel. More than 90 % of the 5′- ^{32}P-^{3}H-Hg-DNA moved as a relatively sharp peak in the range of 20–50 oligonucleotide with an average chain length of 33 nucleotides (Fig. 4.3).

The nascent 5′-^{32}P-^{3}H-Hg-DNA containing 3′- dideoxyAMP termini was subjected to 5′-end analysis. Upon alkaline hydrolysis followed by Bio-Gel P4 gel filtration the ^{3}H-labeled peak remained in the same position, while 95 % of the ^{32}P-label turned to small molecular weight material. High pressure liquid chromatography identified the 5′-terminal small molecular weight material as adenosine 3′,5′-bisphosphate (80 %) and guanosine 3′,5′-bisphosphate (20 %) indicating the presence of ribonucleotides in nascent DNA. The nature of binding between RNA and DNA in the nascent DNA was tested by proving the presence of cis-diols. Boronyl-cellulose column chromatography showed that nascent Hg-DNA contained covalently bound ribonucleotides at its 5′-end. The size of RNA primer was estimated by exploiting the exonucleolytic activity of the DNA polymerase of T4 bacteriophage. This digestion revealed that more than 90 % of nascent DNA molecules carried tri- or tetranucleotide RNA primers at their 5′-end (Fig. 4.4).

The kinetics of the tri- and tetranucleotide formation were similar during the hydrolysis of nascent Hg-DNA by T4 polymerase indicating that the trinucleotides did not originate from the continued hydrolysis of tetranucleotides. We have used relatively high T4 DNA polymerase concentration (1500 units/ml) resulting in the complete removal of nascent DNA from its RNA primer including the deoxyribonucleotide at the site of RNA-DNA attachment. The lower enzyme concentration of T4 DNA polymerase (10 U/ml) used by Kurosawa and Okazaki (1979) could explain why these authors could not remove completely DNA from RNA and why their primer length was estimated to be longer than tri- or tetranucleotides. We have determined the ribonucleotide composition of the RNA primer by the method of Schultz and Wassarman (1980) that included digestion with T2 RNase, phosphorylation with γ-^{32}P-ATP and polynucleotide kinase and separation of 5′-end labeled ribonucleoside 3′,5′-bisphophate digestion products by thin layer chromatography. Based

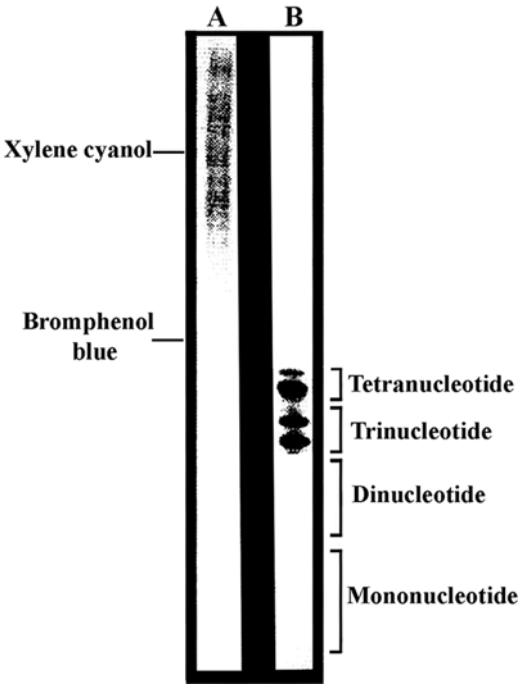


Fig. 4.4 Polyacrylamide gel electrophoresis of 5′-^{32}P-labeled mercurated nascent DNA before and after digestion by the 3′-exonuclease activity of bacteriophage T4 DNA polymerase. *Lane A*: Terminally ^{32}P-labeled nascent mercurated DNA (170 fmol) subjected to electrophoresis in 20 % polyacrylamide gel. *Lane B*: Terminally ^{32}P-labeled nascent mercurated DNA (170 fmol) digested for 2 h at 37 °C with bacteriophage T4 DNA polymerase (6 units) in a volume of 4 µl and then subjected to electrophoresis as described above. Electrophoretic mobilities of various oligonucleotides, bromophenol blue and xylene cyanol are also indicated (With permission Banfalvi and Sarkar 1983, Fig. 6).

on digestions with base specific RNases the composition of RNA primers of *B. subtilis* at the 5′-end of nascent DNA was: (pp)pApG(pC)$_{1\text{-}2}$-pDNA (Fig. 4.5).

The guanylate transferase reaction served to prove the nascent origin of RNA primer. This reaction can be performed only with nascent RNA with a triphosphate at the 5′-end. RNA primer attachment to nascent DNA by covalent bond formation was proved with consecutive steps of the guanylate transferase reaction followed by the capping reaction and resulting in m^{7}GpppG (Fig. 4.6).

4.7.3 Mechanism of RNA Primer Removal in B. subtilis

Next analysis was directed to reveal the mechanism of RNA primer removal from the 5′-end of nascent DNA. The question to be answered was the determination of minimum DNA chain length at which the RNA primer removal begins. When DNA chain growth was increased in the absence of chain terminating dideoxy ATP,

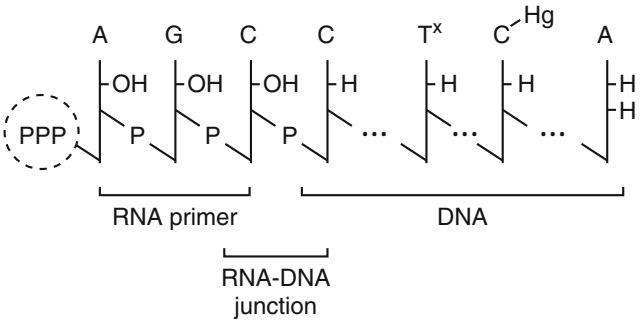


Fig. 4.5 RNA primer containing nascent Hg-DNA of *B. subtilis*. *Asterisk* represents radiolabeled nucleotide, T^x being ^{3}H-labeled dTMP

the length of nascent DNA increased significantly. The ribonucleotide distribution at the 5′ terminus of nascent DNA at increasing chain length from 40 to 200 nucleotides showed that the extent of RNA substitution at its 5′-end declined from 80 % to nearly 0 % (Banfalvi and Sarkar 1985). Only the early replicative intermediates (5–40 nucleotide long) carried RNA primers. RNA primer removal took place with medium size intermediates of discontinuous DNA replication (40–200 nucleotides). Immature (200–1000 nucleotide) and mature Okazaki fragments (1000–2000 nucleotide) have already lost their RNA primers (Fig. 4.7).

Conclusions The pioneering use of permeabilized bacterial cells for Hg^{2+}-affinity labeling and isolation of nascent DNA led to its characterization:

- 95 % of nascent DNA contained RNA primers
- 5′-end analysis of nascent DNA demonstrated for the first time that RNA priming took place during the replication of the bacterial chromosome
- early intermediates (5–40 nucleotide long) of DNA replication were identified
- RNA primer mechanism was clarified, the size, composition and sequence of RNA primer were determined
- the mechanism of RNA primer removal was described and included the elongation of lagging strand and Okazaki fragment formation (Banfalvi and Sarkar 1983, 1985).

4.7.4 Nascent DNA Synthesized in Reversibly Permeabilized Murine Thymocytes

The extension of study of the bacterial DNA replication from permeabilized bacterial cells to permeable murine lymphocytes followed a similar strategy of analysis of nascent Hg-DNA chains. The method of permeabilization (Halldorson et al. 1978) proved to be suitable to apply it to mouse thymus, murine spleen and human

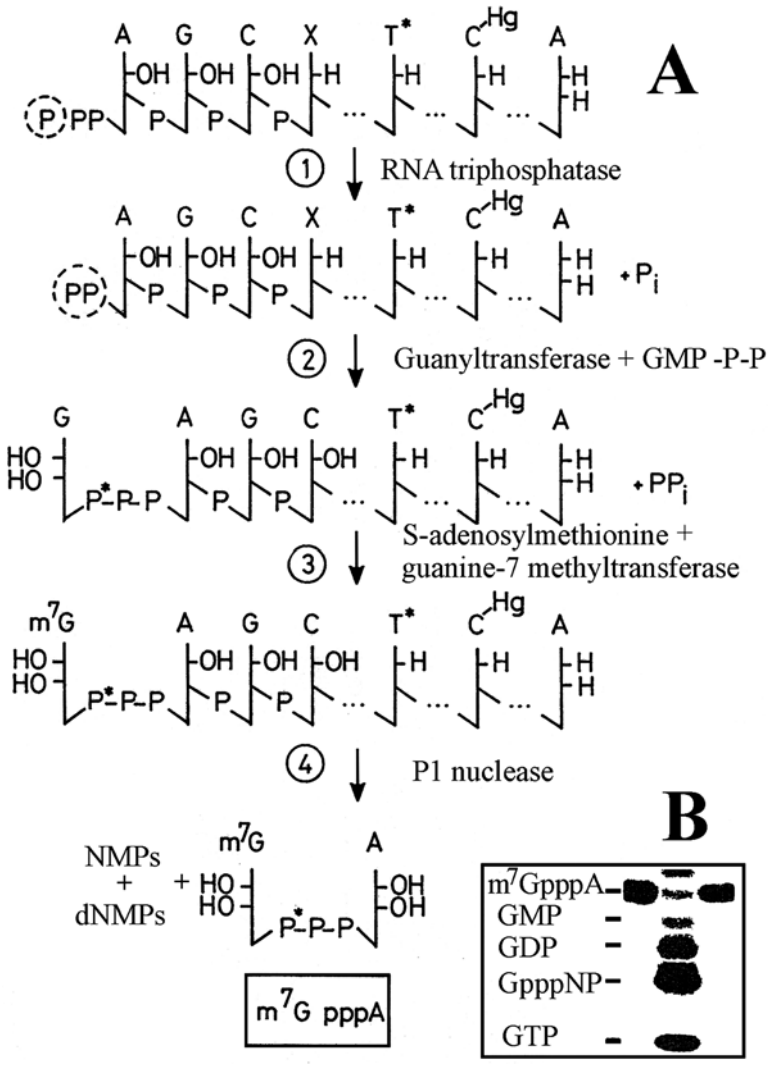


Fig. 4.6 Guanylation of nascent Hg-DNA at the 5′-end using the eukaryotic capping enzyme complex. (**A**) Capping enzyme complex consisted of three enzymes: RNA triphosphatase, guanyltransferase and guanine-methyltransferase. Radioactive labeling is indicated by the *asterisks*: TMP being labeled with ^{3}H and phosphorus ^{32}P-labeled. (**B**) Polyacrylamide gel electrophoresis and autoradiography of digestion products of guanylated nascent Hg-DNA and yeast 5S RNA (as positive control). Left lane of gel electrophoresis: P1 nuclease digested guanylated 5S RNA. Middle lane: Alkaline hydrolysis of guanylated 5S RNA. Right lane: Hydrolysis product of P1 digested guanylated nascent Hg-DNA (Boxed area at the bottom was reproduced with permission Banfalvi and Sarkar 1985, Fig. 1)

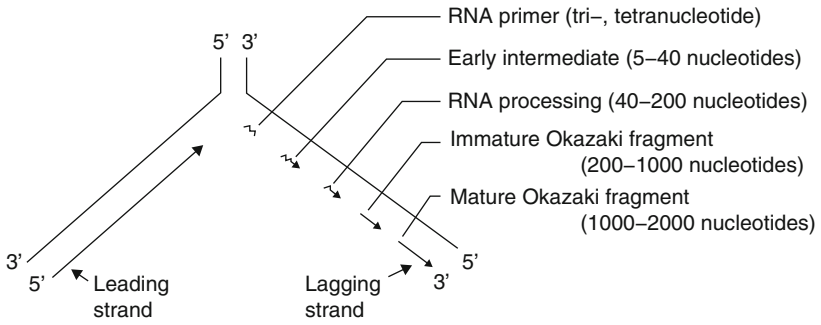


Fig. 4.7 Intermediates of DNA synthesis at the lagging strand of the replication fork during discontinuous replication of *B. subtilis*

tonsillar lymphocytes at $2-3\times10^7$ cells/ml in hypotonic solution. The hypotonic solution consisted of 9 mM hepes pH 7.8, 5 mM dithiothreitol, 4.5 % (w/v) dextran approx. mol. wt 110,000, l mM EGTA, 4.5 mM $MgCl_2$. Not only lymphocytes, but cells from different sources could be permeabilized up to 5.5×10^7 cells/ml. About 95 % of cells became permeable rapidly, allowing the reduction of permeabilization time from 15 to 2 min at 0 °C. After permeabilization the hypotonic solution was replaced by serum enriched medium, resulting in the restoration of the integrity of the cell membrane in the majority of the cells seen by trypan blue dye exclusion (Fig. 4.8). The kinetics of cell regeneration was followed by the incorporation of [^{3}H]-thymidine incorporation characteristic to intact cells, while permeable cells were unable to utilize this nucleoside for DNA synthesis. Permeable cells have lost their ability to incorporate [^{3}H]-thymidine, but ~60 % of permeabilized cells regained their capacity to incorporate it after 4 h of regeneration. There was an approximately 10 % loss of viability after 22 h accounted for by the extended incubation *versus* the 5 % loss in the control experiment where no permeabilization was performed (Fig. 4.8).

Scanning electron microscopy confirmed the regeneration of permeable cells. Figure 4.9a–f shows the morphology of intact, permeable and sealed cultures of murine thymocytes. Control and sealed cells are similar, but still distinguishable by the somewhat larger size and scarred appearance of regenerated cells. Drastic changes in the surface structure of permeabilized cells are indicated by their oval shape, tendency to stick together and hollows indicating the loss of membrane integrity.

4.7.5 Measurement of Replicative and Repair DNA Synthesis in Permeable Cells

The analysis of DNA synthesized in permeable cells has been extended to distinguish and to simultaneously measure the two types of DNA synthesis in fractions of synchronized cells. Synchrony was obtained by centrifugal elutriation of

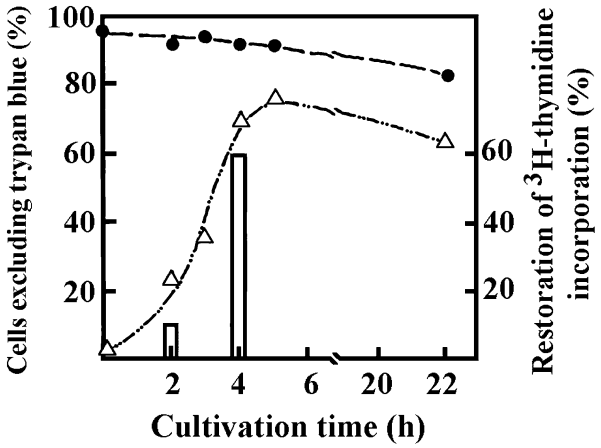


Fig. 4.8 Sealing of murine thymocytes treated with dextran T-150. Cells were permeabilized in hypotonic solution and then diluted with Parker-199 medium enriched with 10 % human AB serum. The viable cells were counted during the incubation by trypan dye exclusion test and expressed as percentages of total cells (Δ). Simultaneously, the viability of intact cells (●), and the [^{3}H]thymidine incorporation (□) after permeabilization were followed and expressed as percentages of [^{3}H]thymidine incorporation of intact cells (With permission Banfalvi et al. 1984a, Fig. 1)

logarithmic-phase Chinese hamster ovary cells. DNA replication in permeable bacteria (Banfalvi and Sarkar 1983, 1985) and mammalian cells was found to be replicative in the presence of ATP, whereas the nucleotide incorporation without ATP represented mainly repair synthesis (Banfalvi et al. 1984a; Basnakian et al. 1989). In major repair processes such as nucleotide excision repair and base excision repair the nucleotide incorporation with repair polymerases takes place without ATP and needs only dNTP substrates for gap filling (Hanawalt 1995; Seeberg et al. 1985). This ATP-independent repair step is generally used as nick-translation. Analogous to nick-translation, repair DNA synthesis in permeable cells could be performed in the absence of ATP and in the presence of substrates (dNTPs). However, to measure exclusively ATP-independent dNTP incorporation, i.e. repair synthesis in permeable cells, the residual ATP production has to be removed completely. We have introduced in the *in vitro* incubation mixture of repair synthesis glycerol (1 %) and an accessory enzyme *E. coli* glycerokinase, which is specific for ATP, but not for dATP. The presence of glycerol and glycerokinase ensured that ATP produced by permeable cells was completely used up for the phosphorylation of glycerol and no ATP remained for residual replicative DNA synthesis. Replicative synthesis in the presence of ATP and repair synthesis by the complete elimination of replication in the incubation mixture allowed the side-by-side measurement of the two DNA synthetic processes in cells synchronized by counterflow centrifugal elutriation.

Exponentially growing CHO cells in suspension culture were subjected to counterflow centrifugal elutriation to collect synchronized cell fractions. The resolution power of elutriation was increased from low to medium and high grade (Fig. 4.10a–c).

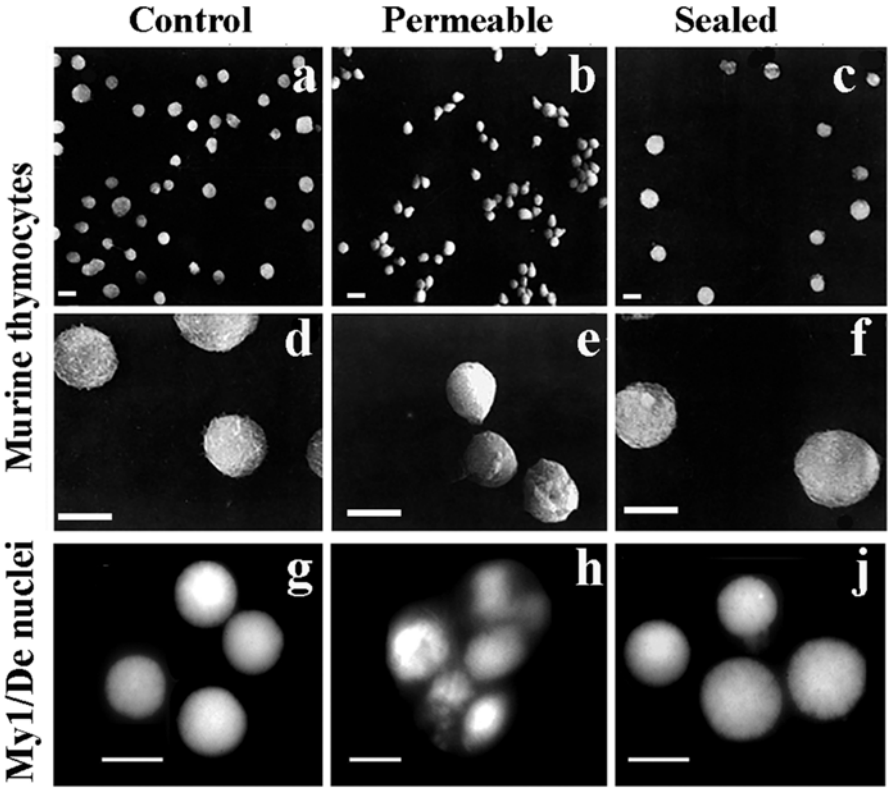


Fig. 4.9 The effect of permeabilization on the shape of cells and nuclei. **a–f** Scanning electron microscopy of intact (**a**, **d**), permeable (**b**, **e**) and sealed (**c**, **f**) murine thymocytes (With permission Banfalvi et al. 1984a, Fig. 2). DAPI staining of nuclei isolated from control non-permeabilized myeloid leukemia (My1/De) cells (**g**), nuclei isolated from permeabilized leukemia cells (**h**), nuclei of reversibly permeabilized (My1/De) cells (**j**). Bar, 10 μm each

As a result the biphasic replication profile has been resolved and subphases of replication were distinguished as multiple replication peaks (Fig. 4.10d–f). These replication peaks termed replication checkpoints were distributed evenly throughout the S phase (Banfalvi et al. 1997b).

The work in permeable cells made possible the independent measurement of replicative and repair synthesis and was utilized to compare the two types of macromolecular biosyntheses in control and cadmium treated cells upon subjecting them to cell cycle synchronization (Fig. 4.11). The following conclusions were drawn concerning the relationship of replicative and repair DNA synthesis:

(i) The rates of ATP-independent repair and ATP-dependent replicative DNA synthesis performed in reversibly permeabilized CHO cells were inversely correlated. When the rate of repair synthesis was high in the cell cycle, ATP-dependent replicative synthesis was low and *vice versa* (Banfalvi et al. 1997a).

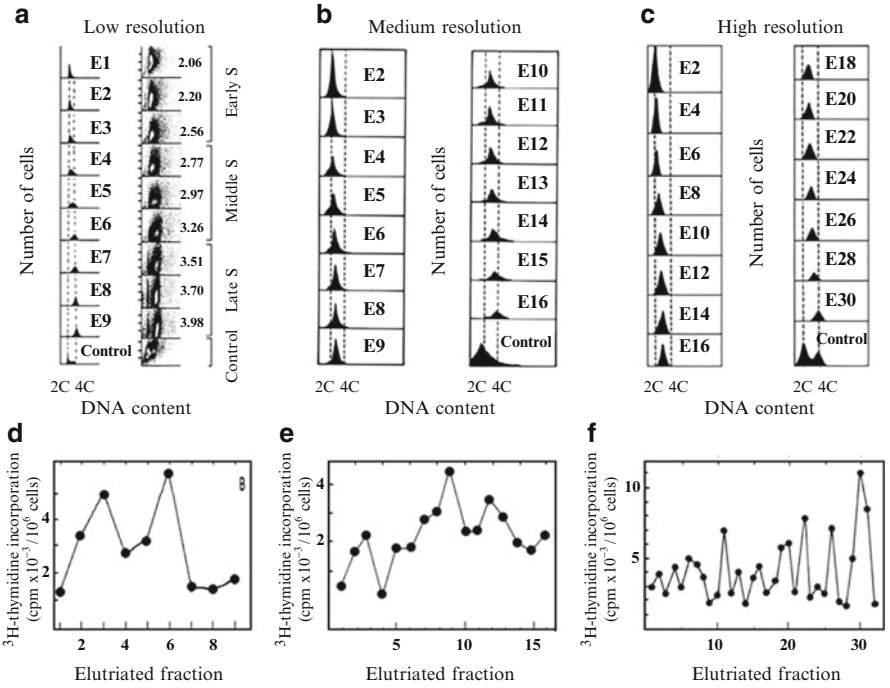


Fig. 4.10 Cell cycle analysis and replication profiles at increasing resolution of centrifugal elutriation. Flow cytometric analysis at low resolution of centrifugal elutriation by collecting nine fractions (**a**). Cell cycle analysis on Coulter Channelizer at medium resolution of elutriation and collecting 16 fractions (**b**). Cell cycle analysis with Coulter Channelizer at high resolution and collecting 32 fractions (**c**). DNA synthesis was measured in permeable cells. Replication subphases at low (**d**), medium (**e**), and high resolution of centrifugal elutriation (**f**) (With permission Banfalvi et al. 1997b, combination of Figs. 1-4)

(ii) Several replication and repair subphases seen as peaks have been distinguished, evenly distributed throughout the S phase (Banfalvi et al. 1997a).

(iii) The number of replication subphases and repair peaks corresponded to the haploid number of chromosomes in *B. subtilis* (Banfalvi et al. 1997b).

(iv) Correlation between DNA replication subphases and the number of chromosomes has been confirmed in *Drosophila melanogaster* excluding the possibility of coincidence between replication subphases and chromosome number (Rehak et al. 2000).

(v) Upon Cd^{2+} treatment, repair synthesis was elevated in certain subphases while replication in these subphases were suppressed providing a valuable permeable system for testing new carcinogens (Banfalvi et al. 2000).

(vi) The existence of subphase-specific repair synthesis was confirmed in permeable murine preB cells after gamma irradiation (Offer et al. 2001).

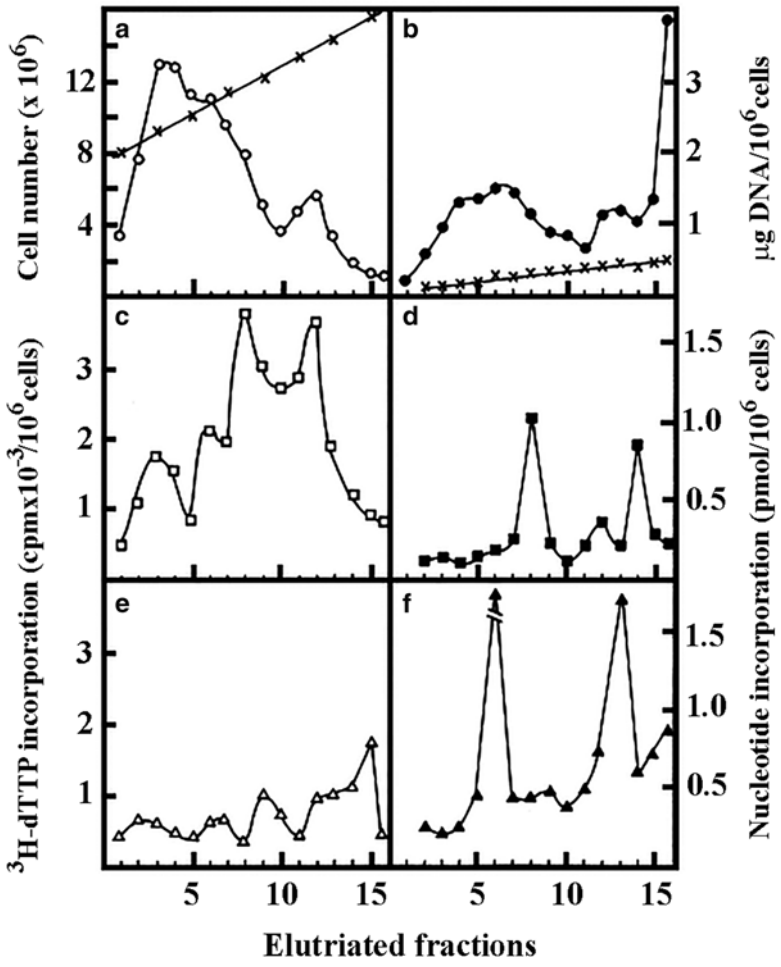


Fig. 4.11 Elutriation profiles of DNA synthesis in control and Cd-treated cells. (**a**) Cell number in elutriated fractions of control cells (O); (x) DNA content. (**b**) Cell number in Cd-treated cells (●); (x) DNA content. (**c**) DNA replication in control cells. (**d**) DNA replication after 9 h of Cd treatment (0.5 μM). (**e**) Repair synthesis in control cells. (**f**) Repair synthesis after 9 h of Cd-treatment (0.5 μM) (With permission Banfalvi et al. 2000, Fig. 2).

4.7.6 DNA Excreted by Stimulated Lymphocytes

We have found that newly synthesized DNA, 8–60 nucleotides in length contained more than 80 % RNA-linked DNA. The sequence of RNA primers was not specific and was predominantly 7–9 nucleotides in length (Banfalvi et al. 1984a). A related analysis concerned the origin of short DNA fragments (6–60 nucleotides) excreted

by phytohemagglutinin stimulated human peripheral blood lymphocytes. These DNA fragments did not contain RNA primers at their 5′-termini and suggested that DNA excreted by stimulated lymphocytes is of degradation rather than replication origin (Banfalvi et al. 1984b).

4.7.7 Early Replicative Intermediates Shorter than Okazaki Fragments

After the characterization of Hg-DNA synthesized in reversible permeable murine thymocytes we have analyzed the 5′-ends of oligonucleotides isolated from thymocytes 48 h after a single dose emetine administration to mice. This small-molecular-weight population of nascent DNA was absent in normal replication (Fig. 4.12a). More than 90 % of the 5′-end ^{32}P-labeled oligonucleotides carried terminally phosphorylated RNA primers. The size of the short nascent DNA fragments ranged between 9 and 50 nucleotides with an average chain length of 15 nucleotides (Fig. 4.12b). These oligomers are regarded as the carriers of the RNA primers and the precursors of the Okazaki fragments. The average chain length of Okazaki is about 200 nucleotides in mammalian cells.

Conclusions The techniques used for the isolation and analysis of nascent DNA synthesized in permeable bacteria have been extended to the study of DNA replication in reversibly permeabilized animal cells. By inhibiting the *in vivo* lagging strand synthesis in murine cells by emetine treatment, we have identified early replicative intermediates, shorter than Okazaki fragments, which are the real carriers of RNA primers (Csuka and Banfalvi 1997).

4.8 Temporal Order of Gene Replication and Gene Expression in Permeable Cells

An experimental system using permeable cells was developed to investigate the replication order of various genes. Exponentially growing CHO-KI cells were separated into populations at different stages of the cell cycle by centrifugal elutriation and analyzed for cell cycle status by flow cytometry (Taljanidisz et al. 1989). The replication of specific genes in each elutriated fraction was measured by labeling with 5-mercuri-dCTP and [^{3H}]dTTP under conditions of optimal DNA synthesis after cell permeabilization. Hybridization probes for specific sequences of genes were described (Taljanidisz et al. 1989). Nascent mercurated DNA was isolated from each elutriated fraction by affinity chromatography on thiol-agarose

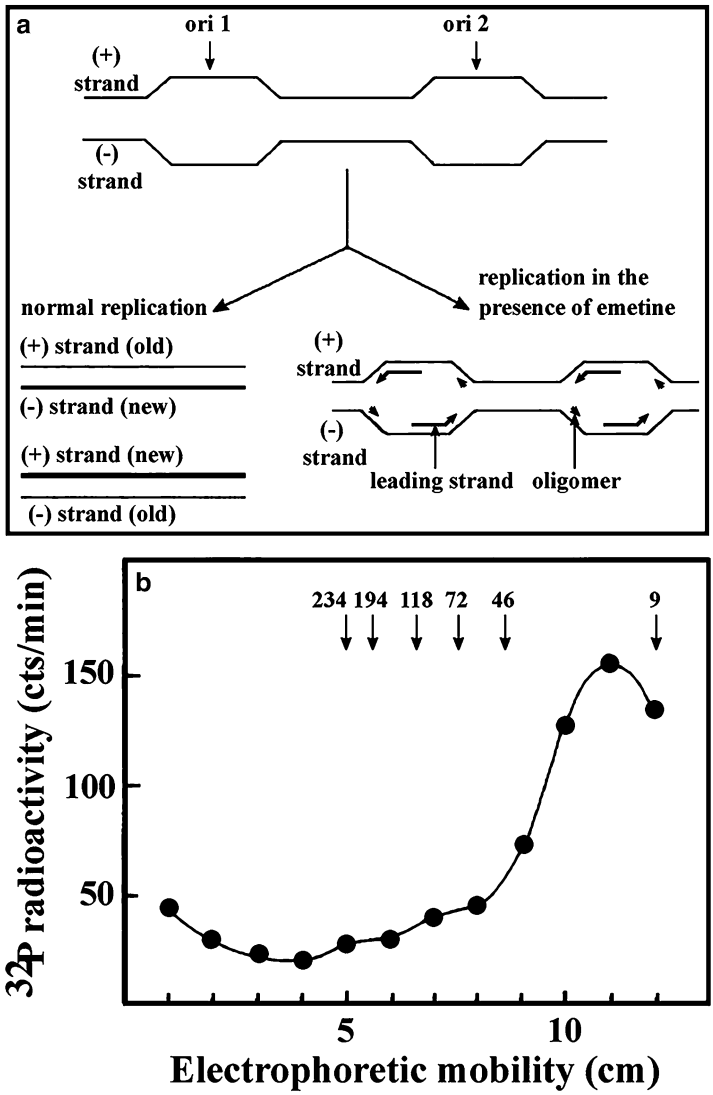


Fig. 4.12 Accumulation of short DNA fragments in thymocytes after emetine treatment of mice. Thymocytes were isolated from saline-treated (control) and emetine-treated mice. Nascent Hg-DNA was synthesized in permeable cells (Banfalvi et al. 1984a, b). DNA was isolated as described (Csuka and Banfalvi 1997). (**a**) Schematic view of bidirectional replication at ori sites leading to newly synthesized DNA of the same chain length as the old parental DNA. Replication in the presence of emetine prevents the synthesis of Okazaki fragments, but allows the synthesis of oligomers carrying RNA primers. (**b**) Agarose (1.8 %) gel electrophoresis of the 5′-end labeled nascent oligomers compared to molecular weight standards (With permission Csuka and Banfalvi 1997, combination of Figs. 4 and 5)

and labeled by using [α-^{32}P]dATP and random primers. The ^{32}P-labeled DNA representing various stages of cell cycle was hybridized to plasmid DNA containing specific cloned genes. From these hybridizations the hybridization profiles were plotted with their peaks representing only a rough estimate of nuclear content and the time of replication of that particular gene expressed in haploid genome content (C-value). The C-values of genes (single-copy, multifamily, oncogenes, repetitive sequences) were found to be distributed all over the S phase. Some of the genes studied in human erythroleukemia (K562) cells were also examined by others (Epner et al. 1981; Furst et al. 1981; Goldman et al. 1984) and showed similarities, but were not identical with the replication pattern of our results given in Table 4.3. The reason of discrepancies could be explained by the overlapping replication curves with their peaks and C-values difficult to determine precisely. Results shown in Table 4.3 confirmed that qualitative observations could not be turned to quantitative results. The relationship between the replication order and gene expression was also traced to decide whether or not transcriptionally active genes replicate in early S phase, or in a later phase of the cell cycle in other transcriptionally less active tissues (Goldman et al. 1984; Gunita et al. 1986; Dhar et al. 1988; Hatton et al. 1988). To estimate the transcriptional potential of different genes replicating at various times, we have isolated total poly(A) RNA from exponentially growing K562 cells. Purified poly(A) RNA was immobilized on nylon membranes and hybridized separately with DNA replicating at various stages of S phase. ^{32}P-labeled replicas of Hg-DNA synthesized in permeable K562 cells were isolated from elutriated fractions. These experiments related to the temporal order of gene replication and gene expression (Table 4.4) led to the following conclusions:

1. To obtain information on regulatory mechanisms of DNA replication and transcription different agents could be introduced into permeable cells that would not enter intact cells.
2. The replication order of various genes (39 genes) in K562 cells could not be determined exactly.
3. The timing of replication of genes expressed in C-values was only a rough estimate, nevertheless indicated a continuous distribution throughout the S phase (from 2.0 to 4.0 C).
4. Expressed (transcribed) genes were thought to be replicated early; however, we did not find such correlation. We found poly(A) RNA expression from all varieties of genes: early, middle and late replicating genes.

Table 4.3 Replication times (C-values) of various genes in human erythroleukemia (K562) cells

Genes	Plasmid probes	Relatives rates of replication in cells from elutriated S-phase fractions									Average replication time of gene
		1 (2.06C)	2 (2.20C)	3 (2.56C)	4 (2.77C)	5 (2.97C)	6 (3.26C)	7 (3.51C)	8 (3.70C)	9 (3.98C)	
Alu repetitive seq	BLUR 8	15.5	28.8	8.7	1.5	7.4	16.3	9.7	7.4	4.6	2.15
r DNA	28 S	44.0	16.7	13.6	4.3	3.1	8.1	3.1	3.7	3.1	2.18
Thymidine Kinase	pH 5.2	6.8	33.8	20.1	5.9	1.8	4.6	3.7	1.4	21.9	2.33
Repetitive sequence	pB4	17.4	24.4	9.4	12.7	9.0	4.0	1.3	4.30	17.7	2.33
TSHα	Ratα	13.7	20.9	12.7	11.8	8.2	7.3	6.4	7.3	11.8	2.36
Prolactine	pRL	13.1	19.7	16.8	11.7	9.5	6.6	5.8	6.6	10.2	2.37
IB-apoferritine		1205	28.4	27.7	11.7	6.6	2.9	7.4	1.5	3.3	2.38
C-myc oncogene	pJay1 phc	16.4	34.3	29.0	17.0	4.9	0.9	11.0	0.9	0.4	2.38
Histon 3-2	pRAH 3-2	12.9	15.9	19.0	11.4	8.4	7.6	9.1	8.4	7.6	2.39
HPRT	pHpT-20	14.4	16.9	26.0	16.9	5.2	5.2	3.9	6.5	2.6	2.43
α-globin		4.7	22.1	21.9	8.5	3.8	7.0	9.2	10.6	12.3	2.44
Collagen α_2	pHF_{32}	13.6	16.5	28.6	24.2	4.4	3.3	3.3	5.5	4.4	2.46
β-tubulin	β-TPT	16.3	20.8	18.2	13.0	12.4	9.1	6.5	3.3	1.3	2.46
TK	pHATK1	6.6	16.9	18.8	15.9	9.4	3.8	5.6	10.3	12.2	2.50
CAD	pCAD41	11.2	16.5	18.9	15.9	11.2	6.5	4.1	10.6	5.3	2.51
N-Ras oncogene	pNR-Sac	10.9	15.3	18.9	12.8	13.9	8.8	13.7	9.2	4.4	2.52
Myosin heavy chain	pMHC	3.4	21.1	32.9	28.9	7.0	1.4	1.1	3.1	2.5	2.54
R-albumin		13.1	15.8	18.0	22.1	14.9	6.3	3.6	3.2	3.2	2.54
Collagen α_1	$pHUC_1$	3.5	19.2	20.7	15.1	10.6	4.5	7.0	1.5	2.5	2.57
X chromosome gene	XNH-3	9.8	14.8	31.9	21.3	11.5	1.6	0.8	2.5	5.7	2.61
Encephalin	pHpE-9	6.6	15.4	28.1	22.4	16.1	3.4	1.6	2.3	3.0	2.63
Hydroxymethyl gluCoA reductase	pRed	9.1	10.3	11.9	22.2	21.7	5.1	6.8	6.8	5.7	2.66

X chromosome gene	X-D	8.9	13.9	18.8	22.8	17.8	4.9	6.9	2.9	2.9	2.66
DHFR	pB67HI	9.2	10.9	17.6	25.2	15.9	7.6	3.4	5.9	4.2	2.67
V-Ki-ras oncogene	pHi-hi3	6.7	11.4	21.2	24.5	18.7	3.4	1.2	7.4	5.3	2.68
V-Ha-ras oncogene	pHB11	3.4	4.7	20.2	20.2	12.2	6.2	7.2	1.5	17.7	2.74
Immunoglobulin	pYJV	1.1	5.5	6.6	47.3	25.3	2.2	6.6	2.2	1.1	2.76
Cytochrome P1-450	pHav-cat	5.2	6.1	18.1	25.7	20.2	3.3	4.3	2.0	12.3	2.77
Nebulin		2.9	6.5	8.9	41.3	21.8	2.9	4.1	4.1	7.1	2.80
Immunoglob. h.c.	pHCG β	1.9	3.5	7.4	35.8	29.1	7.7	4.2	3.5	6.1	2.86
Immunoglob. h.c.	pHCG α	8.7	2.5	4.2	36.7	28.8	11.6	3.6	2.4	3.2	2.92
V-Sis oncogene	pV-Sis	9.1	8.3	13.3	11.6	31.5	13.3	2.5	6.6	4.2	2.92
p21 protooncogene	pCDck-76	7.6	3.5	4.7	20.6	17.7	9.6	8.8	18.2	8.5	2.94
β-globin	pVa1	3.0	5.3	5.3	15.2	24.3	12.9	6.8	9.1	17.5	2.98
C-fos protooncogene	pc-fos	10.0	8.8	6.8	16.8	18.5	17.5	13.0	8.0	2.0	3.10
Muscular dystrophy	pDMD	12.2	15.8	12.4	12.3	12.2	19.1	11.8	3.1	2.4	2.34 and 3.25
APRT	pH2	4.8	6.0	7.2	11.8	12.9	13.9	14.2	15.1	12.2	3.37
N-Myc protooncogene	pN6-6	4.4	0.9	0.9	4.4	12.3	29.0	22.0	15.8	9.7	3.44
β-actin	P221	8.9	9.4	9.4	8.5	9.2	13.4	15.5	17.5	8.6	3.51

Table 4.4 Expression of genes in human myelogenous leukemia (K562)cells

Genes	Plasmid probes	PolyA-RNA (cpm)	Expression of mRNA non-polyadenylated RNA (cpm)	Hybridized DNA (cpm)	Ratio of RNA/DNA hybridization
Alu repetitive seq.	BLUR 8	456	18	7466	0.063
rDNA	28S	1990	2199	161	26.0
Repetitive sequ (SINE)	pB4	38	21	299	0.197
TSHα	Ratα	n.e.	n.e.	110	–
Prolactin	pRL	n.e.	n.e.	137	–
IB-apoferritin		217	47	1456	0.181
C-Myc oncogene	pJay 1 phc-myc	1040	270	5746	0.228
Histon 3-2	pRAH 3-2	14	34	132	0.363
HPRT	pHpT-20	n.e.	n.e.	75	–
α-globin		212	24	530	0.445
β -tubulin	β TPT	n.e.	n.e.	155	–
TK	pHATK1	14	0	106	0.132
CAD	pCAD41	0	3	170	–
N-Ras oncogene	pNR-Sac	3150	563	26,992	0.138
Myosin h.c.	pMHC	n.e.	n.e.	564	–
R-albumin		n.e.	n.e.	222	–
Collagenα_1	pHUCα_1	101	0	198	0.510
X-chromosome	XNH-3	0	16	122	0.131
Encephalin	pHpE-9	n.e.	n.e.	1237	–
Hydroxymethyl gluco-reductase	pRed	87	6	175	0.531
X-chromosome	X-D	n.e.	n.e.	101	–
DHFR	pB67HI	n.e.	n.e.	119	–
V-Ki-ras oncogene	pHi-hi3	944	1196	1247	1.72
V-Ha-ras oncogene	pHB11	n.e.	n.e.	1209	–
Immunoglobulin JV chain	pYJV	39	60	89	1.11
Cytochrome P1-450	pHav-cat	48	10	1215	0.048
Nebulin		24	0	169	0.142
Immunoglobulin h.c.	pHCG α_2	n.e.	n.e.	925	–
V-Sis oncogene	pV-Sis	n.e.	n.e.	121	–
p21 Protooncogene	pCDck-76	130	9	901	0.154
β -globin	pVa1	212	0	131	1.62
C-Fos protooncogene	pc-fos	120	371	405	1.21
Muscular dystrophy	pDMD	56	0	1693	0.033

(continued)

Table 4.4 (continued)

Genes	Plasmid probes	PolyA-RNA (cpm)	Expression of mRNA non-polyadenylated RNA (cpm)	Hybridized DNA (cpm)	Ratio of RNA/DNA hybridization
APRT	pH2	5	0	409	–
N-Myc protooncogene	pN6-6	n.e.	n.e.	113	–
β –actin	p221	119	0	559	0.213
V-Neu oncogene	pSV-neu	0	556	n.d.	–
Erb a oncogene		34	38	n.d.	–
C-Abl oncogene	p102	n.e.	n.e.	n.d.	–
V-Mos oncogene	pHT10	n.e.	n.e.	n.d.	–
V-Myc oncogene	pmyc3	n.e.	n.e.	n.d.	–
Repetitive sequ. (LINE)	pC-1	74	44	n.d.	–

n.e. no measurable expression
n.d. not detected

4.9 Visualization of Replicons in Reversibly Permeabilized Cells

Newly synthesized DNA can be visualized in cells by the incorporation of radioactive nucleotides, affinity and fluorochrome binding to non-radioactive label or label with immunological systems. Pyrimidine analogues substituted at C-5 position proved to be particularly suitable in nucleic acid research. These analogues allowed the introduction into permeable cells bulky groups pointing toward the major groove of the double stranded DNA without the drastic reduction of DNA polymerase activities (Dale et al. 1973; Dale and Ward 1975; Langer et al. 1981). The introduction of biotinylated nucleotides in permeable cells did not significantly disturb normal DNA replication *in vivo* (Hiriyanna et al. 1988) or *in vitro* (Blow and Watson 1987; Langer et al. 1981). Biotinylated nucleotides could be incorporated in the DNA of permeable cells (Hunting et al. 1985; Nakayashu and Berezney 1989). The cytochemical advantages of non-isotopic visualization of biotin-labeled nucleotides (Van der Ploeg and Raap 1988) have been exploited by the development of a highly sensitive immunofluorescent method for the visualization of replicons in mammalian cells (Banfalvi et al. 1989). The visualization of replicons in mammalian cells by confocal microscopy revealed only six major replication foci at the beginning of S phase and not a very large number of putative replication origins that had been postulated. This highly sensitive technique gave sufficient time resolution to allow to identify a single site, the “master ori”, where the replication process started and then other replicons joined in and spread to other sites of the nucleus as shown in Fig. 4.13.

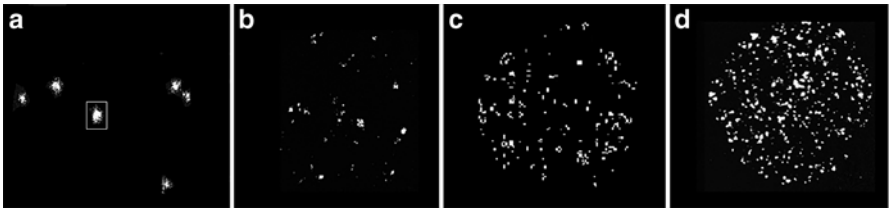


Fig. 4.13 Confocal imaging of replication sites at the unset of S phase. Pulse-label of nascent DNA synthesized in permeabilized cells took place after removal of hydroxyurea from synchronized cells and allowing cells to enter S phase. (**a**) Optical sectioning in eight steps started from the top of the nucleus in 0.5 μm sections (fourth section is shown). Framed *white box* contains the largest master ori. (**b**–**e**) Enhancement of early replication signals by optical Kalman filtration. (Modified with permission Banfalvi et al. 1990, Fig. 3)

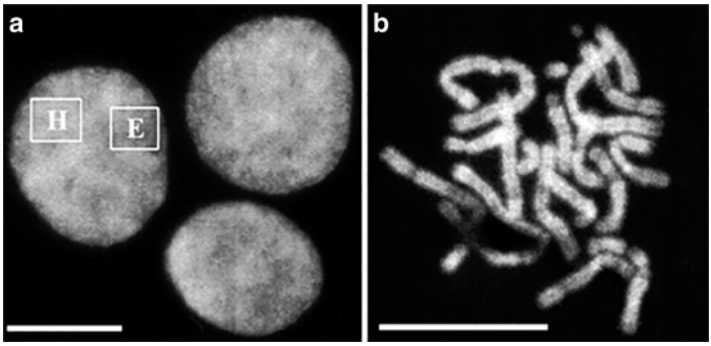


Fig. 4.14 Interphase nuclei of CHO cells (**a**) and metaphase chromosomes (**b**) isolated from Chinese hamster ovary cells. *H* heterochromatin, *E* euchromatin

4.10 Visualization of Intermediates of Chromosome Condensation Isolated from Nuclei of Reversibly Permeabilized Cells

The scarcity of evidence regarding chromosome condensation is related to the fact that individual chromosomes cannot be seen during their development in the interphase nucleus. The two basic nuclear structures seen under the microscope are interphase nuclei (Fig. 4.14a) and metaphase chromosomes (Fig. 4.14b).

Chromatin condensation resembles rope (chromosome) spinning from a long string (double stranded DNA). The importance of chromatin condensation, chromosome arrangement in the nucleus, chromatin models and models of chromosome condensation have been reviewed earlier (Banfalvi 2009). Nevertheless, the interphase chromatin folding that is poorly characterized, needs some explanation. The structure of interphase chromatin was deduced mainly by extrapolations from fully condensed metaphase chromosomes. The major obstacles in distinguishing among different structural forms were that chromatin was not studied:

– under physiological conditions in intact cells,
– during the development of chromosomes in interphase and
– by imaging chromatin structures providing insufficient microscopic resolution.

As earlier models did not come to a generally acceptable conclusion regarding chromatin condensation, the development of new imaging techniques was expected to shed light on this long standing problem in cell biology (Mora-Bermúdez et al. 2007). It was observed that chromatin condensed either into a formless chromatin mass in one cell line reflecting apparently an early apoptosis and in another cell line the late apoptosis with apoptotic bodies already visible (Buendia et al. 1999).

We have chosen a new approach to study chromosome condensation and to confirm the existence of a flexible folding pattern through the isolation of a series of transient geometric forms isolated from nuclei of Chinese hamster ovary cells. Exponentially growing cells were permeabilized reversibly and nascent DNA was synthesized in the presence of the four dNTPs, dTTP being replaced by biotin-11-dUTP (Banfalvi et al. 1989). Biotinylated nucleotides did not perturb DNA replication (Langer et al. 1981; Hunting et al. 1985; Blow and Watson 1987; Hiriyanna et al. 1988; Nakayasu and Berezney 1989; Banfalvi et al. 1989), but their interference with chromosome folding (Banfalvi et al. 1989, 1990) was exploited to accumulate intermediates of the condensation process (Banfalvi 1993).

By continued application of novel techniques for refining analysis of mammalian chromosome replication, synchronization of cells by centrifugal elutriation (Banfalvi 2008a, b), hyposmotic driven reversible permeabilization of synchronized cell fractions (Banfalvi et al. 2000) led to:

– fluorescent analysis of replication (Banfalvi 1993)
– chromatin image analysis and
– computational methods introduced to resolve structural differences invisible for the human eye (Gacsi et al. 2005).

The chromatin image analysis of precondensed CHO chromosomes is shown in Fig. 4.15.

4.10.1 Intermediates of Chromatin Condensation in CHO Cells

Reversible permeabilization driven by hypotonic buffer containing dextran T-150 was originally used for murine lymphocytes (Banfalvi et al. 1984a, b) and has been adapted to CHO cells. Permeabilization lasted for 2 min at 0 °C. After permeabilization the hypotonic solution was replaced by F-12 medium containing 10 % fetal bovine serum and the cells were allowed to regenerate their membrane in a CO_2 incubator at 37 °C and 5 % CO_2 for 3 h. After reversal of permeabilization cells (10^6) were resuspended in growth medium and treated with 0.1 μg/ml colcemid for

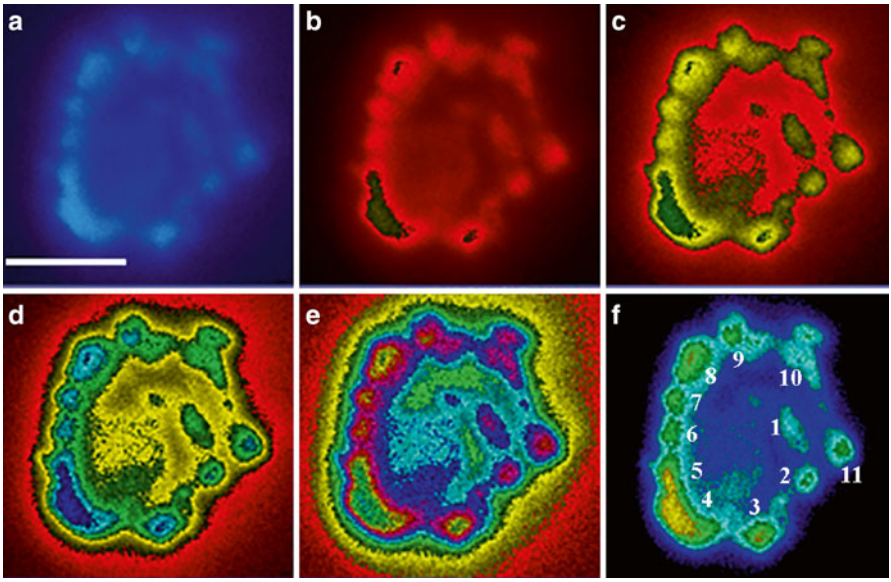


Fig. 4.15 Chromatin image analysis of precondensed CHO chromosomes. (**a**) A nucleus from Elutriation Fraction 7 stained with DAPI. (**b**–**f**) Image analysis of the nucleus shown in panel (**a**) by fitting a precalculated intensity mask to the chromatin image. Chromosome bodies are numbered consecutively by the white numbers on the outside of the circular arrangement and in order of size (**f**). Bar, 5 μm (Modified with permission Gacsi et al. 2005, Fig. 10)

2 h at 37 °C under 5 % CO_2. Chromatin structures were isolated by the addition of fixative (methanol:glacial acetic acid, 3:1) corresponding to the isolation and visualization of metaphase chromosomes (Gacsi et al. 2005).

The chromatin condensation pattern was compared in Indian muntjac, CHO, murine preB and human K562 cells (Fig. 4.16) during the cell cycle. Synchronized cells covering the whole spectrum of the cell cycle were reversibly permeabilized and used to isolate interphase and metaphase chromosomes. Fluorescent microscopy showed that chromosome condensation follows a common pathway irrespective of the mammalian cell type. Major structural categories of intermediates of chromatin condensation include: veil-like decondensed chromatin at the unset of replication, increasingly compact forms such as supercoiled, fibrous, ribboned structures, chromatin fibers, round chromatin bodies (earliest visible forms of chromosomes), elongated prechromosomes, precondensed linear forms and metaphase chromosomes (Fig. 4.16).

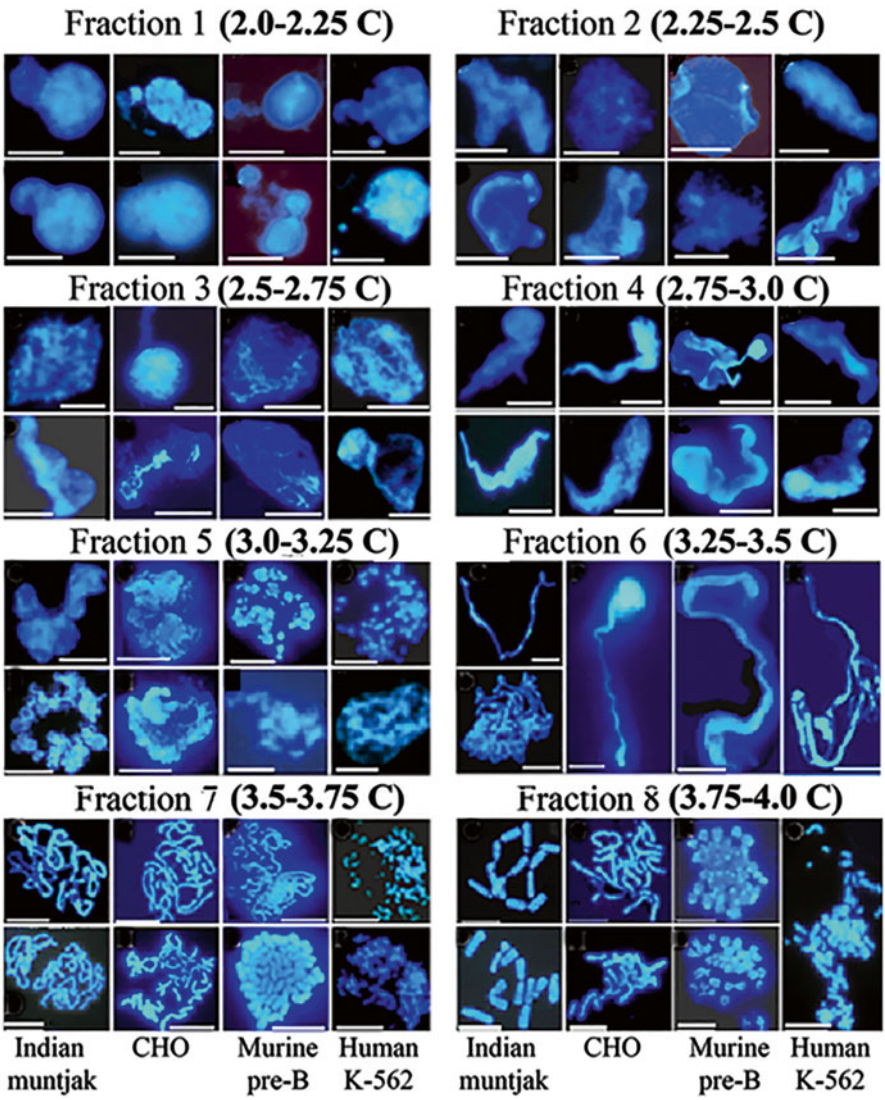


Fig. 4.16 Common pathway of chromosome condensation. Structures of interphase chromosomes isolated from four different mammalian cells (Indian muntjac, CHO, murine preB, human K562). *Upper* and *lower* figures in each pair show the same or similar intermediates of chromosome condensation during different stages of the cell cycle characterized by their C-values. *Bars*, 5 μm each (Modified with permission Banfalvi et al. 2006, Figs. 1+ 2 combined)

4.10.2 Chromatin Condensation in Resting, Tumor and Stem Cells

Chromatin structures were isolated from

- resting rat liver cells (Trencsenyi et al. 2007)
- rat acute myelogenous leukemia cells (Trencsenyi et al. 2012),
- widely used HaCaT cell line that consists of spontaneously transformed aneuploid immortal keratinocyte cells from human skin (Nagy et al. 2011a, b),
- human limbal stem cell line (Turani et al. 2015).

Results showed that the nuclear material in nuclei of normal resting liver cells (Go) contained mainly decondensed veil-like chromatin, chromosomes clustered in six lobular domains, and supercoiled chromatin (Fig. 4.17a).

Distinguishable morphological features of nuclei of rat acute myeloid leukemia cells with enlarged size and distributed nuclear chromatin indicated incomplete chromosome condensation and elevated gene expression. The enlarged nuclei allowed improved fluorescent visualization of common intermediates of chromatin condensation and so far unidentified chromosomal structures named chromatin funnel, seal-ring, spiral shaped and circular chromosomal subunits (Fig. 4.17b).

The panels of Fig. 4.17c show chromatin structures of HaCaT cells corresponding to regular intermediates of chromatin condensation involving veil like chromatin, fibrous chromatin, ribboned chromatin, prechromosomes and chromosomes approaching metaphase.

Nuclei of naturally occurring limbal stem cells contained the same intermediates of chromosome condensation as seen in other mammalian cells and follow the common pathway of chromosome condensation (Fig. 4.17d).

4.10.3 Chromatin Condensation in Regenerating Rat Liver Cells

The regenerating liver following two-thirds partial hepatectomy in the rat is considered one of the best models to study the cellular changes occurring *in vivo* in different phases of the cell cycle during cell proliferation. Because of the relevant synchronism of the first cell cycle, the timing has been well established (Rabes et al. 1976). After partial hepatectomy, most remaining hepatocytes, which are normally quiescent, rapidly reenter the cell cycle accompanied by the induced expression of a number of growth responsive genes. There is a sequential and regulated induction of gene expression, including the induction of immediate early genes such as c-*fos* and c-*jun* (Morello et al. 1990). The elevated gene expression could explain the elevated frequency supercoiled chromatin structures seen in regenerating liver cells (Fig. 4.18).

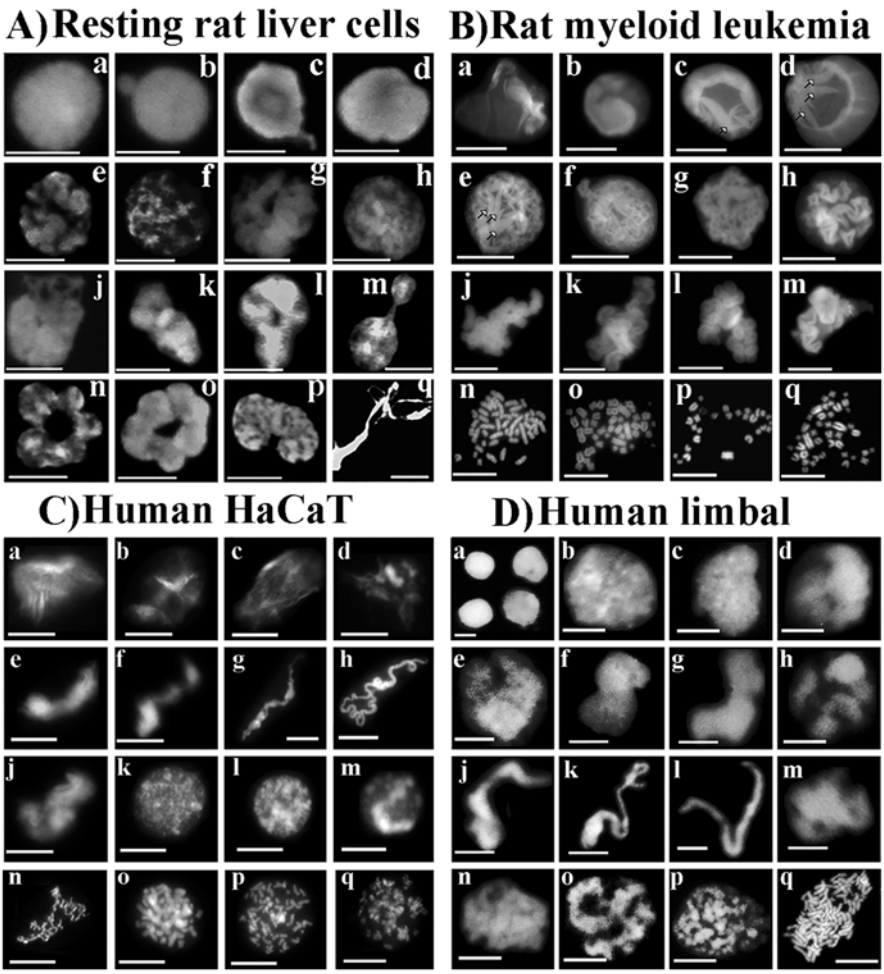


Fig. 4.17 Chromatin structures isolated from rat liver resting cell, rat leukemia, human keratinocyte and stem cell lines. Nuclei and chromatin structures were isolated from these cells after reversal of permeabilization. (**a**) Hepatocytes were obtained (Paragh et al. 2005) from Fisher 344 inbred rats (With permission (Trencsenyi et al. 2007, Fig. 2). (**b**) Myelomonocytic leukemia was originally induced in Fisher 344 rats by 7,12-Dimethylbenz[a]anthracene (DMBA) (Kozma et al. 1993). Cells were obtained from the bone marrow of chemically induced leukemic rats and turned to My1/De myelocytic cell line (Modified with permission Trencsenyi et al. 2012, Fig. 1). (**c**) The HaCaT cell line was derived from human skin keratinocytes (Boukamp et al. 1988) that transformed spontaneously *in vitro* during long term incubation (With permission of Nagy et al. 2011a, b, Fig. 9.6. (**d**) Corneal epithelial limbal cells were obtained from the enucleated eye of the cadaver of a 56-year-old female patient (With permission Turani et al. 2015, Fig. 4)

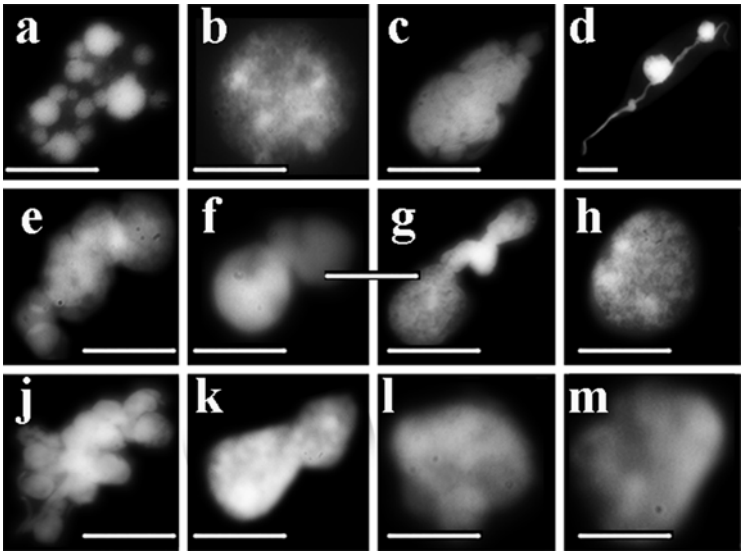


Fig. 4.18 Intermediates of chromatin condensation isolated from regenerating rat liver cells with supercoiled structures dominating the pictures. Bar, 10 μm each

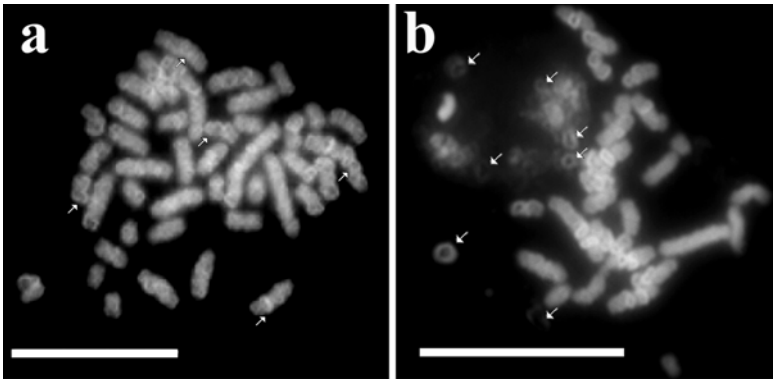


Fig. 4.19 Linear chromosomes of rat myeloid leukemia (My1/De) cells composed of spiral subunits. (**a**) Figure 4.17b/n at higher magnification. Elongated prechromosomes consist of circular chromosomal subunits. (**b**) In the somewhat less condensed prechromosomes circular forms appear as spiral subunits. *White arrows* indicate circular and spiral chromosomal subunits. Bar, 10 μm (With permission (Trencsenyi et al. 2012, Fig. 3)

4.10.4 Subunits of Metaphase Chromosomes

Because of the common dysplastic changes, the enormously enlarged nuclei (average diameter 12 mm) of the rat myelomonocytes (myelomonocytic leukemia 1 cells isolated at the University of Debrecen [My1/De]; diameter: 20–25 mm) were

chosen to visualize additional structural chromatin transitions during the interphase compared to earlier isolates (Banfalvi et al. 2006) seen in Fig. 4.16. Similarly to the rodlets of *Drosophila* cells (Banfalvi et al. 2007) smaller units of chromosomes were observed in leukemia My1/De cells. The linear chromosomes of myeloid leukemia cells consisting of spiral subunits are shown in Fig. 4.19. When panel n of Fig. 4.17b was scrutinized at higher magnification, a large number of spirals with uniform size became visible, indicating that chromosomes consist of smaller chromosomal parts (Fig. 4.19a). The size of chromosomes seems to be related to the number of these spiral units (Fig. 4.19b).

4.10.5 Comparison of Chromatin Condensation in Insect, Mollusc and Mammalian Cells

The presence of many small subchromosomal particles around the nucleus of *Drosophila* cells led to the hypothesis that chromosomes of *Drosophila* cells consisted of smaller units called rodlets (Banfalvi 2008b). Rodlets seen as bulges outside the almost completely folded and linearly arranged chromosome (Fig. 4.20a/1) were subjected to computer image analysis (Fig. 4.20a/2) resulting in an irregular array of chromatin fibers consisting of strings with a topology of 12 nm and an internucleosomal distance of 33 nm, condensed loops of fibers (of about 30 nm diameter). The length of loops corresponded to 300 nm (Fig. 4.20a/3). This analysis resolved not only the fiber structure that was originally invisible but also served as a basis for the plectonemic model of chromatin condensation. Regarding the mechanism of chromosome condensation in My1/De leukemia cells, the circular and spiral subunits observed at the end of the condensation process suggested that they are wound around the nonhistone scaffold of protein axis.

Regarding the mechanism of chromosome condensation, we have known only the diameters of chromatin structures at increasing compaction. Our observations are in agreement with the helical coiling model, where the double helical DNA is turned around the octameric histone core. This creates disc-shaped nucleosomes of 11 nm diameter, the attachment of which is known as “beads on string” or chromatin string. Nucleosomes are packed together to form the 30-nm chromatin fiber, whose exact structure was not known. The 30-nm fiber is thought to form chromatin loops of an estimated 300-nm-diameter. These loops turn around the nonhistone protein core or scaffold (Jackson 1991), bringing about the 600–700-nm condensed chromatin as depicted in Fig. 4.20b. Finally, the isolation of metaphase chromosomes from the gastropod garden snail (*Helix lucorum*) resulted in relatively thick chromosomes (Fig. 4.20/c). The wide and nearly round shape of *Helix lucorum* metaphase chromosomes could be explained by steric considerations in conformity with the notion that the compaction of chromosomes may be related not only to the length of DNA, but also to the availability of space relative to DNA content.

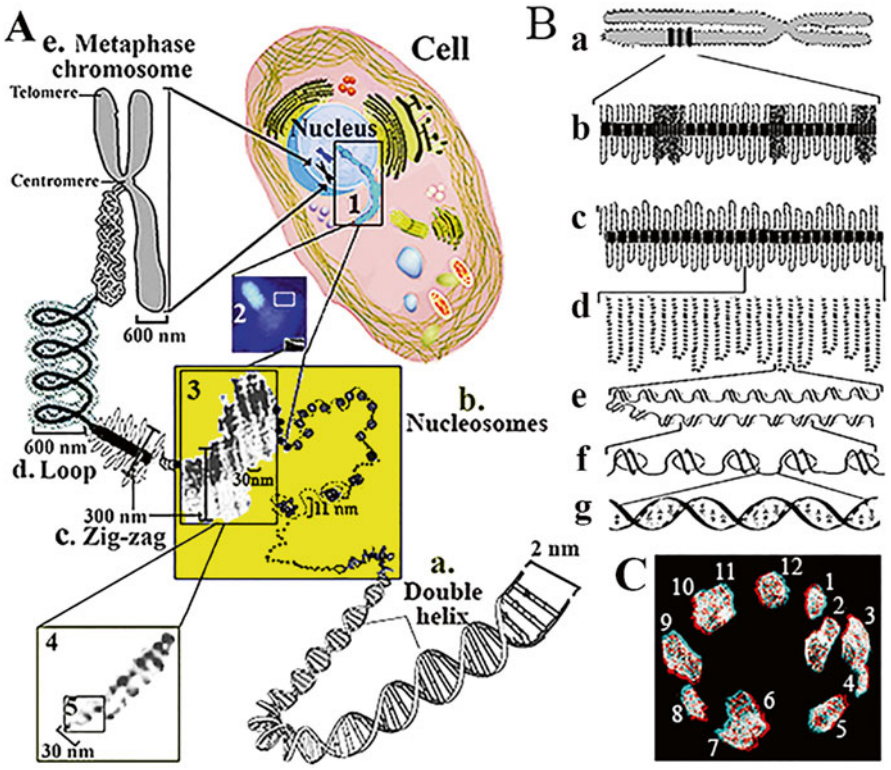


Fig. 4.20 Possible mechanisms of chromosome condensation. **A** Plectonemic model of chromatin condensation in *Drosophila* cells starting from the upper right to the lower left side of this panel. *1* Chromosomes escaping the nucleus of a permeable cell. *2* Small chromosomal unit termed "rodlet". *3 Boxed area* of 2 converted back to black-and-white negative image corresponding to 300 nm loop structure subjected to computer image analysis. *4* "Beads on string" (30 nm) structure forming hairpin structures. *5 Boxed area* of 4 showing nucleosomal (11 nm) arrangement. Opposite diagonal arrangement, from *lower right* to *upper left* direction: (**a**) double-stranded DNA (diameter 2 nm), (**b**) nucleosomes (11 nm), (**c**) plectonemic nucleosomal arrangement (30 nm fiber), and zig-zag loops of plectonemic fibers (300 nm), (**d**) formation of axial rodlets (600 nm), (**e**) metaphase chromosome (diameter 1400 nm) (With permission Banfalvi 2008b). **B** Helical-coiling of chromosome condensation in rat myeloid leukemia cells. (**a**) Chromatids of metaphase chromosome (1400 nm). (**b**) Chromatin loops corresponding to spiral units of chromosomes (700 nm). (**c**, **d**) Helical coiling of loops (300 nm). (**e**) Plectonemic folding of "beads on string" forming chromatin fibrils (30 nm). (**f**) Nucleosome "beads on string" (11 nm). (**g**) Double helical DNA (2 nm) (With permission Trencsenyi et al. 2012). **C** Anaglyph-transmission electronmicroscopy visualization of the 12 haploid chromosomes in the type B spermatogonium of the garden snail. Transmission electron microscopic images of the numbered *Helix lucorum* chromosomes (1–12) made by T. Roszer and G. Nagy. TESLA BS 540 transmission electron microscope was used in digital processing mode (dTEM) (With permission (Banfalvi 2014, Fig. 5c).

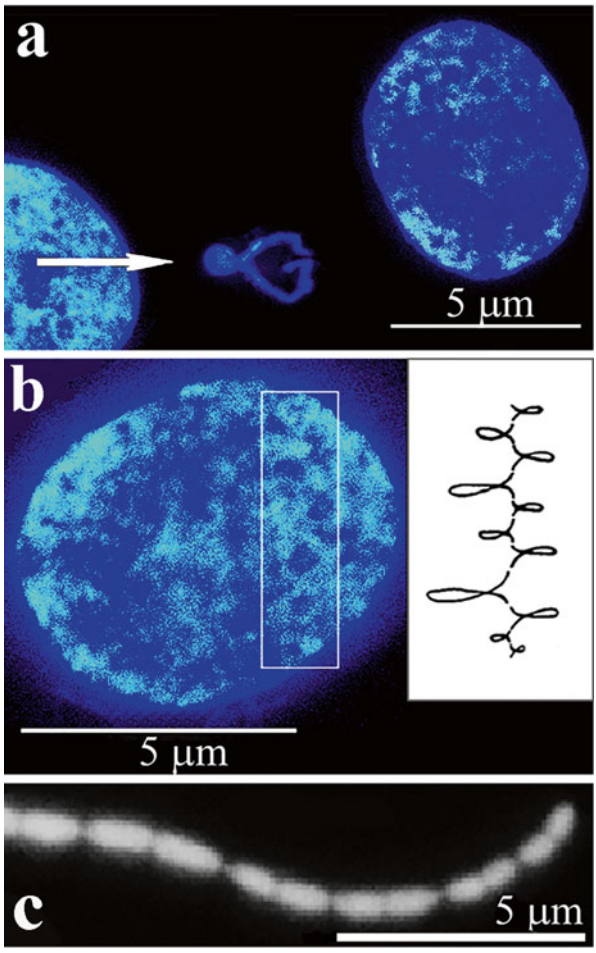


Fig. 4.21 Linear and spatial arrangement of CHO chromosomes. (**a**) Escape of an early chromosomal form from the left nucleus of a CHO cell. The original location of this chromosome inside the nucleus is indicated by the *white arrow* (With permission Banfalvi 2006a, Fig. 1). (**b**) Linear connection of chromosomes inside the nucleus. The zig-zag arrangement of chromatin bodies is shown inside the nucleus in the framed box and outside as a schematical model (*white area*) (With permission Banfalvi 2006b, Fig. 2). (**c**) Linear connection between metaphase chromosomes (With permission Gacsi et al. 2005, Fig. 9)

4.10.6 Linear and Spatial Arrangement of Chromosomes

During visualization chromatin structures were often seen outside the nucleus as escaped early chromosomal forms. Such an escaped CHO chromosome is seen in Fig. 4.21a, where the white arrow is indicating the nuclear origin of this chromosome. After visualizing many isolated chromatin structures outside the nucleus it became easier to distinguish and recognize them and observe their linear arrangement inside the nucleus (Fig. 4.21b). Spatial considerations dictate that decondensed chromosomes are folded into loops and bent forms and arranged in such a manner that neighboring chromosomes are *trans* oriented relative to each other resulting in a zig-zag ladder (white box in Fig. 4.21b). The linear attachment of chromosomes is maintained throughout the cell cycle up to the metaphase (Fig. 4.21c). The controversy between linear connection and telomere ends could be resolved by the formation of non-canonical tetraplex structures at the telomeric regions of chromosomes (Pramanik et al. 2012).

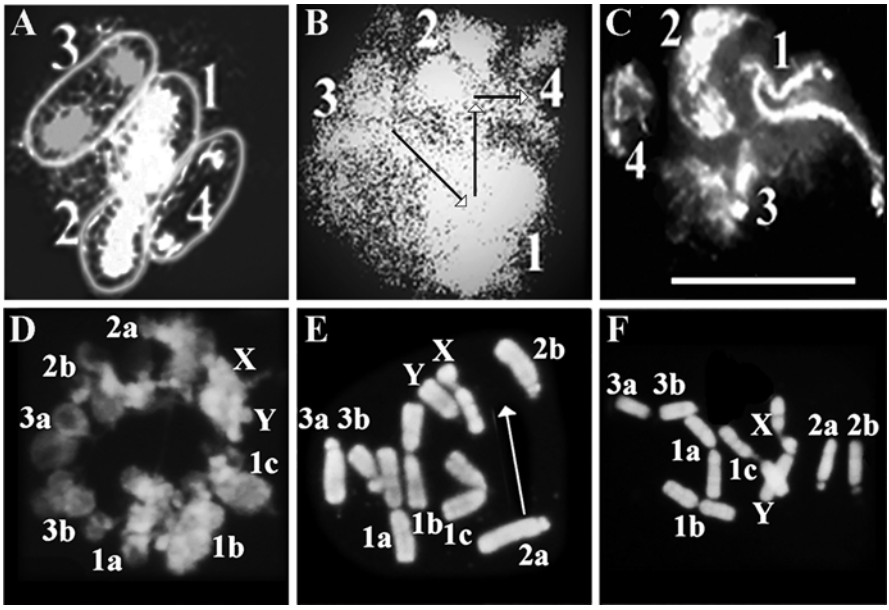


Fig. 4.22 Linear arrangement of chromosomes. (**A–C**) Linear order of chromosomes in nuclei of *Drosophila* cells (With permission Banfalvi 2006c, Fig. 4). (**D–F**) Linear order of Indian muntjac chromosomes

4.10.7 Linear Order of Chromosomes

Interphase chromosomes can be distinguished based on their fluorescence intensity and size of compacted chromatin. This allowed to perform the first chromatin body karyotyping of the earliest forms of chromosomes referred to as chromatin bodies isolated from mid-S-phase murine preB cells (Trencsenyi et al. 2007). As linearity is assured, the size, fluorescent intensity and the ordered tandem arrangement of chromosome pairs allowed the identification of chromosomes.

Exponentially growing cells (*Drosophila*, Indian muntjac) were pulse permeabilized for 2 min at 0 °C in a slightly hypotonic solution in the presence of dextran. Reversibly permeabilized cells could be used not only to follow chromatin condensation but also to determine the linear order of chromosomes. Nuclei of exponentially growing cells were isolated after reversible permeabilization and could be opened in interphase. The linear connection of chromosomes throughout the cell cycle allowed the determination of the chromosome order. For this purpose chromosomes being in mid- to late-S interphase seemed to be the most suitable. Fluorescence intensity and the size of chromatin structures allowed to distinguish among chromosomes. Figure 4.22A–C shows three sets of *Drosophila* interphase chromosomes and the attachment of their chromosome pairs. By following the rules to start the numbering of chromosomes from that end, which is closest to the largest chromosome (chromosome 1), the linear order of *Drosophila* chromosomes is

3 (XX), 1, 2 and 4. Black arrows in Fig. 4.22B show the connections between the chromosomes (Banfalvi 2006c).

The aneuploid Indian muntjac DM male cell line (2n = 9♂, with the trisomy of chromosome 1 was obtained from Prof. Karl Sperling (Pillidge et al. 1986). The extra copy of trisomic chromosome 1 contributed to the identification of chromosomes and to the determination of the linear order. The linear order of chromosomes of the trisomic interphase muntjac cells has been determined by the numbering from that end that was closer to the largest chromosome and was given as 3a, 3b, 1a, 1b, 1c, Y, X, 2a, 2b (Fig. 4.22 D–F). The numbering of the 11 pairs of CHO chromosomes was shown in Fig. 4.15F. To determine the linear order of chromosomes, Drosophila, Indian muntjac and CHO cells were chosen due to their relatively low chromosome number. For species with higher chromosome number beside the isolation and staining with DNA specific dyes (DAPI, Yoyo-1), the use of fluorescently labeled chromosome-specific probes is recommended. The determination of the linear order of human chromosomes with a much higher chromosome number would be more difficult. Nevertheless structural alterations between pairs of homologous chromosomes that remain hidden in condensed metaphase chromosomes could be detected in an earlier stage of interphase.

4.11 Heavy Metal Induced Chromatin Changes

Characteristic distortions of intermediates of chromatin condensation have been observed relative to the common pattern of chromosome condensation in reversibly permeabilized cells. These changes occurred regularly, were characteristic to the genotoxic agent, and independent of the cell type. Among the genotoxic agents heavy metals (Cd, Pb, Ni, Hg, Ag) and irradiation (alpha, gamma, UV-B) have been tested for their chromatin toxicity particularly for apoptosis and necrosis.

4.11.1 Cadmium (II) Induced Chromatin Damages

Low concentration (1 μM) $CdCl_2$ treatment caused significant cellular changes (Banfalvi et al. 2000). These changes included reduced cell growth, generation of DNA strand breaks affecting the amount of isolable DNA, reduced replicative DNA synthesis, increased repair DNA synthesis, in a cell cycle-dependent manner.

Characteristic Cd-induced chromatin damages in CHO cells were seen as:

(a) the absence of decondensed veil-like structures and the presence of premature chromatin condensation in the form of apoptotic chromatin bodies in early S phase,

(b) the absence of fibrous structures, lack of supercoiled chromatin, the appearance of uncoiled ribboned chromatin and perichromatin bodies in early mid S phase,
(c) the presence of perichromatin fibrils and chromatin bodies in mid S,
(d) early intra-nuclear inclusions, elongated forms of premature chromosomes later in mid S,
(e) the exclusion of chromatin bodies and large-sized perichromatin granules in late S (Banfalvi et al. 2005).

In Fig. 4.23a only the most characteristic chromatin changes caused by Cd(II) are shown in CHO cells. These chromatin changes were seen as large extensive disruptions and holes in the nuclear membrane and stickiness of incompletely folded chromosomes.

4.11.2 Chromatin Toxicity of Lead(II)

At low (2–20 μM) $Pb(NO_3)_2$ concentrations the extrusion of polarized chromatin resembled that of micronucleus formation in K-562 cells (Fig. 4.23B). The lack of supercoiling seems to prevent the formation of chromatin bodies, which are regarded as the earliest visible forms of interphase chromosomes. At low concentration of Pb(II) a typical feature was the early condensation into lobular chromatin ribbons arranged in a semicircular manner (Fig. 4.23B at 2 μM). At 5 μM $Pb(NO_3)_2$ concentration beside chromatin ribbons, large chromatin clusters were generated. At higher (10–20 μM) $Pb(NO_3)_2$ concentrations bent lobular chromatin ribbons became visible. These characteristic distortions allowed to make distiction between Cd and Pb toxicity at micromolar concentrations. The visualization of these chromatin changes is relatively easy, fast, selective and sensitive with the potential of early differential diagnostics.

4.11.3 Cellular and Chromatin Toxicity of Nickel (II)

The size of K562 cells varied only slightly upon treatment within a wide range (0.2–100 μM) of $NiCl_2$ concentration. Cellular movement indicating the toxicity of Ni(II) was seen as vigorous cellular motion monitored at relatively high 100 μM Ni(II) concentration by time-lapse microscopy (Fig. 4.24A). Although, initially the Ni-treated cells moved faster than control cells, this motion was less intense than the so called “apoptotic dance” observed after Pb-treatment of Ha-CaT cells. The rapid motion of cells gradually slowed down and resulted in a sticky cell aggregate. Low (0.2–0.5 μM) concentrations of Ni(II) caused the polarization and the rejection of apoptotic bodies. In addition to these changes 1 μM $NiCl_2$ generated larger apoptotic chromatin circles. At 10 μM Ni(II) most of the nuclear material was condensed and the rest remained in a highly decondensed fibrillary stage. Necrotic changes took place at $\geq$50 μM $NiCl_2$ with less fibrillary chromatin, moderate increase in nuclear size and in the preservation of relatively round shaped nuclei (Fig. 4.24B).

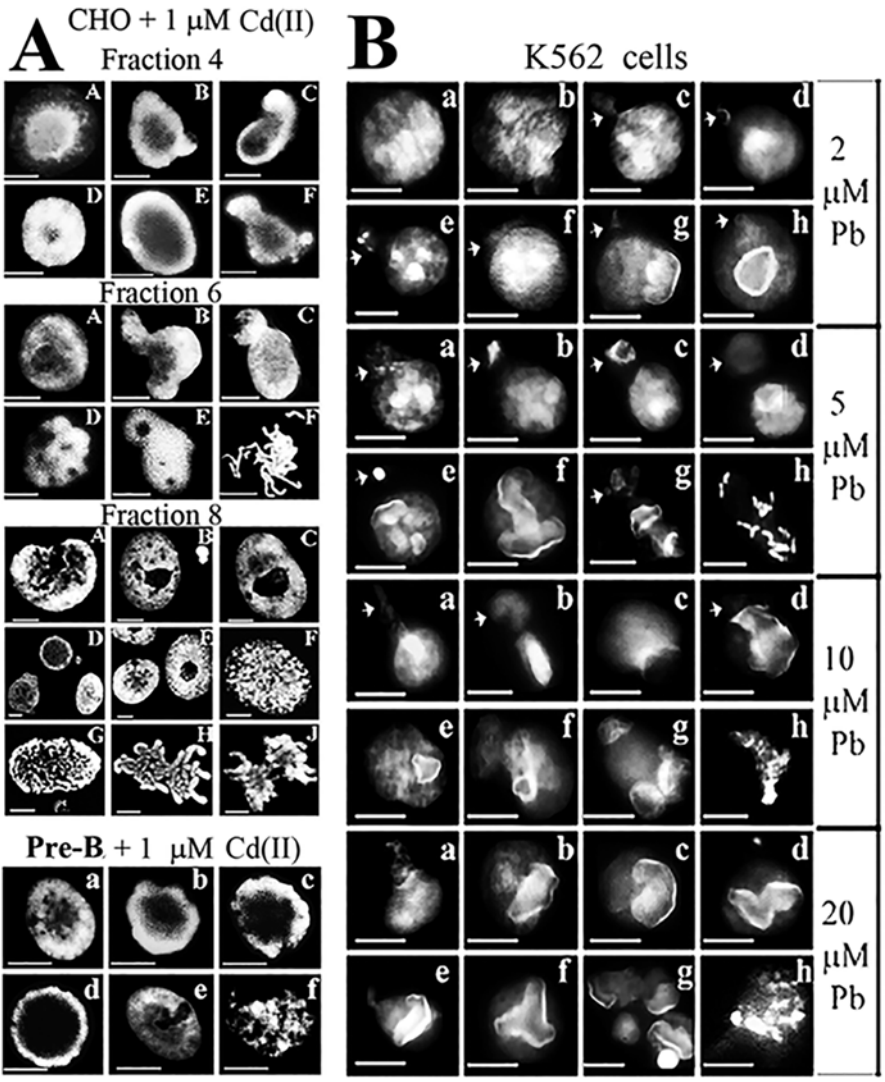


Fig. 4.23 Comparison of Cd and Pb induced chromatin changes. (**A**) After treatment of CHO and murine preB cells with $CdCl_2$ (1 μM) for 15 h, cells were synchronized by elutriation and eight fractions collected. Each cell fraction was permeabilized and then the membrane integrity was restored by the reversal of permeabilization. Typical chromatin changes are shown relative to the control shown in Fig. 4.16. Typical chromatin changes seen as large holes in the nuclei of CHO cells correspond to those seen in preB cells (With permission Banfalvi et al. 2005, parts of Figs. 9–11). (**B**) $Pb(NO_3)_2$ treated K562 cells were subjected to a similar procedure. Control chromatin structures are the same as for cadmium treatment shown in Fig. 4.16 (With permission Banfalvi et al. 2012, Figs. 3 and 4)

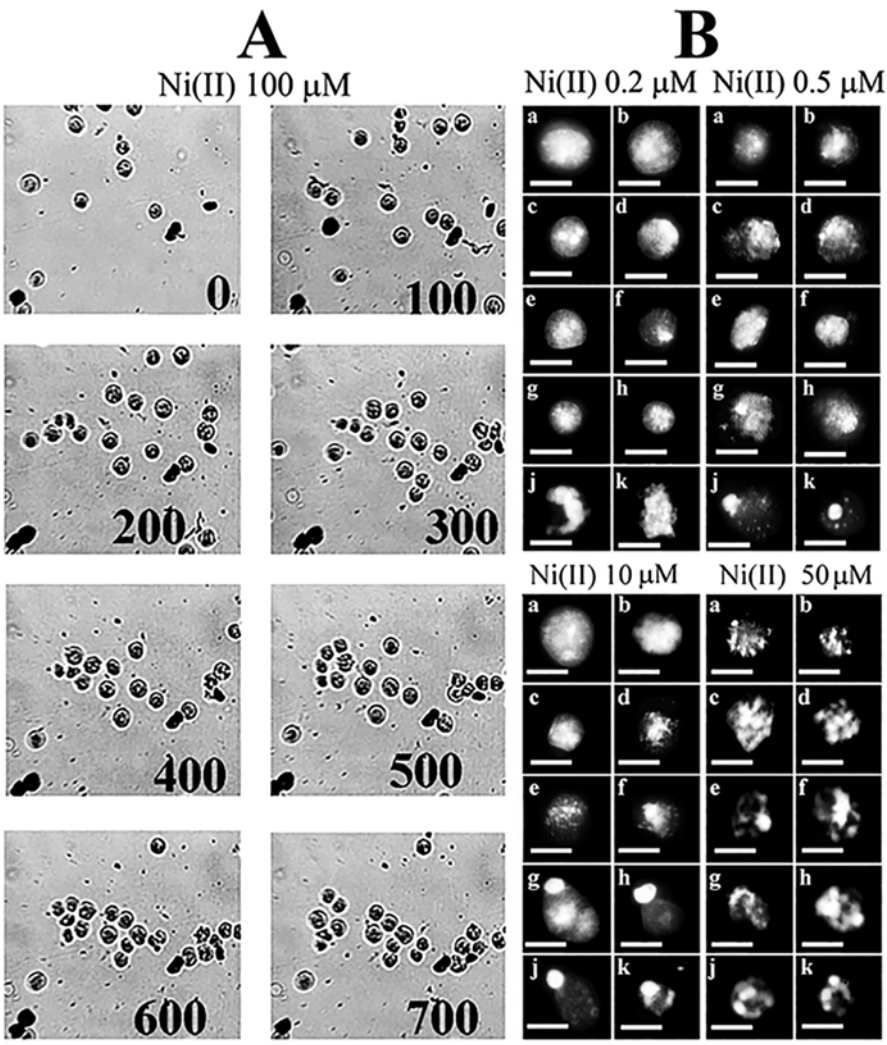


Fig. 4.24 Cellular and chromatin toxicity of Ni(II). (**A**) K562 cells were grown in 25 ml T-flasks and treated with 100 μM $Ni(Cl)_2$, placed on inverted microscope sitting in a carbon dioxide incubator. Time-lapse microphotography was performed by taking pictures every minute by a custom built video camera attached to the microscope and connected to the computer. The motion of individual cells is indicated by their changing position. *Black numbers* indicate the time of photography in minutes after heavy metal treatment. (**B**) K562 cell treated with different concentrations of Ni(II), then subjected to reversible permeabilization and isolation of chromatin structures. Fluorescence microscopy was performed after staining with DAPI. Bars, 5 μm each (With permission Nagy et al. 2011a, Figs. 7.3, 7.5 and 7.6)

4.11.4 Chromatin Changes Caused by $HgCl_2$

Time lapse video photography showed that cellular changes induced by mercuric ions were characterized by their properties of causing reduced cellular motility (10–50 μM), and complete lack of cellular movement at higher concentrations (100–1000 μM). Micromolar concentrations of Hg(II) that did not cause noticeable alterations in the cellular morphology of K562 cells, initiated chromatin changes and were the earliest visible signs of cytotoxicity. Major types of distortions in the nuclear material were highly condensed supercoiled and decondensed veil-like chromatin, decondensed chromosomes rejected as clustered puffs and often the nuclear material was broken down to apoptotic bodies. Nuclear changes caused by Hg(II) acetate at 10 μM were characterized by apoptosis seen as broken nuclei and apoptotic bodies (Fig. 4.25B).

4.11.5 Silver (I) Induced Chromatin Changes

After $AgNO_3$ treatment at low concentrations, that were regarded earlier as subtoxic levels (<1 μM), chromatin changes were the earliest signs of cytotoxicity. Typical nuclear changes induced by silver nitrate involved the polarization of precondensed and the extrusion of decondensed chromatin seen as chromatin tailing. The tail ("comet") formation seen with HaCaT cells was dependent on silver nitrate concentration (Fig. 4.25B). Elevated silver nitrate concentrations (5–10 μM) caused nuclear shrinkage with infrequent formation of apoptotic bodies. Higher Ag^+ concentrations (20–50 μM) allowed the expansion of the nuclear material without necrotic disruptions (not shown). The chromatin tail formation could be accounted for by the decreased chromatin supercoiling and could be related to a dose dependent reduction of ATP content, cell viability and increased production of reactive oxygen species.

4.12 Effect of Irradiation on Chromatin Structure

4.12.1 Cellular and Chromatin Alterations Generated by Gamma Irradiation

Cellular and chromatin changes were followed in a cell cycle dependent manner in non-irradiated murine preB cells and after 400 rad Co^{60} irradiation. Fluorescence microscopy of chromatin structures confirmed the existence of specific geometric forms in nuclei of non-irradiated cells (Fig. 4.16 serving as control).

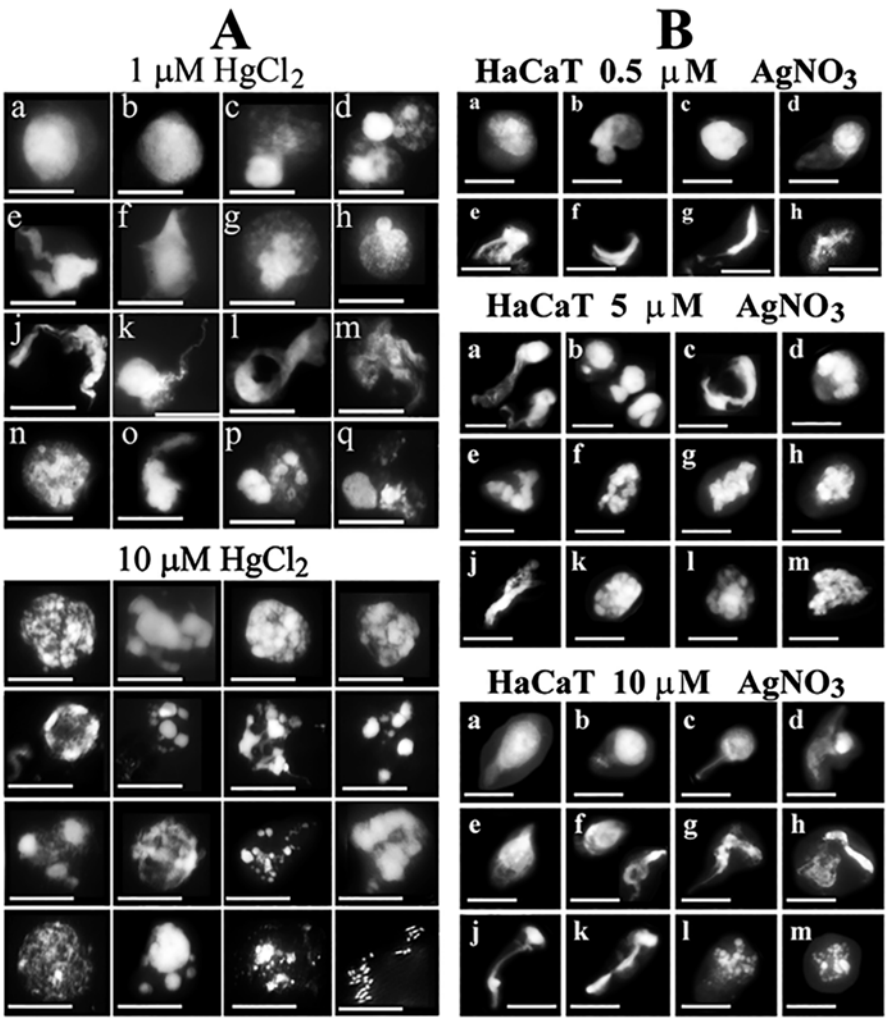


Fig. 4.25 Hg(I) and Ag(I) induced chromatin changes. After heavy metal ($HgCl_2$, $AgNO_3$) treatment, K562 cells were subjected to reversible permeabilization, isolation, DAPI staining and fluorescent visualization of chromatin structures. (**A**) Chromatin structures after Hg(II) treatment (With permission (Farkas et al. 2010, Fig. 3 and 4). (**B**) $AgNO_3$ treatment (With permission Nagy et al. 2011b, Fig. 9.6 and 9.7). Bars, 5 μm each

Results showed that relative to the control upon γ-irradiation:

(i) the cell growth was reduced (left panels, Fig. 4.26A/a),
(ii) the cellular and nuclear sizes were increased (left panels, Fig. 4.26A/b and c),
(iii) the DNA content was lower in each elutriated subpopulation of cells. The progression of the cell cycle was arrested in the early S phase corresponding to ~2.4 C value, where the chromatin condensation was blocked between the fibrillar chromatin and precondensed elongated chromosomal forms (Nagy et al. 2004) and

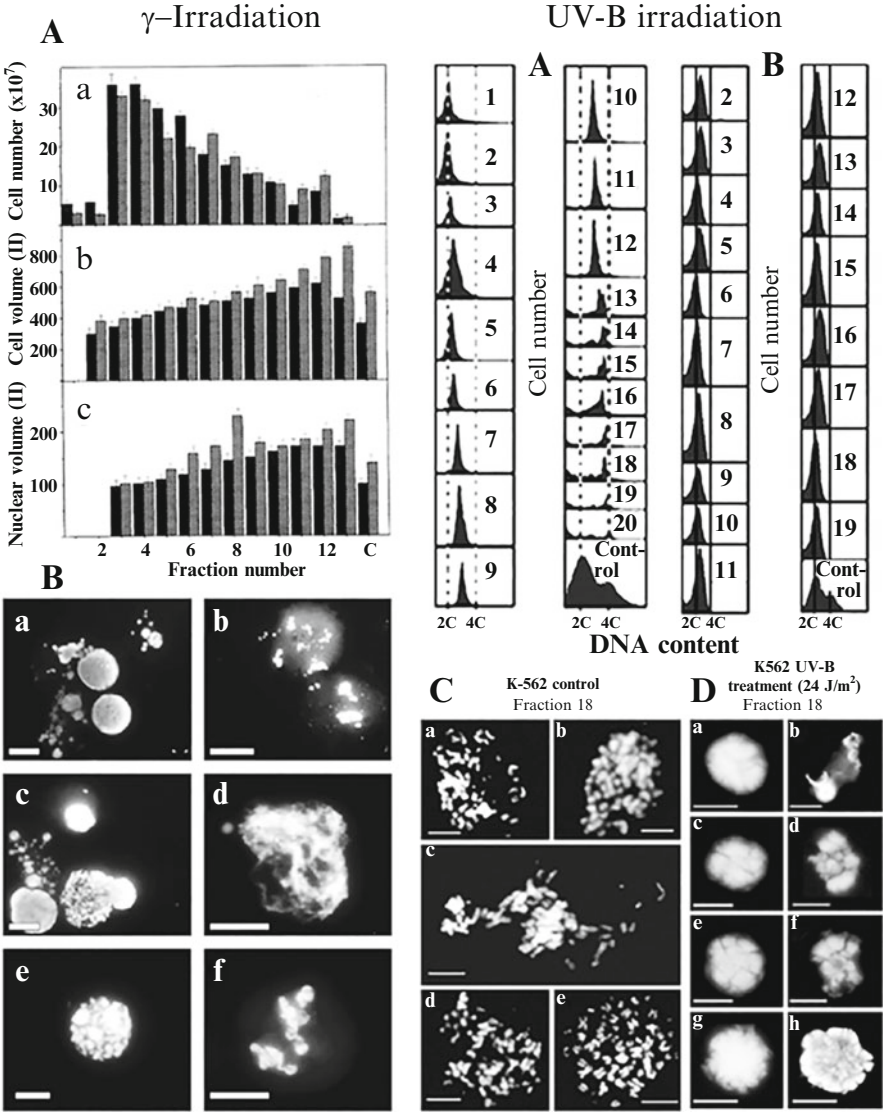


Fig. 4.26 Characteristic chromatin changes caused by γ and UV irradiation. Gamma irradiation (*left panels*): (**A**) The effect of γ-irradiation on cell number (*a*), cell volume (*b*), and nuclear volume (*c*) in elutriated fractions. *Black columns* indicate non-irradiated cells, striped columns correspond to values obtained from irradiated cells (With permission Nagy et al. 2004, Fig. 3). (**B**) Intermediates of chromatin condensation in preB 70Z/3 cells γ-irradiated with 400 R, incubated for 2 h and synchronized by centrifugal elutriation and collecting nine fractions. Chromatin structures were isolated and visualized after DAPI staining. Apoptotic bodies in nuclei from fractions 4, 5 and 6, correspond to panels *a*, *b* and *c*, respectively. Fibrous chromatin (fraction 7, *d*). Incomplete transition from fibrous chromatin to precondensed chromosomes (fractions 8 and 9; *e*, *f*, respectively) (With permission Nagy et al. 2004, Fig. 5). Characterization of UV-B irradiated K562 cell populations (*right panels*). Cells were synchronized and 20 fractions were collected by centrifugal elutriation. K562 cells were UV irradiated with 24 J/m2. (**A**) Cell cycle patterns of non-irradiated cells. (**B**) Irradiated cells were analyzed by FACS after propidium iodide staining. (**C**) Complete formation of metaphase chromosomes in non-irradiated cells. (**D**) Segregation of nuclear material in irradiated cells. Chromatin structures isolated from irradiated cells (fraction 18) covered by chromatin fibrils did not reach the metaphase stage. Bars, 5 μM each (With permission Ujvarosi et al. 2007, Figs. 2 and 5)

(iv) the number and size of apoptotic bodies after "γ bombardment" were inversely correlated with the progression of the cell cycle, with many small apoptotic bodies in early S phase and less and larger apoptotic bodies in late S phase (left panels, Fig. 4.26B/a–f).

When flow cytometric forward scatter analysis was used to determine the proportion of apoptotic cells relative to unirradiated ones, apoptosis was low in unirradiated cells (~1 %) and increased significantly in irradiated cells from 5 % to 13–14 % in 24 h. To estimate the average value of apoptosis, non-elutriated control populations were also subjected to γ-irradiation and grown in culture medium for 2 and 24 h, respectively. The number of apoptotic cells seen in the sub-G_1 marker window increased from 4.8 to 15.6 %. Beside the accumulation of apoptotic cells, the increase in G_1/G_0 and G_2/M phase and the decrease of S phase cells were observed. These changes confirmed the γ-irradiation-induced cell cycle arrest (Banfalvi et al. 2007).

4.12.2 Chromatin Damages Caused by UV Irradiation

Control cells were subjected to centrifugal elutriation and the flow cytometric profiles of the 20 fractions were used to estimate their DNA content (Fig. 4.26A, upper right panels) ranging between 2.07 and 3.88 C-values. The separation of 20 fractions of UV-B irradiated cells (24 J/m^2) was carried out in the same manner. Unexpectedly their C-values remained between 2.2 and 2.4 C-values corresponding to the fibrillary chromatin structure (Fig. 4.26B, upper right panels). At lower doses of non-ionizing UV light (6, 12 J/m^2) chromatin changes occurred only occasionally. Higher UV light dose (24 J/m^2) manifested as an increased fibrillary cloud covering and blurring the condensing chromatin structures.

Although, occasionally small apoptotic bodies were seen, but they were not characteristic to UV irradiation. Neither chromatin bodies, nor completely folded chromosomal forms could be observed. Relative to control metaphase chromosomes (Fig. 4.26C, lower right panels), primitive, early forms of chromatin condensation were covered with a faint chromatin halo that did not reach the stage of metaphase (Fig. 4.26D, lower right panels) without progression of supercoiling that would lead to compact structures (Ujvarosi et al. 2007). The early block of chromatin condensation at ~2.4 C value is probably related to photoreactivation and nucleotide excision repair of pyrimidine dimers and photoproducts.

4.12.3 Chromatin Damages Generated by Alpha Irradiation

The water soluble uranyl acetate (UA) compound often used as a stain in electron microscopy was applied at 5, 10 and 20 μM doses and chromatin changes were followed in reversibly permeabilized K562 cells. Lower dose (5 μM) of UA caused

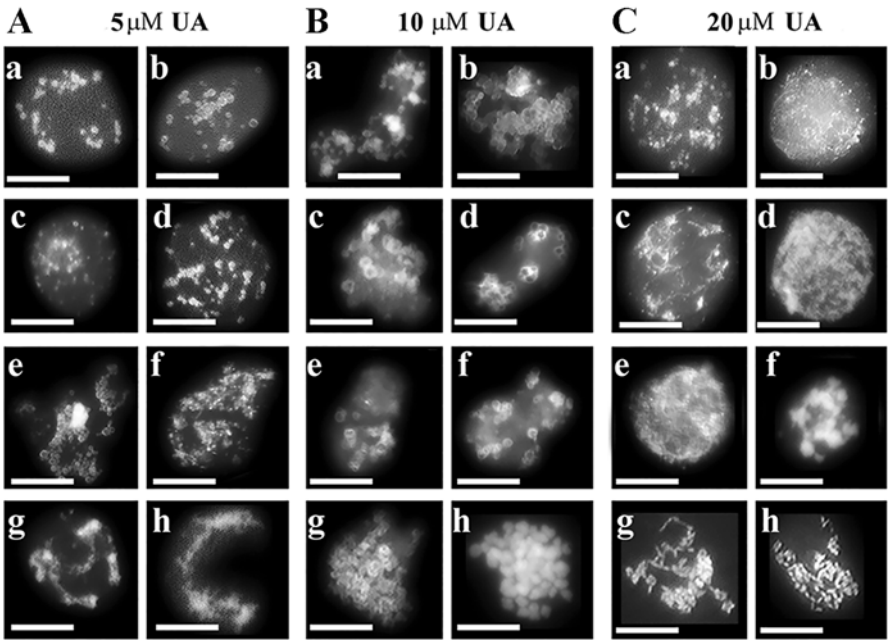


Fig. 4.27 The effect of alpha radiation on chromatin structure of K562 cells. Low (5 µM) uranyl acetate (**A**), higher UA concentration (10 µM) (**B**), and high (20 µlM) UA concentration (**C**). Bars 5 µm each

characteristic chromatin changes seen as small round, often circular bodies resembling chromatin bodies (Fig. 4.27A/a–d), and the appearance of chromatin ribbons containing dots corresponding to apoptotic bodies (Fig. 4.27A/e–h). The chromatin distortions at higher concentration of uranyl acetate (10 µM) resembled those observed at lower UA concentration, with the notable exception that fewer and larger apoptotic bodies were formed and covered by a fine fibrillary network (Fig. 4.27B/a–h). Incoherent large chromatin associations containing many small apoptotic dots or circles dominated the pictures were seen at high (20 µM) uranyl acetate concentration (Fig. 4.27C/a–f). Sticky chromosomes did not reach the metaphase (Fig. 4.27C/g–h).

Conclusions Related to Chromatin Condensation Reversibly permeably permeabilized cells have been useful to visualize common intermediates of chromosome condensation by fluorescence microscopy. These investigations revealed:

(i) temporally distinct intermediates including

- highly decondensed, veil-like structures in early S phase
- supercoiled chromatin later in early S phase
- fibrous chromatin in early mid-S phase
- continuous chromatin fibers in late mid-S

- elongated prechromosomes in late S
- precondensed chromosomes at the end of S phase
- completely condensed chromosome in metaphase

(ii) Temporally distinct structures that followed the general pathway of chromatin condensation.

(iii) Enlarged nuclei of rat myelocytic leukemia cells revealed further intermediates of chromatin folding such as chromatin funnels (early intermediates) and spiral units in the chromonemic folding of metaphase chromosomes. The presence of spiral subunits of chromosomes among the intermediates favors the helical coil model of chromosome condensation.

(iv) *Drosophila* chromosomes also consist of smaller subunits named rodlets. Nevertheless, there are differences in chromosome condensation seen in nuclei of the diptera *Drosophil*a, the gastropod garden snail and mammalian cells.

(v) Interphase and metaphase chromosomes are linearly connected, forming a semicircle.

(vi) Typical genotoxic alterations absent in control cells were caused by various genotoxic treatments excluding the possibility of artifact formation.

(vii) Abnormal chromosomal forms were found after heavy treatment, specific to individual metals. Chromatin changes caused by different types of irradiation (α, γ, UV) generated characteristic, differential diagnostic distortions in chromatin structures.

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