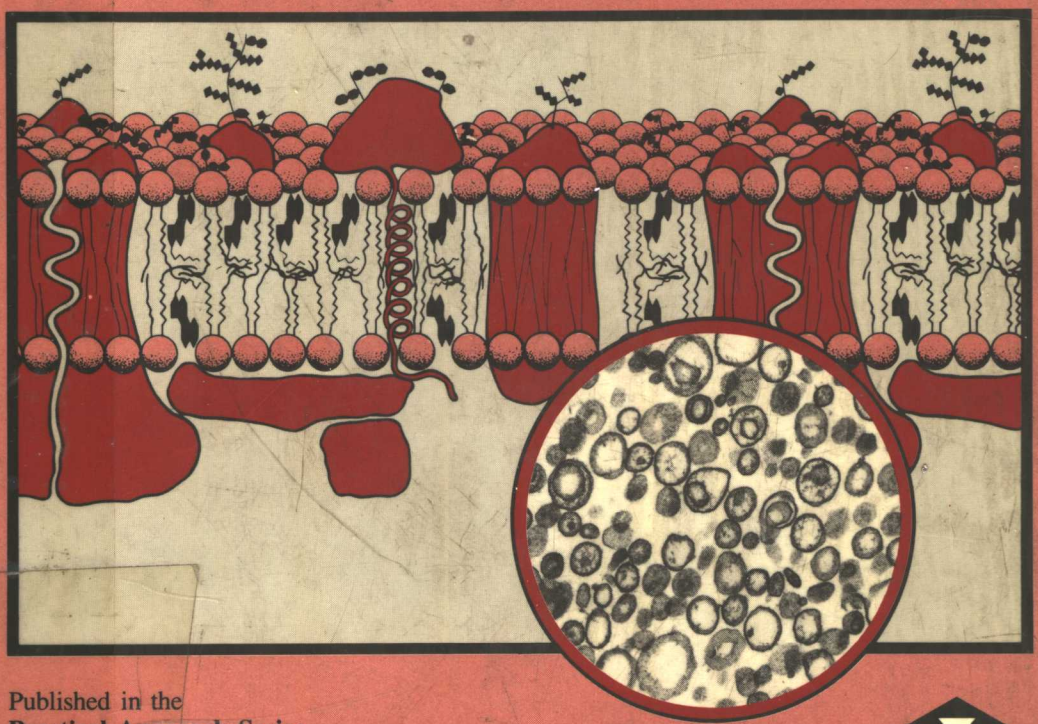
Biological membranes

a practical approach

Edited by
J B C Findlay & W H Evans



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Preface

After decades of relative immobility, the 70's and 80's have seen a dramatic increase in our understanding of membrane phenomena arising from major advances in the selective isolation of membrane fractions and in the discovery and analysis of membrane components. Today's membranologist must possess a wide inter-disciplinary knowledge, ranging from the detailed chemistry and biochemistry of lipids and proteins, through immunology, to complex physical methods and their underlying theory.

Unlike most previous advanced texts on membranes, this volume focuses more on the methodological aspects, allowing a very much wider range to be covered. The inevitable consequence of such a practically-oriented approach is that the theoretical aspects are restricted to the minimum necessary for understanding and explaining the new methodologies. Nevertheless the text, through the examples quoted and the discussion, should supply a bolus of scientific information for the more general reader.

The volume aspires to cover most of the principal approaches for the study of biological membranes. In many cases, it has been possible to provide step by step protocols. In others, general methods are described which can be readily adapted to help achieve a particular goal. Emphasis is placed on extensive referencing so that any small gaps remaining in the text can be plugged. This is particularly important in such a vast area as membrane isolation and characterization where the various methods will require tailoring to the tissues or cells under question. However, methods now exist for isolation of the 10 and 13 biochemically differentiated membrane systems in animal eukaryotic and plant cells respectively.

The rapid advances in the application of mono and polyclonal antibodies to membrane research are detailed, including immunoaffinity purification methods and the use of antibodies in screening cDNA libraries, an approach that is leading to the molecular cloning of an increasing number of membrane proteins. The methods for lipid analysis of membranes are more routine but even here there have been important recent developments, especially with regard to analyses of phosphoinositides and the topology of lipids in membranes. The fractionation and manipulation of proteins have provided fresh scientific challenges and have required the development of new systems, which are given here. Also covered is the art of reconstitution, one of the newer and more important strategies employed to further our understanding of the functional abilities and mechanisms of action of this new class of protein. A comprehensive description of carbohydrate analysis has been omitted since an entire volume in this series deals with the subject.

The wide range of subject matter covered identifies this volume as rather different from most others in the series and this is especially obvious by our inclusion of biophysical approaches. Although we appreciate that it is not possible to detail each of the highly specialized and sophisticated techniques, we felt it important that these approaches, which have contributed so much to our knowledge of membrane structure and function, should not be neglected. We hope that the text in Chapters 7 and 8 will illuminate an otherwise difficult area and spark the experimental imagination into new areas for exploration.

Inevitably, there will be areas which have been omitted or overcondensed. We would appreciate, therefore, receiving any comments on the text and the correction of any errors, subtle or glaring, that might have escaped our notice.

J.B.C.Findlay and W.H.Evans

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Abbreviations

AChR	acetylcholine receptor
ANS	1-anilino-8-naphthalene sulphonate
BSA	bovine serum albumin
CFM	continuous fluorescence microphotolysis
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio] 1-propane-sulphonate
CMC	critical micelle concentration
CTAB	cetyltrimethylammonium bromide
DDISA	3,5-diido-4-diazobenzene sulphonate
DEPC	dielaidoylphosphatidylcholine
DMEM	Dulbecco's modified Eagle's medium
DMPC	dimyristoylphosphatidylcholine
DMSO	dimethyl sulphoxide
DOC	deoxycholate
DOPC	dioleoylphosphatidylcholine
DSC	Differential Scanning Calorimetry
DTAB	dodecyltrimethylammonium bromide
DTNB	dithionitrobenzoate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
e.s.r.	electron spin resonance
FACE	formic acid: acetic acid: chloroform: ethanol
FCCP	carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone
FP	flavoprotein
f.p.l.c.	fast protein liquid chromatography
FRAP	fluoresence recovery after photobleaching
HAT	hypoxanthine, aminopterin, thymidine
h.p.l.c.	high performance (pressure) liquid chromatography
IMP	intra-membranous particle
INT	2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyl-2*H*-tetrazolium chloride
NEPHGE	non-equilibrium pH gradient electrophoresis
NPA	N-1-naphthylphthalamic acid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PC	phosphatidylcholine
PEG	polyethylene glycol
PMSF	phenylmethylsulphonyl fluoride
PN	pyridine nucleotide
POPC	palmitoyloleoylphosphatidylcholine
PTA	phosphotungstic acid
PVP	polyvinylpyrrolidone
SDS	sodium dodecyl sulphate
TFA	trifluoroacetic acid
TID	3-trifluoromethyl-3-(*m*-iodophenyl)diazirine
t.l.c.	thin layer chromatography

Contents

CHAPTER 1

Organelles and membranes of animal cells

W.HOWARD EVANS

1. INTRODUCTION

The membranes of animal cells are organized to encompass morphologically distinctive organelles with well-defined functions, or appear to extend through the cytoplasm as reticular, or vesicular structures. Approximately 10 categories of membranes have been isolated and biochemically characterized from nucleated animal cells. By the late 1960s the major organelles and membranes were prepared and characterized especially from liver tissue, and their relative areas or volumes derived by morphometric techniques (1). However, analyses of nuclei-free liver homogenates by centrifugation in sucrose gradients using zonal rotors indicated that an appreciable amount of membrane protein remained unassigned. Studies have now shown the complexity of the Golgi apparatus (2), especially the extensive trans-tubular axis (3), the functional mosaicism of the plasma membrane of tissue (4,5) and cultured cells (6,7). Also the identification of 'coated' membranes and studies on the labyrinthian complexity of the endocytic compartment (8) have resulted in most, if not all, of the cellular membranes being functionally characterised.

This chapter addresses in general terms the strategies underlying the isolation and characterization of animal cell membranes. Amplified information is available in specialized monographs (9) or in the original papers referenced. The isolation and characterization of plant membranes and organelles, where similar general principles apply, are addressed in Chapter 2.

2. ISOLATION METHODS

2.1 **Non-disruptive methods**

2.1.1 *Cell surface shedding*

In these methods, patches of the cell surface become detached from cells mainly as vesicular structures. Cells, especially tumour cells (10), release spontaneously into media microvesicles originating from the plasma membrane. For example, leukaemic cells release vesicles enriched in tumour antigens (11), mouse mastocytoma cells release H-2 antigens (12), reticulocytes release vesicles containing transferrin receptors (13) and A-431 cells release vesicles containing epidermal growth factor receptor–kinase complexes (14). The shedding of microvesicles enriched in plasma membrane marker components can also be induced by exposing cells to a variety of chemical reagents. Thus, fibroblast monolayers and myoblasts release plasma membrane vesicles after exposure for 15 min to 2 h to protein-free media containing 25 mM formaldehyde – 2 mM dithiothreitol (DTT), or 10 mM N-ethylmaleimide (15). Lymphocytes incubated

in the presence of colchicine or cytochalasin release vesicles containing IgM and IgD (16). Erythrocytes incubated in EDTA − $CaCl_2$ media release, without haemolysis, vesicles impoverished in actin and spectrin (17), and platelets in the presence of low amounts of detergents, for example, dilaurylglycerol phosphocholine (Sigma) also release vesicles (18). Isolated hepatocytes release vesicles, originating mainly from the plasma membrane into saline media when subjected to centrifugation at high speed (19). In addition to cells, whole organisms, for example schistosomes, trematode worms, release membrane fragments from their tegumental surface, a process accelerated by mechanical treatment (e.g. vortexing the worms) (20).

At the practical level, released vesicles are recovered simply by allowing cells, etc., to settle at unit gravity or at low centrifugal forces, for example 500 *g* for 2 min, and collecting the supernatants.

2.1.2 *Attachment to solid supports*

This is a rapid method for preparation of plasma membranes from cells and one that avoids the need for subcellular fractionation. The generalized procedure is described in *Table 1* and is illustrated in *Figure 1*.

This method, used to prepare membranes from red cells, hepatoma and HeLa cells (21−24), has a wide potential. Although the initial experiments with erythrocytes indicated that the plasma membranes attached to the negatively-charged beads after washing off the cells and cellular debris have their cytoplasmic aspect exposed to the medium, the proclivity of plasma membranes of nucleated cells to form vesicles with a right-side-out configuration should be borne in mind. Membranes attached to beads can be analysed directly enzymically and chemically; however, the release of membrane fragments from the beads is difficult to achieve and usually requires detergents or extremes of pH that can destroy biological activities.

Other solid supports used besides polylysine-coated beads include nitrocellulose-treated Sephadex beads to prepare mouse L-cell plasma membranes (25), nylon wool fibres

Table 1. Membrane isolation on polylysine-coated beads[a].

1. Derivatize Bio-Gel P-2 beads with polylysine (mol. wt. 84−91 K, Miles Laboratories) or polyethyleneimine (mol. wt 50−100 K, Polyscience) as described in ref. 24. Alternatively polylysine-coated beads can be purchased from Biorad. Cytodex 1 beads (Pharmacia) may be used as a substitute.
2. Attach the cells to the beads as follows. Wash the beads (~0.5 ml) five times in 10 vol of 0.15 Tris pH 7.4 (bench centrifuge) and four times in 0.14 M sorbitol, 20 mM acetate pH 5.0 (buffer 1). Add the beads suspended in 5 ml of buffer 1 to an equal volume of cells also suspended in buffer 1. Leave for 10 min at 4°C in a centrifuge tube, gently inverting the tube to ensure constant mixing.
3. Dilute the tube to about 50 ml with buffer 1 and allow the beads to settle. Remove the supernatant and repeat this washing procedure twice to remove unbound cells.
4. Vortex tube vigorously for 5−10 s, add ice-cold 10 mM Tris-HCl, pH 7.4 (buffer 2), and mix by inversion. Wash the beads a further four times in buffer 2 to release cells. The number of cells still bound can be assessed by scanning electron microscopy; further washings are necessary if cells are still bound to the beads.
5. After a final wash, suspend the membrane-coated beads in buffer 2 and sonicate at 20 W (or lowest setting) for 5 s. Wash the beads a further three times in buffer 2. Beads may now be used for biochemical analysis of attached membranes.

[a]Taken from ref. 21; see *Figure 1*.

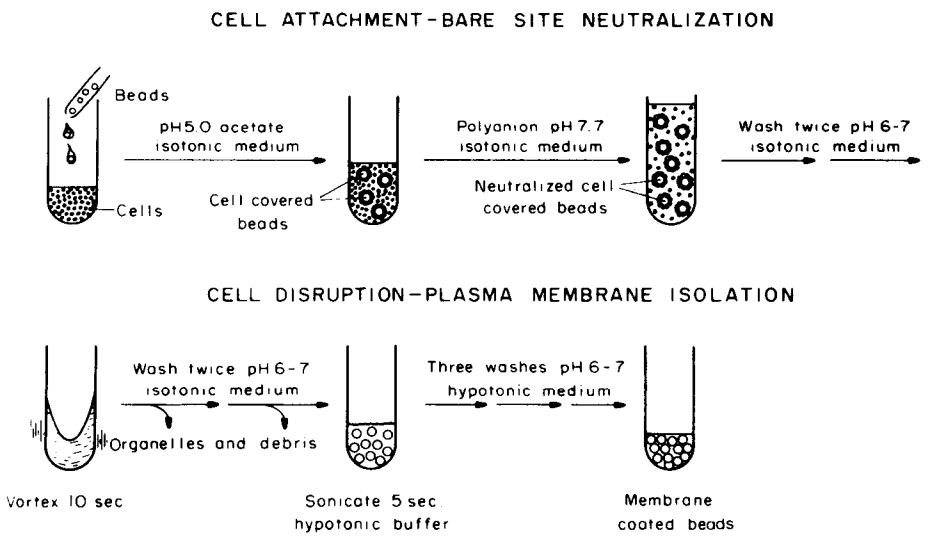

Figure 1. Isolation of plasma membranes on polycation-coated beads. For full details, see *Table 1*. Figure reproduced from ref. 24.

(Baxter-Travenol Labs, Deerfield, IL 60015, USA) to prepare peritoneal leucocyte plasma membranes (26), colloidal silica to isolate *Dictyostelium* plasma membranes (27) and various lectins attached to Sepharose beads to isolate plasma membranes from erythrocytes (28) and mouse lymphoma cells (6). The biological specificity of this approach is open to further development by attachment of antibodies to solid supports, and the immunoabsorption technique for isolation of cell membranes is described in Chapter 3.

2.2 Methods involving cell disruption

2.2.1 *Tissue and cell breakage*

A wide range of methods have been described for the breakage of tissues (see *Table 2* for their relative merits).

(i) Isolated cells, especially cultured cells are, in general, more difficult than tissues to disrupt efficiently and to prepare subcellular fractions of acceptable quality in good yield. The simplest and most common method involves the application of tight-fitting Dounce homogenizers of small capacity (5−12 ml) to disrupt cells first subjected to hypotonic shock; this involves suspending cells for approximately 10 min at 4°C in 5 mM Tris-HCl, pH 7.6. The extent of cell breakage after homogenization is monitored by phase-contrast microscopy and should exceed 80%. Cells grown in spinner cultures are easier to disrupt, for geometrical reasons, than monolayers.

(ii) Of greater utility for disruption of cultured cell lines has been gas cavitation. Cells are suspended in media in a nitrogen-filled metal cylinder for 5−30 min at high pressures (7−65 atmospheres). When the cells are rapidly returned to atmospheric pressure, nitrogen gas dissolved in the cytoplasm is released and