

Dynamics of Biological Membranes

Influence on Synthesis, Structure and Function

MILES D. HOUSLAY

and

KEITH K. STANLEY

Dynamics of Biological Membranes

Influence on Synthesis, Structure and Function

MILES D. HOUSLAY

Department of Biochemistry

The University of Manchester Institute of Science and Technology

P. O. Box 88, Manchester M60 1QD, UK

and

KEITH K. STANLEY

European Molecular Biology Laboratory,

Postfach 10.2209, Meyerhofstrasse 1,

D-6900 Heidelberg, W. Germany

A Wiley-Interscience Publication



JOHN WILEY & SONS

Chichester · New York · Brisbane · Toronto · Singapore

Copyright © 1982 by John Wiley & Sons Ltd.

All rights reserved.

No part of this book may be reproduced by any means, nor transmitted, nor translated into a machine language without the written permission of the publisher.

Library of Congress Cataloging in Publication Data:

Houslay, Miles D.

Dynamics of biological membranes.

'A Wiley-Interscience publication.'

Includes index.

1. Membranes (Biology) I. Stanley, Keith K.

II. Title.

QH601.H686 574.87'5 81-14759

ISBN 0 471 10080 3 (cloth) AACR2

ISBN 0 471 10095 1 (paper)

British Library Cataloguing in Publication Data:

Houslay, Miles D.

Dynamics of biological membranes.

1. Membranes (Biology)

I. Title II. Stanley, Keith K.

574.87'5 QH601

ISBN 0 471 10080 3 (cloth)

ISBN 0 471 10095 1 (paper)

Typeset by Preface Ltd, Salisbury, Wilts.

Printed in the United States of America

Preface

Books on biological membranes have, in the main, discussed this topic primarily in terms of the detailed composition of membranes and the reactions, such as transport and enzyme functions, that characterize them. Over the past few years, however, the functioning of biological membranes at the molecular level has begun to be understood. In particular, biological membranes have emerged, not as static inert boundaries to the cellular and intracellular compartments, but as dynamic structures intimately involved in many, if not most, of the biochemical processes of the cell. The emphasis of this book is therefore on structural and functional aspects which contribute to our concept of the dynamic membrane. In view of this we do not aim to provide a catalogue of reactions that have been observed in biological membranes. As such we give no detailed description of the kinetics of transport of material across membranes as details of these, like that of enzymes, are inappropriate to the aims of this book and can be found in other standard biochemical texts. Hopefully, this approach will give the reader an insight into our current understanding of the many aspects of biological membranes and the direction future research is likely to take.

Much of what we describe in this book is the fruit of biochemical and biophysical techniques that have been developed over the last decade or two. We are conscious, however, that the information regarding the structure and function of biological membranes is going to increase at an enormous rate with the application of genetic engineering techniques. Rarely does a month pass without the sequence of another membrane protein, deduced using these techniques, appearing in the literature. Not only do these techniques provide a means of deducing a protein sequence without having to purify the protein and analyse the amino-acid sequence directly (both difficult and tedious processes), but there is also scope for the investigation of structure and function of membrane protein using systems in which genetically engineered DNA molecules are expressed *in vivo*.

We have compiled this book with the needs of scientific and medical students in mind, as well as those of research workers wishing to be acquainted with current developments in this fast-moving field. A rapid

appreciation of the contents of each chapter may be made by reading only the summary and figure legends of each section. For those wishing more details and explanation, the full text may be read. Finally the references at the end of each section and those cited in figure legends will lead the interested reader to some of the key reviews and published papers. We hope that the concepts which we have tried to lay down will be clear and might even stimulate some to further investigations into the fascinating properties of biological membranes.

We wish to thank all those who gave helpful comments and criticism on the manuscript and Rhian Houslay and Elizabeth Wright who helped in the production of the manuscript.

During an historical period, there have been two main branches of the study of membranes, one primarily in terms of the detailed composition of membranes and the other in terms of their function and properties. The first branch, the study of the detailed composition of membranes, has been the domain of biochemistry and molecular biology. The second branch, the study of the function and properties of membranes, has been the domain of physiology and cell biology. The two branches have converged in the last few decades, and the study of membranes is now a truly interdisciplinary subject. This book is primarily concerned with the function and properties of membranes, but it also includes a chapter on the detailed composition of membranes. The book is written for students of physiology and cell biology, and it is hoped that it will be of use to them. It is also written for those who are interested in the function and properties of membranes, and it is hoped that it will be of use to them. The book is written in a clear and concise style, and it is hoped that it will be of use to all who read it.

During the last few decades, there has been a rapid development in the study of membranes. This has been due to a number of factors, including the development of new techniques for the study of membranes, the discovery of new membrane proteins, and the development of new models for the structure and function of membranes. The study of membranes is now a truly interdisciplinary subject, and it is hoped that this book will be of use to all who are interested in it. The book is written in a clear and concise style, and it is hoped that it will be of use to all who read it.

The book is written for students of physiology and cell biology, and it is hoped that it will be of use to them. It is also written for those who are interested in the function and properties of membranes, and it is hoped that it will be of use to them. The book is written in a clear and concise style, and it is hoped that it will be of use to all who read it.

Contents

Preface	vii
1 Membrane components and their organization	1
1.1 The importance of membranes in cell structure and function	1
1.2 The morphology of membranes	9
1.3 Preparation of membrane fractions	16
1.4 The structure of membrane components	17
1.5 A model for biological membrane structure	24
1.6 Solubilization and purification of membrane proteins	29
1.7 Extraction and separation of membrane lipids	35
2 Mobility of the lipid and protein components of biological membranes	39
2.1 Modes of mobility of the phospholipid components	40
2.2 Fast lateral diffusion of phospholipids	41
2.3 Phospholipid flip-flop in membranes	43
2.4 Cholesterol flip-flop in membranes	48
2.5 Mobility gradients within the bilayer	48
2.6 The lipid phase transition	51
2.7 The nature of the lipid phase transition	57
2.8 The pre-transition of phosphatidyl cholines	61
2.9 Phase transitions in bilayers of negatively charged phospholipids	63
2.10 Phase separations in mixtures of pure phospholipids	65
2.11 The integration of cholesterol into phospholipid bilayers	71
2.12 Modes of mobility of integral membrane proteins	81
2.13 Restraints upon the rotational and lateral diffusion of membrane proteins	85
3 Lipid-protein interactions	92
3.1 Introduction	92
3.2 Peripheral proteins and their association with biological membranes	94

3.3	The modulation of integral protein function by lipids of the bilayer	96
3.4	Methods of manipulating membrane fluidity	125
3.5	The relationship of bilayer fluidity to biological processes . .	138
4	Asymmetry in biological membranes	152
4.1	Introduction	152
4.2	Maintenance of membrane asymmetry	153
4.3	Analysis of membrane asymmetry	154
4.4	Protein asymmetry in the mammalian erythrocyte membrane .	171
4.5	Lipid asymmetry in the erythrocyte membrane	178
4.6	Carbohydrate asymmetry in the erythrocyte membrane . . .	180
4.7	Protein asymmetry in other membranes	180
4.8	Protein asymmetry and cell function	194
4.9	Phospholipid asymmetry in membranes from other cells . .	196
4.10	Phospholipid asymmetry in liposomes	199
4.11	The function of lipid asymmetry	202
5	Reconstitution of defined membrane functions	206
5.1	Introduction	206
5.2	Methods of reconstitution	207
5.3	Aspects of reconstitution studies	212
5.4	Methods of manipulating the lipid environment of integral proteins	217
5.5	Examples of reconstituted systems of particular interest . .	221
6	Membrane turnover and circulation	230
6.1	Introduction	230
6.2	The synthesis and insertion of membrane proteins	231
6.3	Glycosylation of membrane proteins	244
6.4	Synthesis of membrane lipids	248
6.5	Turnover of membrane proteins	250
6.6	Turnover of phospholipids	254
6.7	Membrane circulation	258
7	Dynamic membrane-associated processes	281
7.1	Introduction	281
7.2	Passive diffusion	282
7.3	Ionophores	286
7.4	Membrane channels and pores	290
7.5	Facilitated diffusion	301
7.6	Active transport	310
7.7	Dynamic membrane events in hormone action	317
7.8	Lateral diffusion of cytochrome b_5 in microsomal electron transport	322
	Index	326

Chapter 1

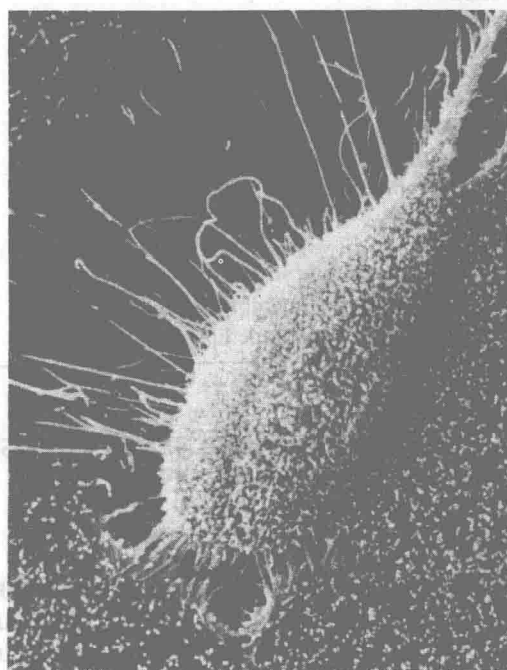
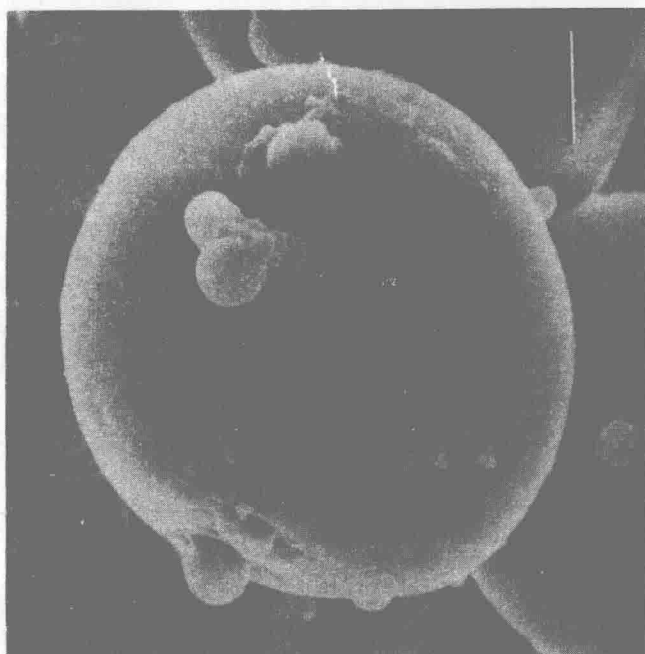
Membrane components and their organization

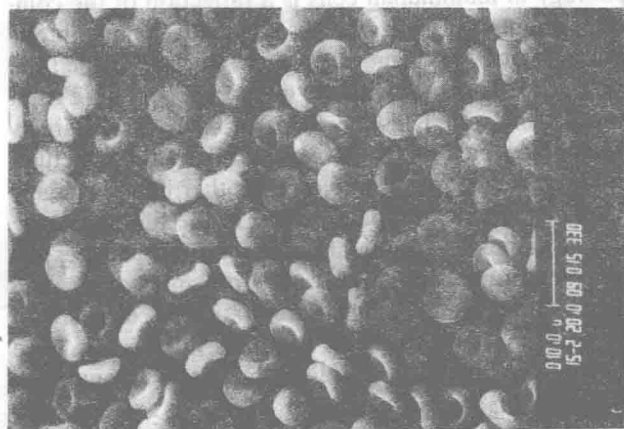
1.1	The importance of membranes in cell structure and function	1
1.1.1	Definition of the cell surface	1
1.1.2	Control of the intracellular environment	5
1.1.3	Communication between cells	5
1.1.4	Cell adhesion and immunogenicity	6
1.1.5	Function of intracellular membranes	8
1.2	The morphology of membranes	9
1.2.1	The plasma membrane	10
1.2.2	The nucleus	13
1.2.3	The endoplasmic reticulum	14
1.2.4	The Golgi apparatus	14
1.2.5	The lysosome	15
1.2.6	The mitochondrion	15
1.2.7	The peroxisome	15
1.3	Preparation of membrane fractions	16
1.4	The structure of membrane components	17
1.4.1	Lipids	18
1.4.2	Proteins	21
1.5	A model for biological membrane structure	24
1.6	Solubilization and purification of membrane proteins	29
1.7	Extraction and separation of membrane lipids	35

1.1 THE IMPORTANCE OF MEMBRANES IN CELL STRUCTURE AND FUNCTION

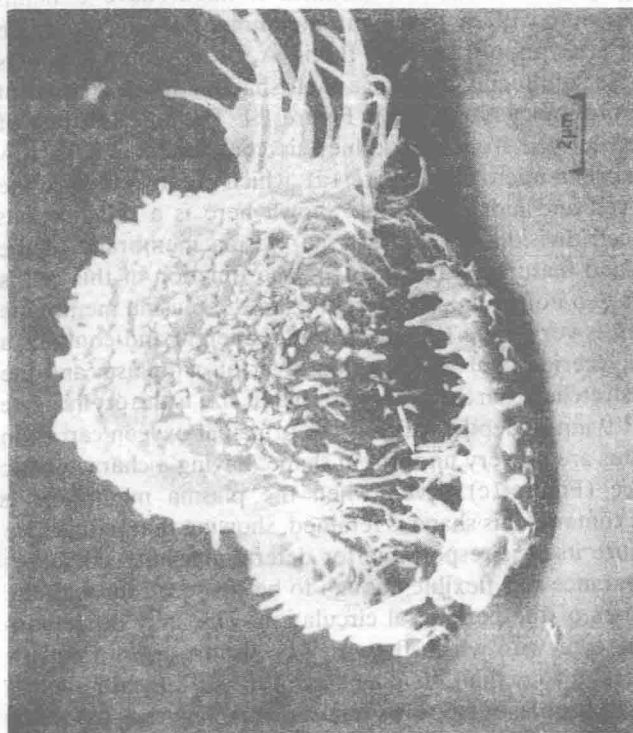
1.1.1 Definition of the cell surface

A living cell, by definition, consists of a discrete mass of cytoplasm separated from its environment by a membrane. The most obvious function of this membrane, called the plasma membrane, is to define the boundaries of the cell. No single structure for this membrane can be described, however, since it





(c)



(d)

Figure 1.1 The morphology of some mammalian cells. Scanning electron micrographs reveal the different surface structures of four mammalian cell types: (a) a MDC cell rounded up during division showing large number of microvilli on the cell surface, scale bar = 10 μm . (Photograph kindly given by Hubert Reggio.) (b) Epinephrine-stimulated adipocyte showing smooth surface with occasional blebs, scale bar = 10 μm . (Photograph kindly given by Robert Smith and Leonard Jarrett.) (c) Human erythrocytes showing biconcave shape. Scale bar = 10 μm . (Photograph kindly given by Dr. S. W. Hui.) (d) Primary rabbit carcinoma cell with extending lamellipodia (arrow), bar = 2 μm . (Photograph kindly given by the division of Cancer Research, Institute of Pathology, University of Zurich, Switzerland)

can take on a number of forms according to the physiological role of the cell. A single cell can also have several different areas of plasma membrane with different morphology and function. For instance it might have domains specialized for interaction with a basement membrane, interaction with adjacent cells and for absorption from body fluids. A further complication is that some cells are able continually to change their shape. Some examples of the diversity of cell morphology are shown in Figure 1.1. The apical surface of the MDCK cell line, derived from dog kidney, is convoluted into narrow cylindrical structures called microvilli (Figure 1a), which greatly enhance the surface area of the cell and aid absorption. Shown here is a cell that has rounded up during cell division. In contrast the plasma membrane of the adipocyte is smooth and featureless (Figure 1b). The function of this cell is principally to store fat, so no specialized structures in the plasma membrane are required. The cell is very large (about $50\text{ }\mu\text{m}$ diameter) and contains a central droplet of triglyceride over which a thin layer of cytoplasm and the plasma membrane is stretched. On the other extreme of size is the erythrocyte ($7.7\text{ }\mu\text{m}$ diameter $\times$ $1.9\text{ }\mu\text{m}$ in depth) which is the principal oxygen carrier in the blood. Erythrocytes are all very uniform in shape, having a characteristic biconcave appearance (Figure 1c). Even when the plasma membrane is isolated from the cell contents this shape is retained, showing that in this case the membrane structure itself is responsible for determining the cell shape. Despite its rigid appearance it is flexible enough to be squeezed through the narrow blood capillaries of the peripheral circulation. This may be demonstrated *in vitro* by the ease with which they may be drawn up into narrow capillaries several times smaller than the diameter of the cell. Flexibility of a different sort is seen in fibroblasts and some tumour cells (Figure 1.1d) which can flatten on to a solid surface, extend lamellipodia and crawl along at several μm per minute (see section 6.7.3). Here the plasma membrane is distensible and irregular in shape, quite unlike the erythrocyte.

Such a diverse morphology in mammalian cells is a reflection of the complexity with which the membrane is organized. Close beneath the membrane and in the cytoplasm is a network of polymeric protein structures called the cytoskeleton (see section 6.7.3). Together with the membrane structure itself these determine the shape of the cell. Thus the shape of microvilli is largely governed by the actin filaments which line up along the axis of the structure. The regular shape of the erythrocyte membrane on the other hand is largely governed by cross-linking between membrane proteins. In the fibroblast, the ability to change shape and move is a combination of the fluid and flexible nature of the plasma membrane and the rapid polymerization and depolymerization of cytoskeletal proteins. This differentiation of cell shape to suit its function is unique to animal cells. Bacteria and higher plants also have a plasma membrane analogous to animals, but it is protected by an outer cell wall which is rigid and of uniform shape.

1.1.2 Control of the intracellular environment

Perhaps the most important property of the cell surface or plasma membrane is the formation of a continuous barrier around the cell which limits the diffusion of substances into and out of the cytoplasm, thus protecting the cell from fluctuating or adverse conditions in the environment. The origin of its impermeability lies in the lipid core of the membrane which, being hydrophobic, does not allow the passage of water or charged molecules. It is, however, punctuated with a wide variety of protein pores and transport systems whose function is to regulate the permeability of the plasma membrane according to the particular needs of the cell (see Chapter 7). Thus the lipids and proteins play complementary roles in the control of membrane permeability. Whilst some small molecules are able to gain access to the cell via these transport systems in the plasma membrane, no large particles, proteins or oligopeptides can enter directly in this way. For these a special mechanism exists. First the particle or macromolecule must bind to the cell, usually to specific receptor proteins embedded in the plasma membrane. The ligand-receptor complexes then aggregate into small areas of the plasma membrane which pinch off to form a vesicle in the cytoplasm. This process is called receptor-mediated or absorptive endocytosis. The endocytotic vesicles then usually fuse with the lysosomes where their contents are degraded, although it is also possible for them to be transported intact to other parts of the cell (see section 6.7). Defects in these transport systems severely affect the metabolism of the cell, usually with pathological consequences.

1.1.3 Communication between cells

While an isolated cell is capable of individual existence it does not normally function in this manner in a tissue. The activity of a cell is regulated by those around it, and also by cells in remote locations. For instance cells growing in a monolayer normally stop growing when they come into contact, a phenomenon known as 'contact inhibition'. Presumably this process is involved in the normal process of limiting cell growth in an organ, since cancer cells, which do not exhibit contact inhibition, grow over each other to produce a tumour. The nature of the intercellular communication involved in contact inhibition is not known, but presumably it takes place at the level of the plasma membrane.

Cells at remote locations may also be linked together by the action of hormones. These are trace substances produced in the endocrine glands in vertebrates which act as chemical messages between distant organs. In mammals a large number of different hormones exist which can evoke a wide variety of responses, ranging from long-term growth and maturation, to short-term regulation of metabolic activity. Hormonal control is organized in a hierarchical manner, secretion of hormones by the adrenal cortex, thyroid, gonads and pancreas being itself under the control of hormones released by

the pituitary gland. This in turn is controlled by factors released from the hypothalamus. In each case specific targets are selected by the presence of an appropriate receptor protein on the cell surface which allows the hormone to bind. The hormone may then have a direct effect on the metabolism of the cell by activating a membrane-bound enzyme facing the cytoplasm or facilitating a movement of ions across the membrane. Alternatively, it may have secondary effects which follow absorptive endocytosis of the hormone-receptor complex (see section 7.7).

Another circulating molecule which has recently provoked intense interest is the small polypeptide called interferon, so named after its ability to protect cells from viral infection. Interferon induces two enzymatic activities in the cytoplasm. The first synthesizes unusual oligonucleotides from ATP containing 2'-5' phosphodiester bonds. These oligonucleotides activate an endonuclease that cleaves mRNA. The second enzyme activity is a protein kinase which phosphorylates the initiation factor, eIF-2, thus inhibiting protein synthesis. Although most cells are capable of making their own interferon, it appears that it can also be used to co-ordinate the defence of distant cells against viral infection, rather like a hormone. Similarly when interferon is added to some tumour cells in culture it inhibits their proliferation, a result which has led to widespread claims for its therapeutic use. It is not known how interferon acts on the cell, but presumably it binds to a membrane receptor.

Communication can also be achieved directly through small molecules. For instance through ion movements across membranes which control the excited state of nerve or muscle cells, or the nerve transmitters which couple adjacent synapses.

In each of these examples the communication between cells relies on structures contained in their plasma membranes. In the absence of these structures a cell would be isolated from its neighbours.

1.1.4 Cell adhesion and immunogenicity

Many of the proteins embedded in the plasma membrane have large carbohydrate structures covalently linked on the external face of the cell. These glycoproteins frequently terminate in sialic acid residues giving the cell a negative charge. The carbohydrate side chains on these glycoproteins are also important in the adhesion of the cell to its neighbours or to its substrate. Another important property of the cell surface is that it is very immunogenic, mainly as a result of the exposed glycoproteins and glycolipids. The structure of these cell surface antigens is under genetic control, and is used by the immune system to distinguish between 'self' and 'non-self'. Thus, all cells in one individual bear similar cell surface antigens, but differ from those in other individuals. It is the compatibility of these antigens which determines the success of a blood transfusion or graft transplantation.

The most important surface antigens determining the success of a blood

transfusion are the blood group antigens. The origin of this antigenic behaviour is the precise structure of the carbohydrate moiety of glycoproteins and glycolipids on the erythrocyte cell surface. Many systems of blood-group specificity exist in man, but the one which is best studied and understood is that of the so-called ABH and Lewis (Le^a and Le^b) antigens. Genetic control of these five antigens is made by four separate groups of genes which control the synthesis of specific glycosyltransferases (see Table 1.1). These act sequentially to produce the characteristic A, B, O, Le^a and Le^b blood groups. Both glycoproteins and glycolipids in erythrocyte membranes can carry these blood-group antigens. Thus the major glycoproteins of the erythrocyte membrane (see section 4.4) can carry blood-group specific antigens. For example band III is amongst those proteins which carry blood-group A activity, and Glycophorin may carry antigens of the MN blood group.

A second set of antigens determining the individuality of cell surfaces is the transplantation or histocompatibility antigens. Unlike blood-group antigens, which define (in most individuals) the antigenicity of body fluids as well as cell surfaces, histocompatibility antigens are restricted to the cell surface. The antigenic determinant in this case is the polypeptide chain of a group of transmembrane proteins coded for directly by the major histocompatibility complex (MHC) on the mammalian genome. In mice the MHC is known as the H-2 complex and is present on chromosome 17; in man it is called the

Table 1.1 Genetic control of ABH and Lewis blood group antigens. The four genes Le , H , A and B each code for a specific glycosyltransferase. Expression of different combinations of these genes in different individuals gives rise to five phenotypes with the blood groups A, B, O, Le^a , and Le^b . Sugar residues are as following: Gal = D-galactose, GlcNAc = N-acetyl-D-glucosamine, GalNAc = N-acetyl-D-galactosamine, Fuc = L-fucose. Sugar linkages are omitted for clarity

Gene	Gene enzyme	Product	Blood group
Le	α -4-fucosyltransferase	Gal—GlcNAc— Fuc	Le^a
H	α -2-fucosyltransferase	Gal—GlcNAc— Fuc	O
$Le + H$	α -4-fucosyltransferase + α -2-fucosyltransferase	Gal—GlcNAc— Fuc Fuc	Le^b
$A + H$	α -GalNAc transferase + α -2-fucosyltransferase	GalNAc—Gal—GlcNAc— Fuc	A
$B + H$	α -Gal transferase + α -2-fucosyltransferase	Gal—Gal—GlcNAc— Fuc	B

HLA complex which is found on chromosome 6. The MHC is made up of a number of loci for each of which up to 100 alleles exist in the population. This high degree of polymorphism explains the ability of the antigens to function as markers of the cell surface 'individuality'. The importance of these antigens lies not only in graft rejection but also in their role in the immune recognition of viral antigens, and the linkage of particular alleles with susceptibility to certain diseases.

The plasma membrane of mammalian cells thus expresses, through histocompatibility and blood-group antigens, the genetic individuality of the cell. This complex system, while not essential for the continued existence of a cell (or individual), is valuable in the defence of an organism against invasion by virus containing antigenic determinants picked up from their previous host. The modification or loss of these antigenic markers which occurs in transformed cells might also prove to be a useful way of screening for tumours.

1.1.5 Functions of intracellular membranes

In the electron microscope the most prominent feature of a mammalian cell is the abundance of subcellular organelles partitioned off by intracellular membranes (see section 1.2). All of these membranes conform to the same general pattern as the plasma membrane, having two layers of lipid molecules with a hydrophobic centre, but the metabolic functions differ quite radically according to the proteins which are embedded in the membrane.

One of the simplest functions of these intracellular membranes is to provide a support to which enzymes may bind. Thus, a number of glycolytic enzymes, which one might have assumed would be soluble enzymes, have been found in association with the plasma membrane in erythrocytes (see section 3.2). It is not entirely clear why this should occur. In other cases, where the substrates are lipid soluble a more efficient metabolic pathway is produced by restricting the reactions to the membrane surface.

A second, and very important, function of intracellular membranes is to define compartments within the cell which can maintain a different environment from the milieu so as to support their own specialized function. Thus the lysosomes maintain an acid pH for the function of acid hydrolase enzymes, the mitochondrial inner membrane maintains an ion and proton gradient which is used as an energy store in the synthesis of ATP, and the ratio of ATP to AMP and NADH to NAD can be independently regulated to suit the metabolic activities of the cytoplasm. Intracellular membranes, however, are not just permeability barriers defining intracellular compartments, they also contain many integral and spanning membrane proteins which perform many of the reactions characteristic of each organelle. Thus the mitochondrial inner membrane is composed of protein complexes which themselves act as the proton and electron carriers between the cytoplasmic and mitochondrial compartments. Other proteins exchange ATP or carboxylic

acid ions which directly feed the catabolic pathways inside the mitochondria and generate most of the energy supply for an aerobic cell. Similarly, integral membrane proteins are responsible for recognizing extracellular molecules during hormone action or absorptive endocytosis at the plasma membrane and probably are also responsible for recognizing the leader sequences of secretory and membrane proteins, being synthesized in the endoplasmic reticulum. Thus intracellular membranes play a key role in the function of cell metabolism.

Summary

Biological membranes are essential components of living cells.

Few cellular activities could continue in their absence.

1.2 THE MORPHOLOGY OF MEMBRANES

In the electron microscope the most prominent feature of a mammalian cell is the abundance of subcellular organelles partitioned off by intracellular membranes. These organelles may be classified into a number of types (e.g. Golgi apparatus, endoplasmic reticulum, lysosomes) which are present in almost all eukaryotic cells. The appearance of any one type of organelle, however, is very variable depending on the cell type. Thus the endoplasmic reticulum is very prominent in secretory cells, and the Golgi apparatus which has only 1–3 cisternae in rat liver may have up to 30 cisternae in some plant cells. For simplicity we have chosen one cell type, the rat hepatocyte, in order to illustrate what the intracellular organelles and their membranes look like. It must be remembered, therefore, that other cell types may have similarly named structures with a rather different appearance.

Figure 1.2 shows a thin section through a rat hepatocyte viewed in the transmission electron microscope. In cross-section the hepatocyte has a polyhedral shape and is about 20–25 μm in diameter. Alternate faces of the cell interact with adjacent hepatocytes and contain the bile capillaries. In between these are the blood sinusoidal faces which are separated from the circulating blood by flattened endothelial cells. The adult mammalian liver is thus a sponge-like cellular mass perforated by two systems of communicating cavities.

The staining procedure used to prepare specimens for the electron microscope results in electron-dense osmium and lead compounds being deposited in the membranes (as well as some other structures) of the cell. The complexity of the intracellular structure of a hepatocyte, seen in Figure 1.2 is therefore largely a reflection of the large number of membranous organelles that fill the cytoplasm of mammalian cells. These organelles, which may be identified as morphologically distinct structures, are shown in Figures 1.3 and 1.4.

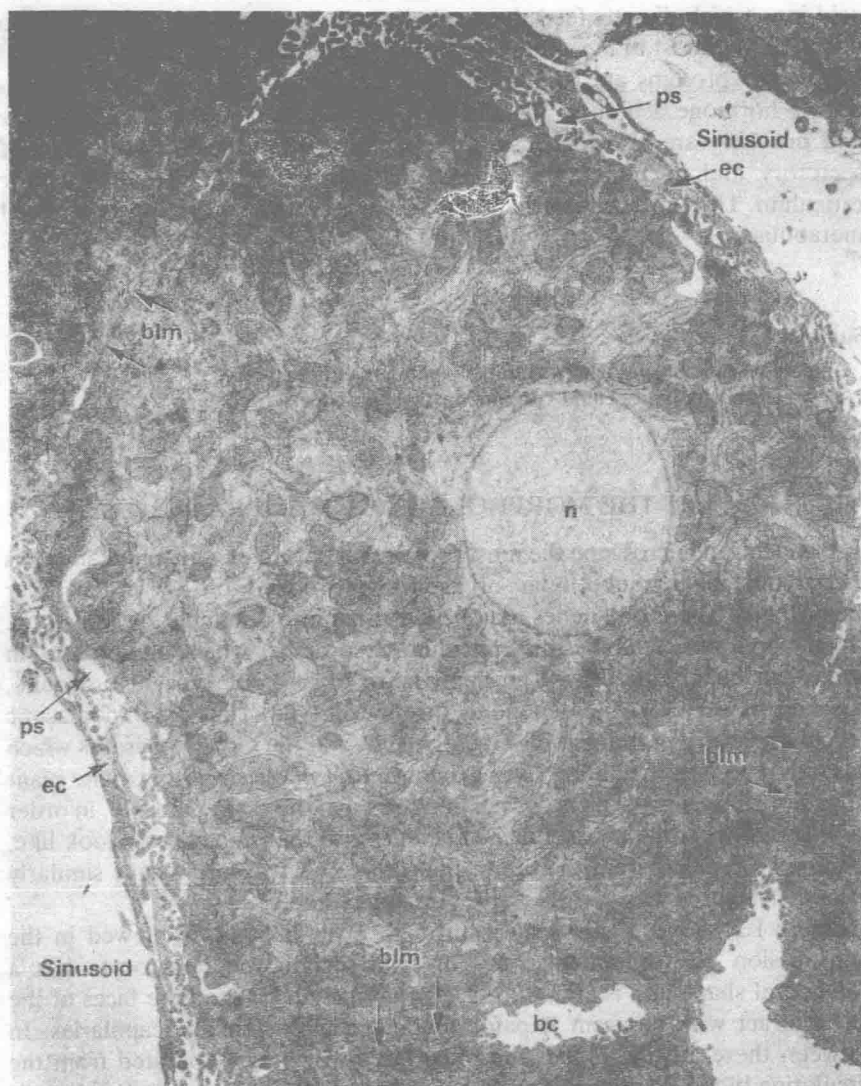


Figure 1.2 Thin section of a whole rat hepatocyte. In the electron microscope the most prominent feature of mammalian cells is the abundance of membranous organelles; ec = reticulo-endothelial cell, ps = pericapillary space (or space of Disse), bc = bile capillary, blm = basolateral membrane, n = nucleus. Magnification $\times 5780$. (Micrograph kindly given by Stephen Massardo and Kathryn Howell)

1.2.1 The plasma membrane

Surrounding the cell is the plasma membrane which in hepatocytes is differentiated into three domains that may be distinguished on the basis of their morphology and composition. Where adjacent cells are apposed, the