THE ROLE *of* CALCIUM *in* BIOLOGICAL SYSTEMS

Volume II

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FOREWORD

Calcium must certainly be the major bioelement of the times. Only a generation ago Ca^{2+} was known to physiologists and biochemists as a component of bone mineral and as a blood plasma constituent required in heart function and blood coagulation, but little more. Only a few, such as Baird Hastings and Walter Heilbrunn, saw more clearly into the future of Ca^{2+}, a future that was a long time coming. Then came the discovery of the role of Ca^{2+} in the contraction-relaxation cycle of skeletal muscle and the recognition that the free Ca^{2+} concentration of the resting sarcoplasm must normally be orders of magnitude lower than that in the blood plasma. Thus it was found that skeletal muscle must possess extremely efficient energy-dependent Ca^{2+} pumps. The discovery that mitochondria can accumulate Ca^{2+}, by my colleagues Vasington and Murphy, was at first regarded by many as an anomaly of in vitro conditions, since Ca^{2+} had earlier been found to uncouple oxidative phosphorylation. How could oxidative phosphorylation and Ca^{2+} transport be compatible? What possible role can mitochondria play in cellular Ca^{2+} distribution? And why does calcium phosphate form insoluble but noncrystalline granules in the mitochondrial matrix?

Answers to these and other questions came slowly at first, but in the 1970s a crescendo of Ca^{2+} research developed. Today we know dozens if not hundreds of different cellular and extracellular processes that are regulated by changes in the level of cytosolic or extracellular Ca^{2+}, in which at least three different membrane systems of the cell take part. Indeed, Ca^{2+} is now emerging as a most important and ubiquitous intracellular messenger, perhaps even broader in function than cyclic AMP, the original second messenger. What is even more remarkable is that cytosolic Ca^{2+} levels can regulate several different activities simultaneously in a single cell, raising fundamental questions regarding spatial and temporal regulatory fluctuations in cytosolic Ca^{2+} concentrations. Also remarkable are the biochemical mechanisms that keep calcium and phosphate, which occur in extracellular fluids and urine in supersaturating concentrations, from precipitating and turning us into stone. Central to all these questions is the chemistry of Ca^{2+}, its special features that endow it alone, of all the common cations, to participate in such a panoply of biological activities.

The papers in this volume address many aspects of these problems in the biochemistry and physiology of calcium and provide an important guide to recent progress.

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PREFACE

The purpose of this review is to summarize and correlate the recent advances in several fields of scientific research related to the involvement of calcium in the structure development and function of biological systems.

Considering the general interest in calcium, this publication which is a comprehensive collection of contributions on the biochemical properties of the ion, is aimed to be of interest to workers in many fields of biology and medicine whose investigations might be related, directly or indirectly to the role of this ion in biological systems. In addition to the benefit of presenting a concise review of the state-of-the-art on each subject, it will provide a useful reference source of the work done in a wide range of scientific disciplines such as biochemistry, analytical chemistry, cell biology, physiology, nutrition, pathology, pharmacology, toxicology, etc.

The text consists of six major divisions. The first deals with the chemistry of calcium and gives both the theoretical and practical basis to interpret the role of this element in the function of normal and pathological biological systems, as described by the other subsequent divisions.

It is not the aim of this publication to provide an exhaustive compilation of all the subjects concerning the biochemistry of calcium, but to give within the limits of the present work the most important and actual highlights related to this bioelement. In most instances the given information has been made as concise as possible to make feasible the coverage of all the different subjects, but without sacrificing the updated bibliographic references which constitute a quick access to the ultimate source of knowledge. To the contributors and publisher who have made possible this publication we are very much indebted.

Leopold J. Anghileri
Anne Marie Tuffet-Anghileri

THE EDITOR

Leopold J. Anghileri, Dr. Chem. is Senior Scientist and Research Group Leader at the Laboratory of Biophysics, Service of Nuclear Medicine and Diagnostic by Ultrasons and Thermography, Medicine Faculty B, University of Nancy, France. Dr. Anghileri graduated in 1951 from Buenos Aires University where he obtained his Dr. Chem. degree in 1957. Dr. Anghileri became a member of the American Chemical Society in 1961. Until 1964 he was Investigator at the Argentine Atomic Energy Commission. From 1964 to 1968 he was a fellow at the Johns Hopkins Medical Institutions, Division of Nuclear Medicine and Radiation Health, Institut de Radium, Laboratoire Curie, Paris, and Institut für Nuklearmedizin, Deutsches Krebsforschungzentrum, Heidelberg, West Germany. During the period 1970 to 1975 he was Investigator at the Tumorforschung, Ruhr Universität, Essen, West Germany.

Dr. Anghileri has presented invited papers at international meetings. He has published more than 210 research papers. His current major research interest includes hyperthermic treatment of cancer and the calcium-magnesium membrane relationship in tumors.

THE CO-EDITOR

The late **Anne Marie Tuffet-Anghileri, Dr. Sci.** was Investigator at the Centre National de la Recherche Scientifique, Paris. Dr. Tuffet-Anghileri graduated in 1969 from Paris University. She worked in carcinogenesis at the Laboratoire Curie, Paris and in Molecular Biology (DNA-metal ions interactions) at the Paris University.

To my wife and dedicated co-worker Anne Tuffet-Anghileri (1937-1981) whose life was sacrificed for and by the Science.

L. J. Anghileri

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Calcium and Membranes

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CALCIUM AND MEMBRANE STABILITY

Larry M. Gordon and Richard D. Sauerheber

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I. INTRODUCTION

Ca^{2+} exerts multiple effects on biological membrane activities. It is well-known that Ca^{2+} participates in a number of membrane-associated physiological functions, including regulation of enzyme activities, transduction of hormonal information, release of secretory products, functioning of transport systems, neuronal conduction, and muscle contraction. Recent studies have sought to define the action of Ca^{2+} on membrane structure and function by employing an increasingly sophisticated armament of biological and physical-biochemical techniques. Although the ability of Ca^{2+} to coordinately modulate such diverse activities is a powerful stimulant to continue these investigations, it nevertheless serves to complicate our understanding of how Ca^{2+} acts on any given function. This is particularly true since many of the above cellular processes are thought to be interrelated.

The present chapter provides a brief review on the role of Ca^{2+} in the stability of membranes and in the regulation of various membrane functions. It is our view that a satisfactory interpretation of these phenomena requires that the underlying structural alterations induced by Ca^{2+} be elucidated. Accordingly, we first survey several models of biological membranes and then consider results from key experiments which tend to either support or refute these models. The specific interactions of Ca^{2+} with model and biological membranes, together with possible mechanisms which have been proposed to link Ca^{2+}-mediated structural perturbations with functional changes, will next be examined.

II. THE HETEROGENOUS COMPOSITION OF BIOLOGICAL MEMBRANES

Biological membranes consist of *lipids, proteins,* and *carbohydrates,* in proportions which depend on the system in question.[1] Phospholipids constitute the major fraction of endogenous lipid, and may be classified according to either headgroup specificity (e.g., isoelectric or acidic species) or length and degree of saturation of the fatty acyl chains extending from the glycerol backbone. The neutral lipid cholesterol is also present in high levels in mammalian surface (or plasma) membranes, but relatively deficient in intracellular organelles. The proteins of biological membranes are exceedingly varied, thus permitting these molecules to play a wide range of structural and functional roles. Lastly, carbohydrates may be covalently attached to either lipid (glycolipid) or protein (glycoprotein).

III. MODELS OF MEMBRANE STRUCTURE

Although the matrix of biological membranes is generally agreed to be a lipid bilayer, there remains uncertainty as to the precise distribution of proteins and carbohydrates and as to the relative motions of the individual components. A number of structural models have been proposed to account for the properties of biological membranes, and the advantages and disadvantages of these models have been reviewed in some detail.[2]

A. The "Fluid Mosaic" Model

The most widely accepted view of biological membranes is that they exist as a "fluid mosaic" at physiologic temperatures.[3] Proteins noncovalently associate with the lipid bilayer, and were broadly classified by Singer and Nicolson[3] as being either integral or peripheral. Integral proteins are firmly embedded in the lipid bilayer and may be extracted only with rather harsh treatments employing detergents or organic solvents.

Contrarily, peripheral proteins are weakly associated with the membrane, and may be removed with less drastic procedures such as the use of high salt or chelating agents.

The protein and lipid components are each arranged so that hydrophobic ("water-repelling") and hydrophilic ("water-seeking") interactions are maximized. For example, phospholipids are amphipathic molecules with one highly polar end and another nonpolar end. The hydrophobic fatty acyl chains of phospholipids are in close physical contact in the bilayer and are unable to interact with water. Conversely, the polar headgroups of phospholipids remain exposed to the aqueous phase, thereby maximizing hydrophilic interactions. Integral proteins are also amphipathic molecules that will be both embedded in and protruding from the bilayer. The amino acid sequence will be folded so that the hydrophobic residues are in contact with the fatty acid chains, while the polar ones are exposed to the aqueous phase. Peripheral proteins, such as cytochrome c or spectrin, are probably linked with endogenous lipids or integral proteins through electrostatic interactions and/or Ca^{2+} coordination.

The lipids and proteins of biological membranes were viewed as being, in general, randomly distributed in the plane of the bilayer and without long-range order. Nevertheless, Singer and Nicolson[3] allowed for several important exceptions, in that specific lipids may be tightly coupled to integral proteins or short-range, nonrandom interactions may occur between certain integral proteins. The vertical (or transverse) positioning of integral proteins is such that these molecules will either partially penetrate or completely span the bilayer width.

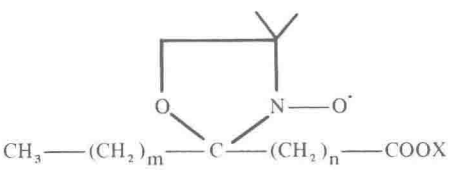
An essential feature of the fluid mosaic model is that lipids and proteins are, for the most part, expected to be in motion at physiologic temperatures. These components may exhibit lateral movement in the bilayer plane (i.e., translational diffusion) or rotational motion about an axis perpendicular to the bilayer plane, but only restricted "flip-flop" from one half of the bilayer to the other (i.e., transverse diffusion), since that requires passing a polar group through a hydrophobic surface.

B. Evidence Supporting the Fluid Mosaic Model

Several aspects of the fluid mosaic model for biological membranes have been supported by the results of various studies. For example, electron microscopic investigations indicate that, to a first approximation, integral proteins are randomly distributed in erythrocyte membranes.[3] Numerous penetrant proteins exhibit rapid motions in the plane of the bilayer, including translational diffusion (diffusion coefficients between 10^{-9} and 10^{-12} cm^2/sec) and rotational diffusion about an axis perpendicular to the bilayer plane (relaxation times between 20 to 400 μs).[2-4]

Differential scanning calorimetry (DSC) and diffraction techniques have also assigned the lipids of biomembranes as existing in a fluid bilayer at physiologic temperatures. Most biological membranes have been found in X-ray diffraction studies to be above the main lipid order → disorder transition under these conditions. Similar results were obtained from electron diffraction experiments on rat liver plasma membranes and erythrocyte ghosts, which indicated transitions at 12 to 18° and 0°C, respectively.[6] DSC also demonstrated that the main lipid transition in such cholesterol-poor membranes as sarcoplasmic reticulum and rat liver microsomes and mitochondria occurred at temperatures much lower than physiological.[7]

The fact that a significant fraction of lipid present in biological membranes is in the fluid state has been indicated by the use of electron spin resonance (ESR) probes sharing the following structure:



I(m,n)

These labels intercalate into the membrane so that their long molecular axes are perpendicular to the bilayer plane and execute rapid axial rotation ($\sim 10^8$ r/sec) at physiologic temperatures. *I(m,n)* may concurrently undergo flexing or bending motions, and this may be quantitated by calculating an order parameter, S, from the ESR spectra.[8,9] S may range in value from 0 to 1, with the extreme order parameters indicating that the probe samples fluidized or immobilized environments, respectively. Since the ESR spectra indicate that *I(m,n)* probes primarily sample the lipid phase of biological membranes, the S may be viewed as a measure of the bulk lipid fluidity. Increasing the distance of the oxazolidine ring from the carboxyl terminus of *I(m,n)* enhances the flexibility of the reporter group and decreases S. Consequently, the carboxyl group is relatively anchored to the polar surface, while the more mobile methyl terminus lies within the membrane interior.[10,11] *I(m,n)* and steroid spin labels also execute rapid translational diffusion in model and biological membranes at physiologic temperature.[2] The use of either extrinsic fluorescence probes or nuclear magnetic resonance (NMR) spectroscopy has indicated results broadly in support of those obtained with spin labels (however, see following text[2]).

The proposal by Singer and Nicolson[3] that integral membrane proteins may be tightly associated with endogenous lipid has been verified with ESR spectroscopy. Spin-label studies on reconstituted lipid-protein mixtures demonstrated that enzymes such as cytochrome c oxidase[12,13] or Ca^{2+}-ATPase[14] immobilized a certain percentage of probe, leaving the remaining label in an environment characteristic of a fluid-lipid bilayer. These integral proteins are apparently surrounded by an immobilized layer of boundary lipid (or "annular lipid"). Annular lipid would be expected to interact with the protein by van der Waal's interactions and hence exchange with the bulk lipid pool at a slower rate than exchange within the bulk pool itself.[14] One can envisage that the primary function of such a lipid annulus is to seal the protein in the bilayer and that the annulus may, depending upon the protein, segregate specific lipid species while excluding others.[14] The presence of annular lipid may explain why integral proteins do not greatly perturb the bulk lipid phase transitions in biomembranes.[7]

C. Evidence Contradicting the Fluid Mosaic Model

Despite the success of the fluid mosaic model in interpreting the above experimental results, more recent studies suggest that this model is oversimplified in that lipid domains of differing structure and/or fluidity may coexist in biological membranes. For example, phospholipids are asymmetrically arranged about the outer and inner lipid layers of the surface membranes of intact cells, with negatively charged (acidic) lipids predominating in the cytosol half of the bilayer.[15] Distinct clusters of phosphatidylserine and phosphatidylethanolamine have been detected in erythrocyte membranes with the aid of cross-linking agents.[16] Furthermore, electronmicroscopic investigations on such biological membranes as rat liver plasma membranes indicate that constituent cholesterol is not randomly distributed.[17] The existence of cholesterol-rich and -poor lipid domains is consistent with the fact that specific phospholipids showing high affinity for the sterol may achieve a lateral segregation of cholesterol in the bilayer.[2]

Contrary to the fluid mosaic model, all of the lipids in biomembranes do not necessarily exist in the same fluid state at physiologic temperatures. Although natural abundance ^{13}C-NMR studies indicate that a significant fraction of lipid in biomembranes undergoes rapid translational diffusion, these experiments also demonstrate that most membrane lipids are immobilized having rotational correlation times much longer than those of incorporated spin probes.[2] Jain and White[2] have criticized the exclusive use of extrinsic spin or fluorescence labels to characterize the bilayer fluidity, since these probes may either concentrate in domains of "high" fluidity or perturb to some extent the organization of neighboring components. In fact, evidence that discrete lipid do-

mains coexist in biological membranes has been provided by more recent spin-label experiments. Tanaka and Ohnishi[18] reported an asymmetric fluidity for intact erythrocytes labeled with various classes of phospholipid spin probes, in which the outer bilayer half is more rigid than the inner half. The coexistence of strongly immobilized and fluid lipid components has also been noted in a number of spin-labeled membranes.[13] Lastly, examination of the segregation of *I(m,n)*, as revealed by enhanced radical interactions, suggests that restricted lipid domains occur in erythrocytes,[19] liver and heart,[8,9] and lymphocyte[20] plasma membranes.

The mere fact that the main order → disorder lipid transitions of biological membranes frequently occur at low temperatures does not rule out the presence of distinct lipid clusters or domains at physiologic temperatures. "Breaks" in Arrhenius plots of the motional properties of spin labels incorporated into such membranes as mitochondria, dioleoyllecithin-substituted Ca^{2+}-ATPase, and *Bacillus stearothermophilus* have been reported at temperatures well above the "bulk" melt.[7] Lee[21] attributed this behavior to the formation of "quasicrystalline" clusters (*QCC*) in a liquid lipid (*L*) matrix; *QCC* were viewed as having both molecular densities and fluidities in between that of freely-dispersed *L* and solid lipid (*S*) domains. The presence of *QCC* would not necessarily be detected in diffraction or DSC experiments, if such structures are short lived or associate on the basis of weak interactions.[2,7,9,21]

Another example of discrete lipid domains existing at temperatures above the main lipid transition has been recently reported for rat liver plasma membranes. A lipid phase separation has been identified in *I(12,3)*-labeled liver membranes at temperatures less than 28°C by examining Arrhenius-type plots of S and empirical parameters sensitive to probe-probe interactions.[8,9] This phase separation was attributed to the formation of *QCC*, inasmuch as electron diffraction studies detected no *S* at temperatures exceeding 18°C.[9] Since rat liver plasma membranes have a relatively enhanced cholesterol content,[22] it seems likely that the high temperature onset at 28°C involves the formation of cholesterol-rich *QCC* and cholesterol-poor *L*, such that the *I(12,3)* probe is restricted to *L*. Several model studies on lecithinolesterol mixtures indicate that short lived, cholesterol-rich clusters may laterally segregate in the bilayer.[9]

Either the main lipid order → disorder transition or the formation of *QCC* may inhomogenously distribute integral membrane proteins. Although there are exceptions, it seems to be a general rule that penetrant proteins are sequestered from *S* lipid during a main lipid phase transition.[7] *QCC* at temperatures well above the "bulk" melt in such membranes as *B. stearothermophilus* and the endoplasmic reticulum of *Tetrahymena pyriformis* also segregate membrane proteins.[7] Moreover, recent reports on model and biological membranes indicate that integral proteins preferentially accumulate into cholesterol-depleted domains.[22] It is tempting to speculate that the penetrant proteins of rat liver plasma membranes may be excluded from cholesterol-rich *QCC* at temperatures below 28°C.[22] Obviously, the fluid mosaic model will not provide an entirely satisfactory description of a biological membrane if *QCC* capable of segregating integral proteins are present.

D. The "Plate Model" of Membrane Structure

The findings of the previous section are not readily accommodated by the fluid mosaic model, and Jain and White[2] have proposed a new model in which the biomembrane continuum is broken up into a number of ordered regions that are not only in motion with respect to one another, but also are separated by relatively disorganized regions. The ordered and disordered domains, or "plates", are viewed as being contiguous and in equilibrium. The various physical characteristics of these plates have yet to be defined, but it is conceivable that their maximal size may be several thousand molecular diameters.[2] Although the "plate" model appears to be more accurate than the fluid