METHODS IN MEMBRANE BIOLOGY · 1

Edited by EDWARD D. KORN

METHODS IN MEMBRANE BIOLOGY

VOLUME 1

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Preface

Examination of the tables of contents of journals — biochemical, molecular biological, ultrastructural, and physiological — provides convincing evidence that membrane biology will be in the 1970s what biochemical genetics was in the 1960s. And for good reason. If genetics is the mechanism for maintaining and transmitting the essentials of life, membranes are in many ways the essence of life. The minimal requirement for independent existence is the individualism provided by the separation of "life" from the environment. The cell exists by virtue of its surface membrane. One might define the first living organism as that stage of evolution where macromolecular catalysts or self-reproducing polymers were first segregated from the surrounding milieu by a membrane. Whether that early membrane resembled present cell membranes is irrelevant. What matters is that a membrane would have provided a mechanism for maintaining a local concentration of molecules, facilitating chemical evolution and allowing it to evolve into biochemical evolution. That or yet more primitive membranes, such as a hydrocarbon monolayer at an air–water interface, could also have provided a surface that would facilitate the aggregation and specific orientation of molecules and catalyze their interactions.

If primitive membranes were much more than mere passive barriers to free diffusion, how much more is this true of the membranes of contemporary forms of life. A major revolution in biological thought has been the recognition that the cell, and especially the eukaryotic cell, is a bewildering maze of membranes and membranous organelles. Parallel with this development has been the realization that these membranes are not just the static barriers and demarcators of cellular space they may appear to be in stark photomicrographs. Rather, membranes are dynamic structures in continual movement, both morphological and molecular. Moreover, the major biochemical and physiological events of life occur in, on, or through membranes. Not just the highly selective, often energy-coupled, transport of ions and molecules but also the complex processes of oxidative phosphorylation, photosynthesis, vision, and nerve conductance; intermediary biochemical events such as protein and lipid biosynthesis, the citric acid cycle, and fatty acid oxidation; hormone–cell and cell–cell interactions; endocytosis and secretory processes

are all membrane phenomena. It is at the membrane that morphology and metabolism unite; that catalytic chaos is organized; that self is distinguished from nonself.

Elucidation of the structure, function, and biosynthesis of membranes thus becomes a major goal of contemporary experimental biology. The tasks are to determine the chemical and enzymatic constituents of the membranes; to study the physical and chemical properties of relevant models; to dissociate the biological membranes into their natural structural and functional units; to recombine these minimal units into membranes that are structurally and functionally identical to the originals; to discover the biological mechanisms of synthesis and organization; to understand the varied roles of membranes in normal and pathological states. In practice, of course, all of these approaches are undertaken simultaneously, and progress in any one area is a tremendous stimulus to success in the others.

Unfortunately, the methodological as well as the conceptual difficulties are immense. Morphological and ultrastructural techniques are inherently static and in any case stop short of revealing information at the molecular level. Physical and chemical methods are averaging techniques providing considerable information about the mean properties of membranes, but only a general guide to the organization of the specific and diverse functionally distinct subregions within them. Traditional enzymology has many weapons with which to fight in the sea of aqueous reactions but possesses few tools with which to dig into the fertile fields of surface chemistry. Despite the enormous difficulties, however, considerable progress is being made in all areas by the use of a wide variety of old and new methods.

The generality of the importance of membranes and the tremendous diversity of experimental approaches to their study create yet another difficulty which it is the purpose of this series to help overcome. In membrane research perhaps more than in any other area of biology, progress depends on methodology, and the methodology can be highly technical and highly specialized. As a consequence, one investigator often finds it difficult to understand and to evaluate the techniques used by another. Authors for *Methods in Membrane Biology* have been urged, and given adequate space, not only to describe their methods in sufficient detail for the reader to use them in his laboratory (or at least to tell the reader where he can find the experimental details when they are readily available elsewhere) but also to discuss fully the theoretical backgrounds of the methods and their applications and limitations in membrane biology. The expressed aim is to enable each of us to evaluate more critically, and to understand more fully, data obtained

by methods foreign to our usual experiences. It is planned that each volume will contain a range of methods varying from the physical to the physiological, thus maximizing the audience for each. "Methods" may at times be interpreted rather broadly, but it is not intended that these articles shall be primarily reviews of the results obtained by the methods under discussion.

It is entirely appropriate that Volume 1 of *Methods in Membrane Biology* should begin with a chapter on liposomes. It is now generally accepted that, whatever the degree of complexity of the arrangement of proteins and carbohydrates in membranes, most of the phospholipids of biological membranes are in molecular bilayers (although there still is debate on the extent of uninterrupted lipid bilayer within some membranes), for which the liposomes are simple and elegant experimental models. Indeed, the liposome may resemble even more closely that putative primitive membrane referred to earlier. In any case, whether or not it is an evolutionary as well as an experimental prototype, the liposome is a fascinating system for structural and functional studies of phospholipid bilayers. Although myelin figures had been known for many years and lipid dispersions had been studied previously, it was A. D. Bangham who first made explicit use of the liposome as a model membrane. In their chapter, Bangham, Hill, and Miller present a succinct and authoritative review of the preparation of multilamellar and single-bilayer liposomes and of their structural and functional applications in membrane biology.

Ultimately, our understanding of lipid interactions will be complete only when it rests on a thermodynamically sound support. N. Gershfeld has modified the experimental approach to the Langmuir trough and performed simple, definitive experiments with lipid monolayers. The data are analyzed so as to reach conclusions that are always interesting and frequently provocative. The surface chemist contributed very much very early to membrane theory, and he still has much to offer the biologist.

At a more complex level of organization, the third chapter deals with spectroscopic analysis of membrane proteins, where it is particularly important to recognize the experimental difficulties. D. Urry was among the first to realize that, valuable though optical rotatory dispersion and circular dichroism measurements were in providing information about the configurations of membrane proteins, the particulate nature of the systems imposed severe limitations on the interpretation of the spectra. Urry and Long have now developed theoretical and experimental ways to circumvent many of these difficulties, allowing a fuller utilization of spectroscopy in studies of membrane structure.

The next chapter in this volume discusses the physiologically, pathologically, and therapeutically vital subject of the antigenic properties of the cell surface. Here, major experimental problems are to design quantitative assays for membrane antigens and to develop methods for the isolation and purification of surface antigens from normal and abnormal cells. Reisfeld, Ferrone, and Pellegrino describe their and others' efforts with respect to the histocompatibility antigens in this rapidly developing field.

Finally, the chapter by Y. Kagawa is a comprehensive presentation of the brilliant achievements in the structural and functional reconstitution of that most complex and integrated system, the inner mitochondrial membrane. While dealing specifically with the dissection and reassembly of the energy transfer reactions of the mitochondrion, Kagawa has developed a set of general principles and techniques that should prove of inestimable value as a rational guide for investigations of all biological membranes.

It is our intention that future volumes in this series will appear at not greater than annual intervals. At the time of this writing Volumes 2, 3, and 4 are at various stages of gestation; their anticipated contents are listed separately. Future volumes will best serve their intended purposes if readers will take the time to communicate to the editor "methods" that they would like to have critically reviewed. If the reader can also suggest the names of possible authors, that would be of additional value. Should they wish to volunteer themselves, so much the better, but acceptance is not guaranteed for then the editor would have to do nothing but read their proofs and collect his royalties.

September, 1973

Edward D. Korn

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Preparation and Use of Liposomes as Models of Biological Membranes

A. D. Bangham, M. W. Hill, and N. G. A. Miller

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1. INTRODUCTION AND HISTORICAL SURVEY

The recognition that biological cells exploit the water–oil interfacial activity of certain lipids to define anatomical boundaries has, in recent years, encouraged many workers in this laboratory* and others to develop and study protein-free model membrane systems prepared with such compounds. A considerable technical advance was made more than 10 years ago, when Mueller *et al.* (1962*a*,*b*) first reported a method for preparing usable preparations of bimolecular membrane from membrane molecules, the so-called black lipid membranes or BLMs. The merits of this powerful technique were quickly realized, and, as with Aladdin's lamp, a property requested was a property acquired! Indeed, it might be said that the skeptics themselves were the holders of the lamp, because it was they who, by dismissing the model as unrealistic for a succession of reasons, indicated explicitly the characteristic implicit (passive) properties of a biological membrane: before very long, the earlier convictions of the Langmuir–Hardy–Rideal schools of surface

* We borrow Kinsky's connotation of the word "laboratory" as a means of indicating chronologically the names of colleagues who have participated in the development of the liposome model: J. C. Watkins, M. M. Standish, G. Weissmann, D. Papahadjopoulos, J. de Gier, G. D. Greville, S. L. Bonting, S. M. Johnson, R. C. MacDonald, R. Klein, M. Moore, M. A. Singer, P. Callissano, R. Lester.

chemistry, relating the role of amphiphilic molecules to biological membrane structure, were vindicated.

Unlike the mechanically supported black lipid membranes, contrived for the first time so very recently, lyotropic smectic mesophases (liposomes) of membrane-like lipids have probably been forming and reforming on the aqueous earth for longer than life itself. Indeed, it is worth remarking, yet again, on the similarity which might exist between the present state of model membrane syntheses and some stage in prebiotic history (Bangham, 1968, 1972). Such a statement might not seem a very flattering assessment of present-day achievements, but in reality it takes us a very considerable distance along the evolutionary trail. Simple principles of electrostatic, hydration, and surface free energies now recognized as being the principal forces governing membrane stability could just as well have applied to a primitive population of compounds, of increasing carbon chain length, acquiring polar or ionic head groups and being synthesized in a continuum of water (Oparin, 1924).

The late A. S. C. Lawrence (1969), in a lifetime spent on this subject, described many ordered-phase systems of simple mixtures of long-chain alcohols, long-chain acids, and water. Together with Dervichian (1964), he recognized the physical similarities of phospholipids to their own simpler analogues. Leathes, too, as was pointed out by Small (1967), demonstrated that lecithins interact physically with water to form what were termed "myelin figures," which were recognized then as being "made up of films two lecithin molecules thick, with the hydrophilic groups facing the water on each surface" (quoted by Small, 1967).

An evolutionary sequence would have involved a selective adsorption of amphiphilic compounds at the air–water interface, their orientation to form a monolayer, and their eventual collapse due to aero- or hydrodynamic stresses to form closed aqueous compartments, i.e., liposomes. A (molecular) form of natural selection, based on surface free energy minima, would ensure the evolution of increasingly more stable bilayers. In the event of a particularly favorable synthetic process of membrane molecules evolving *within* an extant membrane system, a clone of membranes would develop. A factor reinforcing such speculation is the surprisingly long time constant for a molecule on one side of a bilayer to flip to the other (Kornberg and McConnell, 1971). The sequestered, aqueous compartments so formed would become isolated from the bulk primeval soup by a continuous, thin hydrocarbon membrane which would exhibit its very distinctive permeability properties, to be referred to later. The interfacial region, too, between aqueous phase

and thin hydrocarbon film, would present a mosaic of hydrophilic groups such as hydroxyl, carbonyl oxygen, and amide as well as positive or negative ions. Most probably, such a planar array of ordered reactive sites would have both catalytic and template properties. There is also the very real and interesting possibility that simple polypeptides of the type which are now recognized as being selective ion carriers, coexisting in the primeval soup, might have initiated the familiar pattern of an intracellular enrichment of K^+ over Na^+.

In December 1932, a British subject (J. Y. Johnson, 1932) applied for a British patent on behalf of I.G. Farbenindustrie Aktiengesellschaft, who had found that "pharmaceutical preparations for injection into the muscular system or subcutaneously can be prepared by combining medicaments with liquids, such as fats or fatty oils, if necessary together with waxes or wax-like substances, with water, or other liquids, and a dispersing agent, whereby a system, hereinafter called "depot" is found capable of holding any desired doses of the medicament but releasing it over any desired space of time only gradually ... without the slightest detriment to the organism"! One specification particularly singles out lecithins as being a useful lipoid, and the description of a "depot" to contain strophantin reads singularly like a contemporary preparation of liposomes: "An emulsion is prepared from: 25 parts of lecithin; 20 parts of water; 1.5 parts of cholesterin; 0.03 parts of strophantin and 0.5 parts of a 'Nipasol' (*p*-hydroxybenzoic acid normal propyl ester)"; its success can now be ascribed to the fact that at least part of the dose of the drug had been sequestered within a smectic mesophase or liposome system. Unaware of the existence of this patent specification, one of the present authors, while preparing aqueous suspensions of lecithin for electron microscopy (Bangham, 1963; Bangham and Horne, 1964), observed by light microscopy that the structures could be seen to alter shape in response to changes of concentration of solute—whether of electrolytes or nonelectrolytes—in the continuous aqueous phase. Furthermore, it was noted that the presence or absence of charged phospholipids in the system modified the birefringence of the smectic mesophase at a given electrolyte concentration. These observations were followed up and subsequently reported as a possible complementary model to the BLM (Bangham *et al.*, 1965*a*,*b*, 1967*a*,*b*). Rendi, too, had noted that "suspensions" of total mitochondrial lipids in an aqueous phase "extruded" water in an analogous manner to mitochondria when exposed to calcium or albumin solutions (Rendi, 1965), but he seemed unaware of the membranous nature of the lipid material.