PREPARATION AND CHARACTERISATION OF MAMMALIAN PLASMA MEMBRANES

W. Howard Evans

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List of abbreviations

SDS	Sodium dodecyl sulphate
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethyleneglycol bis(β-aminoethyl)ether-*N*,*N*′-tetraacetic acid
ATPase	Adenosinetriphosphatase catalysing in the presence of Mg^{2+} the reaction: ATP + H_2O = ADP + orthophosphate
$(Na^+$-$K^+)$-ATPase	Incremental ATPase activity in the presence of Na^+ and K^+ that is inhibited by ouabain
Tris	2-Amino-2-hydroxymethyl-1,3-propanediol.

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CHAPTER 1

Introduction

This monograph describes methods and the principles underlying them, for preparing, identifying and characterising subcellular fractions containing the cell's plasma (surface) membrane and its constituent parts. The cell surface membrane is increasingly recognised as playing a crucial role in the regulation of cell metabolism, growth and communication; it interprets environmental signals and controls the nature of molecules entering and leaving the cell. The plasma membrane fraction is the in vitro model of the cell surface membrane used to correlate functional and molecular studies of surface events.

Structurally, the mammalian plasma membrane is conceptualised as a mainly fluid bilipid layer containing a variable but usually large number of metabolically dynamic proteins, enzymes and glycoproteins. Proteins bordering the cell's environment interact specifically and in small numbers with a number of biologically-active substances ('ligands') such as hormones, drugs, antibodies, toxins and infectious agents. Those proteins traversing the bilipid layer are thought to provide the vehicle enabling the cell interior to be made aware of environmental perturbations, and to constitute channels for the passage of ions and charged and hydrophilic molecules across a largely hydrophobic barrier. Further, submembraneous elements (microfilaments, microtubules) may allow direct interaction with cytoplasmic structures and events occurring deep inside the cell. The wide range of processes occurring at the cell's periphery has meant that the plasma membrane is not only being investigated to explore its biological organisation per se, but it has also featured in combination with cellular and other approaches in the experimental

designs of endocrinologists, pharmacologists, immunologists, virologists, etc. The plasma membrane is also being studied extensively in attempts to relate primary metastatic lesions to cell surface modifications.

In this account, attempts are made to rationalise and generalise practical guidelines for the preparation of plasma membrane fractions. In Chapter 2, methods for cell breakage are described and assessed. Polydispersity of plasma membrane fragments produced at the crucial cell breakage step constitutes the most common pitfall limiting the recovery of plasma membranes acceptably free of contamination by cytoplasmic components. Although centrifugation is the major technique used to separate subcellular components, attention is also focussed in Chapter 3 on other complimentary methods such as free-flow electrophoresis, the partitioning of membranes between solvents, immunological approaches, and chromatographic techniques. The use of the more conventional markers to identify and characterise plasma membrane fractions, i.e. enzymic, chemical and morphological, is described in Chapter 4. To corroborate the claims made for the quality of the final product, and to monitor the separation of plasma membranes, newer approaches involving the attachment of radio-labelled markers to the plasma membrane are described. The mammalian plasma membrane, especially in cells constituting tissues and organs, is increasingly appreciated to be a highly differentiated organelle constructed of geographically distinct areas interfacing with different environments and specialised for widely disparate physiological functions. Thus, in most plasma membrane preparative procedures (except when cell membrane 'ghosts' are isolated e.g. from erythrocytes and adipocytes), the final product represents an actual subfraction of an idealised anatomical membrane. The low recovery of plasma membranes frequently reported not only may indicate the persistence of contaminating intracellular membranes, but is also likely to betray the selective purification of a restricted and unrepresentative part of the plasma membrane. Finally, in Chapter 5 the general methodological approaches described in Chapters 2–4 are further illustrated by reference to the preparation

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of plasma membrane fractions from a range of organs, tissues and cultured cells. However, no attempt is made to describe intricate experimental details in individual cases, since this falls well outside the brief of a single author and the space available. References are made to the original publications, which have been frequently redescribed and amplified in treatises such as Methods in Enzymology (edited by S. Fleischer and L. Packer) Vols. 31 and 32, and in the various volumes of Methods in Membrane Biology (edited by E. Korn, Plenum Press).

Inevitably, a wide range of techniques is encompassed in a description of methods for preparing and characterising mammalian plasma membranes and these are described in such detail as is applicable in the present context. More comprehensive and detailed descriptions are available in other volumes of this series, namely Density Gradient Centrifugation by Hinton and Dobrota (1976), Electrophoresis of Proteins in Polyacrylamide and Starch Gels by Gordon (1976), Immunochemical Techniques for the Identification and Estimation of Macromolecules by Clausen (1969), Techniques in Lipidology by Kates (1972) and in Affinity Chromatography by Lowe accompanying this volume.

CHAPTER 2

Methods of cell breakage: assessing their suitability and efficacy

The first step in membrane preparation is breakage of the cell membrane so as to liberate the constituent organelles, membranes and soluble components. A number of methods, tailored to a wide variety of cells, tissues and organs accomplish this with varying degrees of success. In all these methods, the major objective is to effect the disruption of all cells to obtain relatively intact organelles and membrane fragments while inflicting the minimum physical (e.g., heat) and biological (e.g., enzyme inactivation) damage to the liberated components and their content. In chosing methods for the disruption of different cell types, the major subsequent requirement is the adequate separation of liberated parts. The more traditional methods that use shear homogenisation (see below), normally of soft tissues, are geared more to the preservation of the subcellular organelles, especially nuclei and mitochondria. In the isolation of intact lysozomes, the monitoring of the extent of organellar damage reached a high degree of sophistication as a wide range of quantitative biochemical parameters were developed to monitor the separations. Methods of cell rupture for preparing plasma membranes, while respecting the necessity for preserving organelles intact, must also attempt to control the extent of breakage of the cell surface membrane so as to produce a class or classes of membrane fragments of similar size and density adequately separable from other subcellular particles, organelles, etc. This requirement for limiting the polydispersity of plasma membrane fragments is especially important when differential centrifugation methods feature in the subsequent separation techniques (see § 3.1.1). Therefore, the methods of cell rupture employed are of prime

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importance in plasma membrane preparation, for this initial procedure represents a critical point for control of the degree of polydispersity of membrane particles and therefore, of the yield and purity of the isolated membrane fragments.

2.1. Apparatus and techniques

Disruption of cells has been effected by a variety of techniques: the most popular involve propelling cells and tissue fragments through narrow gaps or orifices in which the shearing forces generated cause cell breakage. Other methods utilise osmotic forces, rapid pressure changes or ultrasonic energy to break cells. For disrupting soft tissues the mild shearing forces generated by tissue homogenisers are generally used (Potter and Elvehjem 1936). Cultured cells are usually more difficult to disrupt and this has prompted the introduction of methods similar to those originally developed for the disruption of bacteria and micro-organisms, e.g., French press (Milner et al. 1950) Hughes press (Hughes 1951). Pressure homogenisation, originally introduced by Fraser in 1951, has also featured in the disruption of mammalian cells for isolation of plasma membrane fragments and is carried out in the apparatus described by Hunter and Commerford (1961). For homogenising large numbers of cells under controlled and reproducible conditions, cell rupturing pumps that inject cell suspensions through spring or air-loaded valves set at various pressures are currently finding favour (Wright et al. 1974; Crumpton and Snary 1974).

2.1.1. Potter-Elvehjem homogenisers

These homogenisers (Potter and Elvehjem 1936) are extensively used for disruption of tissues and organs and are available from a number of suppliers in a wide range of capacities (see Table 2.1). In its modern form, it consists of a Teflon plunger or piston attached to a motor that rotates it inside a glass cylinder. The number of revolutions per minute can be closely monitored by connecting the piston to a tachometer. A modern Potter–Elvehjem apparatus is

TABLE 2.1
Properties of tissue homogenisers.

A Potter–Elvehjem Type[a]					
Capacity (ml)	1	4	10	30	55
Grinding length (mm)	–	90	75	105	110
Chamber clearance (mm)[c]	0.1–0.15	0.07–0.13	0.1–0.15	0.15–0.18	0.15–0.23
B Dounce homogeniser[b]					
Capacity (ml)	5	14	30		
Clearance[c] (mm)					
'tight' pestle	'tight'	0.076	0.076		
'loose' pestle		0.127	0.127		

[a] Homogenisers at or approximating to these dimensions are available from Kontes, Thomas, Arnold H. Horwell, Jenkons, Braun, Tri-R instruments. Note that chamber clearance varies according to the capacity of the homogeniser. Large scale (up to 200 ml) homogenisers are available from Houston Glass Fabricating Co.

[b] Homogenisers are usually sold with 'tight' and 'loose' pestles. They are available from Blaessig, Kontes, Thomas and Braun.

[c] Clearance is the difference (mm) between the pestle diameter and the vessel's inside diameter. Radial clearance (or gap) is half this measurement.

shown in Fig. 2.1.

Two major parameters are usually varied. The first is the gap or clearance existing between the rotating pestle and the wall of the glass vessel. The clearance adopted varies according to the tissue being homogenised. Although the dimension of the gap between the plunger and container vessel is usually stated by the manufacturer (Table 2.1), this can be increased by trimming down the diameter of the Teflon pestle in a machine shop. Most commercially available Potter–Elvehjem homogenisers have a clearance of 0.10–0.15 mm. This value does not vary very much with temperature during use because of the low coefficient of expansion of Teflon. The second parameter that can vary is the control exercised over the homogenisation conditions, i.e., the number of passages up and down the vessel, the time taken and the speed of rotation. This includes also the

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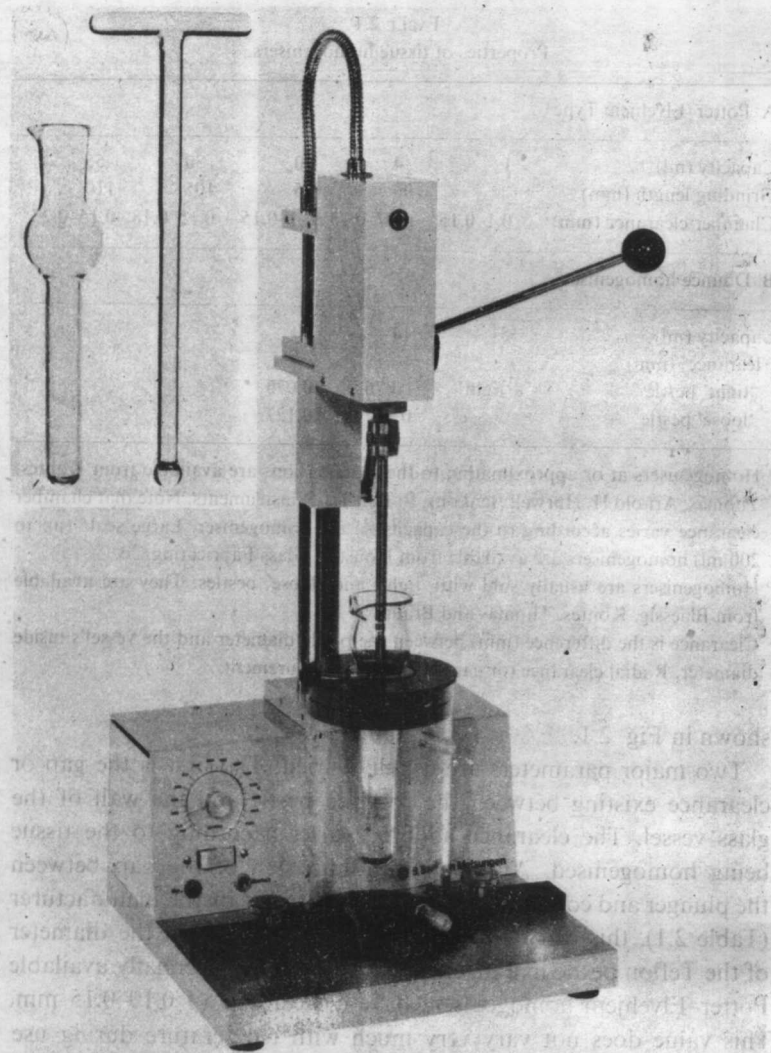

Fig. 2.1. Potter–Elvehjem and (inset) Dounce homogenisers. In this Potter–Elvehjem homogeniser up and down movement of the Teflon pestle inside the water-jacketed vessel is remotely-controlled by the handle.

tissue to homogenising medium ratio and, overall, this is probably the parameter that may vary most amongst users. Thus, a full description of the range of conditions used should include: (a) the tissue to medium ratio (§ 2.2); (b) the necessity for prior removal of connective tissue by filtration through gauze (e.g., cheese-cloth) and for chopping up the tissue into manageable portions (§ 2.3); (c) the clearance between the pestle and plunger; (d) the number of complete passes of the rotating pestle (i.e., passages down and up the vessel) and (e) the speed of rotation, as monitored by a tachometer.

The power-driven Potter–Elvehjem homogeniser can cause problems for the novice operator, since failure to keep in parallel the rapidly rotating pestle and the hand-held container can result in the sudden breakage of the glass. From a safety point of view, it is therefore recommended that a glove is used to hold the glass vessel and a separate control, e.g. a footswitch is present to free the hands to operate the homogeniser. To rotate the pestle at a constant speed, it is useful to have a high-torque (series wound) motor equipped with speed control and a tachometer. A further important consideration is to ensure that homogenisation at high rotation speeds does not result in local heating thereby damaging heat-labile tissue constituents. Homogenisers with water jackets are commercially available, but usually it is more practicable to immerse the homogeniser in ice-water for a few seconds between each passage of the plunger.

The conditions for cell disruption will vary widely between tissues and animal species. Older animals contain more collagenous tissue than younger animals, thus making complete homogenisation more difficult. Thus variation can be expected in the use of Potter–Elvehjem homogenisers to disrupt tissues from animal species of different ages and physiological or pathological states.

For homogenisation of cells of small size, e.g., platelets, a 'no-clearance' Teflon pestle (Kontes No. 12–152–A) may be used (Marcus et al. 1966). The Teflon pestle does not fit into the vessel at 20 °C, but at 4 °C it has contracted slightly. As homogenisation proceeds, local heating causes it to expand and this provides strong shearing forces.

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2.1.2. Dounce homogenisers

This homogeniser (Fig. 2.1, inset), was first introduced to prepare intact nuclei from liver for metabolic experiments (Dounce et al. 1955) and has since featured prominently in many surface-membrane preparative routines. The Dounce homogeniser was first used for plasma membrane isolation by Neville (1960). Compared with the Potter–Elvehjem homogeniser, used under similar conditions (Emmelot et al. 1964) the Dounce hand homogeniser is a far more controllable instrument for tissue disruption, since the operator can obtain a 'feel' of the disruption process as the ball-tipped pestle is moved up and down the vessel. High shearing forces that are characteristic of motor-driven homogenisers are avoided, thus largely eliminating any possibility of damage due to heat generation. Furthermore, in many instances of plasma membrane preparation, the low shearing forces result in the release of large sheets of plasma membrane that can be sedimented at low centrifugal forces. Dounce homogenisers are available in various capacities and with two plungers, tight or loose fitting (Table 2.1). The clearance between the ball on the plunger and the vessel constructed of constant bore glass tubing gradually increases after prolonged use. A rapid method of assessing whether the gap is of the correct order and to compare or standardise loose-fitting homogenisers involves measuring the time taken by the plunger to descend by gravity through the water-filled constant-bore portion of the vessel. For example, the optimal gap for disruption of rat liver for plasma membrane isolation is such that the plunger will descend in 4–5 sec through a water-filled, large, type A Dounce homogeniser (Blaessig Glass Specialities).

Dounce homogenisers are frequently used to disrupt tissue and cells in hypo-osmotic media. An additional parameter involved under these conditions is the amount of swelling of cells prior to use of the homogeniser, as discussed in § 2.2.

2.1.3. Microhomogenisers

As techniques of membrane isolation are extended to include cells or tissues available only in limited amounts, e.g., biopsy material,