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MEMBRANES AND CHANNELS

PHYSIOLOGY AND MOLECULAR BIOLOGY

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INTRODUCTION

Most of the papers in this book discuss the properties and roles of channels in membranes, and the methods needed to investigate them. Work on channels has evolved (in large measure) from older work on the properties of membranes themselves. Since channels are the major pathways for solute movement, the mechanism of solute movement can best be investigated when channels are embedded in as simple a membrane as possible, attached to as simple an apparatus as possible. Indeed, that is why single channel measurements, as described in several chapters in this book, have created such excitement and are so promising for the future of membrane biophysics.

Membranes, however, are not just structures in which interesting channels are found. The structural organization of membranes and nonuniform distribution of channels within membranes are also of considerable biological interest since both play an important role in tissue function. This paper presents a systematic method for analyzing complex membrane architecture. In

particular, I consider in detail the role of restricted extracellular spaces, formed by infolded membranes. From the electrical point of view, these spaces produce a distributed resistance in series with most of the plasma membrane. From the electrochemical point of view, these spaces provided a restricted diffusion space (an 'unstirred layer') in which physiological current flow produces significant changes in solute concentration. I argue that the biological consequences of restricted electrodiffusion are profound and are likely to be widely exploited in the function of many cells and tissues.

While working on this paper, and thinking about the role of cell structure in cell function, I attended meetings on protein structure in honor of Dr. M. Perutz and Prof. J.T. Edsall, the latter my tutor at Harvard College. Both meetings had many lectures devoted to structure/function relations of protein molecules and I was struck by the similarity of these molecular questions, discussed in the language of chemistry, to questions concerning structure/function relations of cells and tissues, usually discussed in the language of physiology. This similarity seemed hardly a coincidence and led to the discussion presented in the second section of this paper. I argue there, in a rather philosophical way, that the investigation of biological systems has a certain unity, whether those systems occur on a length scale of nanometers or centimeters. The unity of biological questions is in contrast to the diversity of the physical sciences, where the nature of the systems and scientific investigation depends profoundly on the length scale being studied. The properties of physical systems are quite distinct, requiring quite different experimentation and physical description if they are elementary particles (on a length scale of femtometers), atoms (picometers), molecules (nanometers), everyday objects (centimeters), or astronomical objects, ranging from solar systems (10^{12} meters), to

galaxies (10^{21} meters), to the universe (10^{26} meters). The mathematical laws describing physical phenomena on these different length scales seem more similar than they really are: when predictions of practical experimental results are made, the different distances scales require quite different treatment of the same 'unified' equations.

I believe biological systems have a great deal of unity because they are all built by the same evolutionary process, the the processes of evolution are much the same no matter what the size of the resulting system. The properties of systems built by evolution seem to be closely related to the 'chaotic' outputs of simple mathematical models used to describe idealized evolutionary systems (May, 1976). I argue that only certain questions can be profitably and efficiently asked of chaotic systems: "What is there?" "What does it do?" "How does it work?" "How did it evolve?" These questions seem to me to be the central ones confronting biologists, no matter what length scale they study.

PART 1 - RESTRICTED EXTRACELLULAR SPACES

Role of extracellular spaces. We begin with a discussion of one aspect of membrane architecture, namely the role of the narrow extracellular spaces commonly found in nerve and epithelia, skeletal and cardiac muscle.

The chemical consequences of extracellular spaces occur because the fluxes of ions across membranes can drastically change the concentration of ions in the space outside membranes. This change in solution composition can change both the properties of channels and the ions which flow through them. Thus, the flow of ions through channels can be regulated by the geometry of the

extracellular space as well as by the intramolecular properties of the channels themselves. In this respect, the accumulation of potassium and depletion of calcium in extracellular spaces will be most provocative, since the changes in concentration of these ions is large and the physiological role of these ions is considerable. In the case of potassium, changes in concentration would modify the 'resting' potential of neighboring cells, modifying all the processes which depend on that voltage. In the case of calcium, changes in concentration would modify the calcium current through calcium channels; it would modify the conductance of channels to other ions; and it would act as a signal to the various membrane enzymes which use extracellular calcium as a trigger signal.

Consider the particular case of cardiac muscle. The natural cardiac function, the cardiac output of blood flow, is closely coupled to the duration of the ventricular action potential. Changes in concentration predicted in narrow extracellular spaces between ventricular cells are likely to change the duration of the action potential. Thus, cardiac output is likely to be regulated by the properties of an extracellular space, as well as by the properties of membrane channels.

Despite these speculations, relatively little attention has been paid to the functional role of extracellular spaces, probably for good reason. They are hard to study, they are not molecular in nature, and their function is not evident. Indeed, much of the progress in physiology in this century has occurred in the study of those tissues without prominent structural complexity. The classical physiological 'preparations' of axons, red blood cells, and neurons (at least in their invertebrate peripheral idealization) may actually have been chosen because of their relative simplicity. The preparations of interest here

(epithelia, cardiac and skeletal muscle) may have been avoided because of their complexity.

One of the classical physiological preparations bridges the gap between the appalling complexity of syncytial structures, like epithelia, and the appealing simplicity of the squid axon membrane. Skeletal muscle fibers have extensive structural complexities, and yet of a periodic kind. These cells are nearly crystalline in the regularity of their structures. Their membranes form a complex network of tubular invaginations, yet these networks occur (to a good approximation) in a regular repeat. Indeed, the complexity of the network occurs at such a small length scale that it can be averaged away, as a statistical mechanic averages over quantum mechanical states he cannot accurately describe, leaving macroscopic properties relatively easy to deal with.

It was through the study of skeletal muscle that a systematic procedure for analyzing structural complexity was created (by Falk and Fatt, 1964; the role of morphological measurements was clearly stated by Mathias et al., 1977) and the entire procedure was generalized and described in Eisenberg and Mathias (1980).

That procedure has now been applied to a number of tissues and cells, most notably syncytial tissues like heart (e.g., Levis, et al., 1983) and epithelia like the lens of the eye (Mathias et al., 1979), the gall bladder (Clausen et al., 1979; Schifferdecker and Frömter, 1978) or gastric mucosa (Diamond and Machen, 1983). It has proven reasonably successful. The procedures have not yet been applied, at least in any systematic way, to their most obvious target, neurons of the vertebrate central nervous system. Here we must expect a structural analysis to be a prerequisite to

the understanding of neuronal integration, the methods by which neurons compute their outputs from their many inputs.

Structural analysis of electrical properties. Structural analysis provides a systematic procedure to analyze tissues and cells with complex membrane architecture. Structural analysis starts with a description of the topology of membranes, which corresponds closely to the mind's eye view of a tissue presented as line drawings in textbooks. The analysis proceeds to measure the amounts of membranes and the spaces they enclose, often using the techniques of stereology. The books of Weibel (1981, 1982) and the papers of Brenda Eisenberg and co-workers (1983) provide details of theory and applications in this area. Suffice it to say here that the problems of measurements of biological structure can be considered solved, at least the measurement of the volumes of cells and extracellular spaces and the measurement of surface areas of membranes. But we must remember that the problem of defining shape quantitatively -- which is part of the problem of pattern recognition outside the human nervous system -- is a formidable task. The problem of measuring that shape is even harder. Both problems may be greatly simplified if attention is paid to only that subset of structures which can arise from self-replicating cells (Dormer, 1980, and Meinhardt, 1979, discuss some of the consequences of self-replication from a morphological and mathematical perspective, respectively).

Tissue preservation. An outstanding problem in structural analysis, indeed in all of morphology, is that of tissue preservation. No methods are known which preserve both physiological structure (i.e., the volume of cells and extracellular spaces) and fine structure as observed in the electron microscope. Chemical fixation takes far too long, and involves too many unknown reactions, osmotic effects, and possible

artifacts, to allow confidence in the resulting image of physiological structure.

Rapid freezing better preserves the physiological structure of cells, provided the structures are not more than some 10 μm from the cooling block. But even close to the cooling block, freezing is not rapid enough to guarantee preservation of the volumes of small structures in which volume could change significantly in milliseconds. And, of course, not all fine structure is accessible to this technique.

New techniques of tissue preservation are clearly needed if structural analysis is to fulfill its biological potential. One possibility involves the use of bifunctional photoaffinity reagents to effect light-activated fixation. Consider a molecule which is membrane permeable and biologically inert prior to irradiation. If the compound became a potent protein cross-linker only after absorption of light, one might be able to obtain rapid fixation and good preservation of physiological and fine structure. Admittedly, the chemical and biological requirements which must be satisfied to make this approach useful are many. The compounds must be membrane permeable to very high concentrations, biologically non-disruptive and non-toxic at these concentrations and they must, upon irradiation, cross link with high efficiency and speed. Of course, cross-linking might take milliseconds, being limited not only by the chemistry but also by the time until Brownian motion brings the relevant proteins close enough together to allow cross-linking to occur. But, cross-linking on even that time scale would importantly increase our capabilities. In fact, I suspect that fixation might be quite rapid because I guess that all fixatives fix by cross-linking soluble proteins to nearby cytoskeleton, although this guess should be clearly identified as the unsupported speculation it

really is. If the cytoskeleton is within a few nanometers of all cell proteins, collisions between it and soluble proteins will occur very often, and photoactivated fixation might be just as rapid.

In collaboration with Jeanne Nerbonne (of Cal Tech), we are presently considering suitable bifunctional photoaffinity reagents which could be utilized for this purpose. The compounds of Mikkelsen and Wallach (1976), and other commercially available reagents seem a logical starting point.

Theoretical predictions. The anatomical description, topological and morphometric, is not too useful biologically until its functional consequences can be predicted. That is the role of theory. A mathematical model of the measured structure is needed to predict the electrical properties expected from that structure, with the minimum of assumptions. The prediction, of course, must involve some assumptions, because of mathematical difficulties and anatomical complexities, and also because the mathematical model includes unproven assumptions of membrane properties. Indeed, to make the analysis tractable, we often must assume that the membrane properties of interest are linear, i.e., independent of voltage or other driving forces.

The assumption of linearity. Although excitable membranes are certainly nonlinear over the extended range in which they function, an assumption of linearity is not as restrictive as it seems. The assumption allows analytical solutions of our mathematical models, analytical solutions which depend quite simply on membrane architecture, on the extracellular spaces and membrane capacitances, and thus give qualitative understanding of the electrical properties of the tissue or cell. In fact, these analytical solutions can often be cast as equivalent circuits

(with no loss of accuracy) and the power and intuition of circuit analysis thus can be applied to our complex biological tissue, without loss of rigor. This simplification to an equivalent circuit can only be done (in an accurate way, with known mathematical error) when the circuit is linear.

The assumption of linearity is also necessary because it allows a drastic reduction in the number of free parameters in our model. Under the condition of linearity the detailed properties of channels have a much smaller role in determining electrical properties than they do in general. Thus, our ignorance of these detailed channel properties will have a relatively minor effect on our analysis of the properties of the extracellular space. Finally, the assumption of linearity is also more biologically relevant than it may seem to workers on excitable membranes. Most cells have a substantial range of voltage over which their properties do not change. Thus, a linear analysis of electrical properties is also an analysis of the properties of cells in a physiologically relevant state.

Properties of channels do influence linear properties, of course; they determine the resting conductance of membranes and they may have a role in determining a component of membrane capacitance as well (see Bezanilla et al., 1982; Fernandez et al., 1982, and papers cited there). These linear channel properties are but a small subset of all channel properties. They are characterized by many less parameters than the full scale behavior of the channel over a wide range of voltage.

For these reasons I emphasize the analysis of the linear properties of cells and tissues. This linear analysis must, of course, be extended to include the full nonlinear properties if we are to confront much of biological interest. However, the

nonlinear analysis cannot be seriously undertaken until the structural analysis is complete, until the role of extracellular space and membrane capacitance is established.

Membrane models. The analysis of linear properties requires a model of a small piece of each homogeneous membrane. Historically, the membrane was usually represented as an ideal resistor in parallel with an ideal capacitor, although more complex models are sometimes used (see the article by Mathias in this volume). With the discovery that a large component of membrane capacitance is markedly nonlinear, varying with both voltage and time (Schneider and Chandler, 1973; Armstrong and Bezanilla, 1974), this assumption is no longer self-evident; indeed, it is obviously unjustified! Fortunately, analyses in the literature (assuming linearity) can still be used, if experiments are done in a narrow range of voltages. The full frequency dependent properties of the membrane capacitance can be included in the structural analysis if an empirical expression for the properties of the capacitance is known. This expression can then be substituted for the appropriate capacitance variable in the expressions already derived.

The voltage dependent properties of the membrane capacitance can be easily accommodated only if experiments are done over a narrow range of voltages, in which an accurate expression for the frequency dependent properties is known. If the experiments are done over a full range of voltage, the general applicability of a circuit approach is not clear. Some circuit modelling of the nonlinear capacitance may be useful (see, for example, the appendix of Mathias et al., 1980, where a circuit model is used to describe the properties of nonlinear capacitance of skeletal muscle fibers), but one must be skeptical of the general utility

of the approach. In the case of steep voltage dependence, one probably must resort to full scale simulations.

The complications caused by the frequency and voltage dependence of membrane capacitance do not change the goals and need for structural analysis, but the strategy and tactics are changed, and the execution of the analysis is made more difficult. At the present time, for example, such a structural analysis, including the voltage and frequency dependence of membrane capacitance has not been completed, even for the simplest preparation, squid axon, because the best measurements there (Bezanilla et al., 1982; Fernandez et al., 1982) still depend on the series resistance in a complex manner not fully understood experimentally or structurally.

Despite these reservations, we now proceed with a traditional linear structural analysis, following the time honored human behavior of activism; it is better to proceed imperfectly, than to sit paralyzed, waiting for perfection.

Field equations and cable theory. Using the assumption of linearity, structural theory derives field equations (which are a combination of conservation laws and Ohm's law) and a boundary condition describing each homogeneous membrane. These field equations are of varying degrees of complexity, depending on the problem at hand. Jack et al., (1975) provides the best physiological introduction to the one dimensional case -- traditional cable theory. Three dimensional field equations in single cells, or a tissue which can be approximated as a single cell, are described in Eisenberg and Johnson (1970), Adrian et al. (1969b), Peskoff and Eisenberg (1973, 1975), and Peskoff et al. (1976).

Three dimensional field equations for syncytial tissues, consisting of many cells electrically coupled one to another, are described in Eisenberg et al. (1979), where a historical discussion of earlier work with an intracellular perspective is presented. Syncytial tissues have been analyzed from an extracellular point of view by Miller and Geselowitz (1978), and Plonsey and Rudy (1980) and earlier workers cited in those papers.

Perturbation analysis. The field equations for both the single cell and syncytial problems appear formidable, particularly when coupled with the appropriate 'membrane' boundary condition (derived in Peskoff and Eisenberg, 1975), which is a mixture of the Dirichlet and Neumann boundary conditions extensively analyzed in texts of potential theory. Nonetheless, these problems admit of pleasingly simple solutions if the techniques of singular perturbation theory are used. Perturbation methods systematically exploit small parameters which appear in differential equations and frequently permit drastic simplifications of forbidding problems. These simplifications correspond to problems of reduced complexity equivalent to classical problems familiar to experimental workers. Kevorkian and Cole (1981) is a quite complete presentation of perturbation techniques and is accessible to those with only a nodding acquaintance with partial differential equations. Kevorkian and Cole also includes a discussion of potential spread in spherical and cylindrical cells.

Perturbation theory is particularly well suited for problems involving membranes, because a small parameter appears naturally in the boundary conditions for these field equations. The membrane resistance is always very large compared to the resistance of the intracellular or extracellular solution (when written in comparable units). The biological function of membranes, their evolutionary reason for being, is to isolate the

interior of cells from the external world. Membranes isolate the interior of cells by preventing the flow of water and solutes which might perturb the cell interior. Thus, membranes by their very nature have high 'impedance' to all natural flows.

The small parameter exploited by singular perturbation theory is just the mathematical expression of the biological role of membranes (Peskoff and Eisenberg, 1973). The small parameter of the membrane boundary condition is the ratio of the 'conductance' of the membrane to the 'conductance' of the intracellular solution (both expressed in similar units). This ratio is small because the membrane always presents a greater impediment to flow than the cytoplasm.

Another small parameter arises naturally in field problems describing syncytial tissues. Those tissues usually consist of two interdigitating media, the intracellular space, which occupies the great majority of the tissue volume, and the extracellular space, which occupies a tiny fraction of the volume. The ratio of these two volumes is then a small parameter which can be exploited in perturbation expansions.

Circuit diagrams of tissues and cells. A successful perturbation analysis of biological field equations produces a set of reduced equations which usually are identical to the equations describing a circuit model of the tissue or cell. This circuit diagram resembles the circuit diagram one might draw directly from the topology of the tissue or cell, without the intervening theoretical trek. Indeed, one's first impression may be that perturbation theory has provided small dividends, given the investment of effort. But first impressions can be misleading -- the historical fact is that few tissues have been so clearly perceived by investigators as skeletal muscle was by Falk and

Fatt, 1964. They did in fact deduce the correct circuit (including point source effects and correction for capacitive artifact) without theoretical analysis. Even in that case, however, the morphometric parameters were not correctly included. It took much later work to describe the effects of branching (Adrian et al., 1969a; Schneider, 1970) and wiggling of the tubules (Mathias, 1975; Mathias et al., 1977; Eisenberg et al., 1977; Mathias, 1983) and it is still not certain that the random branching network of tubules has been completely analyzed or described.

In the case of syncytial tissues, current flow in two interdigitating media, including flow across the membranes which bound the tissue, was incompletely analyzed many times (see historical discussion in Eisenberg et al., 1979) before perturbation expansions were used to resolve the issue.

The reasons for the difficulty in drawing an equivalent circuit, without perturbation analysis, is seen if one looks at the mathematical results of the perturbation analysis in all but the simplest cases. Perturbation analysis was introduced into applied mathematics because most problems were too complex to allow successful approximation without a systematic formalized procedure. It is possible to guess simplified forms of complex problems, and sometimes correction factors for those simplifications, by intuitive methods involving systematic approximation. But it is difficult to avoid logical inconsistencies; it is difficult to include all correction terms of equivalent size; and it is particularly difficult to convince skeptical colleagues of the validity of such approximations. For these reasons, as well as for reasons of rigor, the methods of perturbation theory are needed in biological problems.

Thus, the drawing of a correct circuit representation of a tissue seems to require a theoretical analysis, starting with field equations, exploiting small parameters with singular perturbation theory. Only then, after this tedious but necessary process can a simplified representation of the tissue be convincingly derived.

Measurements of electrical properties. Once the circuit diagram is available to describe the electrical properties of the tissue or cell, sensible experiments can be designed to check the analysis. If the analysis survives experimental check, the linear properties of the different components of the tissue can then be measured. These measurements will at first be made under one set of conditions; indeed, at the present time surprisingly little work has been done under multiple conditions. We will return to this subject later and argue that measurements under multiple conditions are an essential component of structrual analysis and must be performed, if the approach is to be fully exploited. But first things first.

Two classes of measurements have been widely used, measurements either in the time or frequency domain. Measurements in the time domain have obvious advantages. 1) The required equipment is ordinarily available in the electrophysiologist's laboratory. 2) They appear to require no specialized knowledge of circuit theory. 3) The methods can be directly generalized to the non-linear time varying properties which are the biological functions of greatest interest. These complex properties can be recognized and dealt with in traditional and well precedented (hopefully correct) ways: the presence of non-linearities need not corrupt the rest of the analysis.

There are also disadvantages to time domain measurements. 1) Theory in the time domain is in fact often quite difficult, invariably leading to expressions considerably more involved (i.e., convolved) than those in the frequency domain. The output of most systems can only be described by convolution integrals difficult and expensive to compute, and harder to understand, in a qualitative or physical way. 2) Time domain data is notoriously insensitive to different topologies or circuit values in the underlying circuits. For both reasons parameters of individual subsystems (membranes or regions of extracellular space) are often hard to determine uniquely from time domain data. While the last sentences describe widely known 'facts' (see citations in Eisenberg and Mathias, 1980), I realize that the facts may turn out to depend on unconscious assumptions. At the present time, it is not known whether the difficulties in determining circuits and circuit parameters from transient responses are inherent to the mathematics of time domain analysis, or could be remedied by changes in the numerical procedures of curve fitting. Resolution of this problem is eagerly awaited.

Frequency domain methods have advantages and disadvantages which are almost the inverse of those of the time domain. The advantages are: 1) The theory involved is much simpler since convolution integrals are replaced by multiplication or division. 2) Circuit topology and parameters are determined far more precisely for data of (apparently) equivalent accuracy. The disadvantages are: 1) Specialized equipment is needed. 2) The theory, while relatively simple, nonetheless uses complex variables which are not familiar to experimental biologists. 3) The inclusion of time varying and voltage dependent processes requires considerable analysis (see paper of Mathias in this volume) and it is not yet clear how useful or intuitive the frequency domain will be in this case.

A potentially useful method of analyzing time domain data can avoid some of the difficulties of curve fitting. If time domain responses are integrated, they can be directly related to linear properties as usually measured in the frequency domain. This method holds much appeal but it is not yet clear whether improved methods of fitting the transient response will not remove that appeal.

Location of measurements. We now turn to the question of where measurements are made. It is clear that measurements made at just one location, of the 'input' impedance, as it is often called, have inherent constraints. Indeed, many circuits and structures of physiological interest have membrane properties which are in principle unmeasurable when observed from just one location under one set of conditions. Membrane properties cannot be measured in those cases for the same reason that the properties of two resistors in parallel (or series) cannot be measured from measurements at a pair of terminals made under one set of conditions: there are more parameters than there are independent observable quantities.

The way to break this constraint is to take measurements at different locations or under different conditions. If the topology, and perhaps some of the circuit parameters, can be assumed to be unperturbed by changing these conditions (or the location of measurements), a great deal of useful information can be derived. For example, Valdiosera et al. (1974a,b) used this method to check models of current flow in the t system and sarcoplasm of muscle fibers. Mathias et al. (1981) used this method to measure the radial variation of internal resistance in the lens. And related techniques have been used in analyses of epithelia to separate the properties of apical and basolateral membranes.

A particularly useful intervention, which can often be arranged to perturb the properties of the tissue in a known way, is to decrease the conductivity of the bathing solution by replacing sodium ions with osmotically equivalent amounts of an impermeant solute, like sucrose. This procedure should have little effect on membrane potential, no effect on membrane capacitance, and a linear effect on extracellular conductivity. (That is to say, the conductivity of extracellular spaces should be proportional to that of the bathing solution). Changes in extracellular sodium concentration should have minimal effects on membrane conductance -- none, if the membrane is impermeable to sodium and the concomitant change in ionic strength is not too severe.

If measurements can be made on one preparation bathed in solutions of different conductivity, a great deal of additional information should become available. For example, with this approach it should be possible to tell if the complex properties ascribed to the membrane capacitance of squid axon are in fact properties of the membrane or might be properties of an impedance in series with the membrane, arising in unanalyzed structural complexity.

Measurements at different locations are certainly required if systems of considerable complexity are to be studied. Consider, for example, epithelia, consisting of a series combination of membranes. It is hard to believe that enough information will ever be available from transepithelial measurements to determine individual membrane properties (Clausen et al., 1979). Rather, one must place an electrode within the epithelial cells, and perhaps within the lateral intercellular space between cells, if one is to measure all physiologically interesting parameters independently (Schifferdecker and Frömter, 1978).

Structural analysis of neurons. An equivalent problem arises in the analysis of dendritic trees of neurons. Here the cell body is so remote from the dendrites in which interesting integration (i.e., spread of current and potential) occurs that measurements of just input impedance are unlikely to allow unique analysis. Rather, one must seek measurements in the periphery of the dendritic tree as well as in the cell soma. Such measurements can in principle be made by microelectrode penetration, or localized breakdown of dendritic membrane held to the microelectrode by a seal with gigohms of resistance. It seems more likely, however, that optical methods of measuring potential, which would (in principle) allow measurements anywhere in the dendritic tree, will be needed to deal with this problem.

Despite many difficulties, and many anatomical, theoretical, and electrical unknowns, I am confident that all the techniques are already developed to perform a structural analysis of the spread of potential in the dendrites of neurons, particularly neurons in hippocampal slices. Such an analysis will probably be the most significant application of the structural analysis advocated here, because an analysis of spread of potential in dendrites is an analysis of the mechanism of integration in neurons. In other words, structural analysis of dendritic trees is a mechanistic analysis of decision making in the nervous system.

The task will not be simple, however, because the integration of so many techniques into one project remains a combination of motivation, skills, and resources not easy to put together. Furthermore, one must anticipate that interesting aspects of dendritic integration will be mediated by the specific location of channels with specific properties within the dendritic tree. Thus, nonlinear properties will come to the forefront. Certainly,

these cannot be investigated without a previous structural analysis of the underlying linear properties. But it is equally certain that investigation will require direct measurements of channel properties and location (with patch electrode techniques, labelling with monoclonal antibodies, or labelling with specific toxins and pharmacological agents) and direct confrontation of nonlinear simulations with nonlinear experimental measurements.

Summary. This part of this paper has described a systematic approach to the electrical properties of tissues and cells of complex structure. Obviously, this approach has weaknesses as well as strengths. Ad hoc methods (suitable for specific measurements in specific tissues) are often more efficient. Our treatment of complex membrane capacitance and nonlinear membrane properties is obviously incomplete enough to be called unsatisfactory.

Nonetheless, the questions posed by structural analysis remain among the most significant in physiology, at least in my view. We cannot expect to know how a tissue or cell works unless we can synthesize its natural function using the properties of its molecular components. Surely we need to know the properties of the channels which are the most significant molecular components; surely we must also know the arrangement of the channels and membranes which convert the flow of current through one channel into the biological signals and flows of direct functional significance.

Electrical role of restricted extracellular space. The structural analysis of electrical properties is in large measure an analysis of restricted extracellular spaces near and within cells and tissues. These spaces provide the series resistance which isolates one part of a membrane from another, making them

electrically and (perhaps) functionally distinct. The widespread presence of these spaces in so many tissues suggests a common theme to their existence. Do they mediate a common function in all the cells in which they are found? Are restricted extracellular spaces an evolutionary adaptation with a universal function?

To some extent the answers to these questions are obvious, to some extent, obscure. Obviously, restricted extracellular spaces are formed by membranes; thus one obvious general function of these structures is to increase drastically the area of membrane and the number of channels imbedded in the membrane. The significance of this adaptation should not be minimized. It may in fact have been the original evolutionary reason for the existence of restricted extracellular spaces.

In excitable cells, particularly skeletal muscle and the conduction systems of the heart (i.e., sheep Purkinje strands), tubules or clefts serve another purpose directly involved in one of the main functions of the tissue, the conduction of the action potential. In both of those tissues the resistance of the restricted extracellular space serves to isolate the capacitance of the cleft membrane from the surface membrane, particularly when the voltage is changing rapidly, as during the foot and upstroke of the action potential. Because of the extracellular resistance, the sodium current flowing across the surface membrane need depolarize only a small fraction of the total membrane area of the cells. The sodium current (which is limited by the number of sodium channels) is thus able to depolarize the surface membrane much more rapidly than otherwise. Furthermore, at any given time the current can flow a further distance longitudinally down the fiber. For these reasons, the conduction velocity of the action potential in skeletal muscle fibers or Purkinje strands of the

sheep heart is much greater than it would be without the resistance of the restricted extracellular spaces. Of course, this argument assumes the prior existence of the extracellular space. If those spaces did not exist at all, the conduction velocity would be still higher. Thus, the resistance of the extracellular space serves a useful function given their existence, but their existence does not seem to be justified by the resistance itself.

In the case of skeletal muscle fibers, the restricted extracellular space exists for an obvious purpose. It is a necessary property of the tubular system which itself is an essential link in the chain of mechanisms called excitation contraction coupling. This subject is well reviewed in Costantin (1975) and in a forthcoming volume of the Handbook of Physiology (Peachey and Adrian, 1984).

In the case of cardiac muscle, the purpose of the restricted extracellular space is not so obvious. Excitation contraction coupling does not seem to require its existence, so the arguments used for skeletal muscle fail in this tissue. It seems quite possible that the extracellular space of cardiac muscle is a necessary consequence of the syncytial nature of the tissue. Cardiac muscle consists of many cardiac myocytes coupled together, which have not fused early in development, as the myocytes of skeletal muscle do early in embryonic life. The reason for the lack of fusion is a most interesting question, perhaps arising from the need for specialized conduction pathways or from the need of the heart to function even after some cells are injured or die (as suggested to me by Brenda Eisenberg). In any case the absence of fusion immediately implies the existence of extracellular spaces of the type discussed here.

The electrical role of restricted extracellular spaces in other tissues is not so clear; and so we now turn to another class of physiological phenomena, involving both diffusion and current flow, seeking a general significance to the role of restricted extracellular spaces. We will find the electrodiffusion in these spaces has profound consequences which may be central to the functioning of many tissues, skeletal muscle, cardiac muscle, and epithelia.

Electrodiffusion. The analysis of the action potential by Hodgkin and Huxley, which is the paradigm for so much later work in electrophysiology, exploits the difference in time scale between conductance change in the squid axon membrane and the concentration change in its environs. Speaking loosely, the conductance changes, which control and are controlled by membrane potential, take 1-2 milliseconds, whereas the concentration changes which accompany the flow of ions through the membrane take 2-10 milliseconds before they are of comparable effect. The separation of these two time scales is essential if membrane properties are to be analyzed with the assumption of a constant gradient of chemical potential, that is to say, with the assumption that the concentrations of permeant ions on each side of the membrane are reasonably constant.

The analysis of conductance changes (i.e., channel properties in more modern languages) is much more difficult if the time scale of concentration change overlaps the time scale of conductance change. In that case, experiments and analysis must be designed to measure and understand both the concentration change itself and the consequent effects on channel properties.

Necessity and size of concentration change. It is important to remember that concentration changes must accompany current flow

carried by ions. The only questions are "How big is the concentration change?", "What is its time scale?", and "Are the size and time scale relevant for natural function or biological experimentation?"

A simple general expression for the initial rate of change of concentration is given in Levis et al. (1983):

$$\left.\frac{d[X]}{dt}\right|_{t\to 0} \cong 10^3 \quad \frac{V_F}{V_c} \quad \frac{S_c}{V_F} \quad \frac{g_X}{nF} \quad (U-E_X)$$

where the permeant ion X, of charge n, is at concentration [X], with equilibrium potential E_X. The volume of extracellular space is V_c in a tissue volume of V_F. The membrane lining that space has area S_c with a transmembrane potential of U. The conductance of 1 cm^2 of the membrane is g_X; F is the Faraday constant (96,500 coulombs/mole); and the factor 10^3 reconciles chemical units of moles/liter and spatial units of cm. Typical values (for calcium in the clefts of sheep Purkinje strands) would be $g_X \cong 100$ $\mu S/cm^2$; $U - E_X \cong 100$ mV; and morphometric parameters giving a cleft width $w \cong 30$ nm, where the cleft width $w = 2(V_c V_F)/(S_c V_F)$. The resulting initial rate of change of calcium concentration would be ~33 (mmole/liter) per second.

This approximate calculation immediately shows the significance of changes in concentration of permeant ions, in the presence of small extracellular spaces. Extensive numerical simulations (see, for example, Levis et al., 1983) show that the initial rate of change of concentration is maintained for a reasonable period of time (10-100 msec in that case); thus, the transmembrane flow of ions has a drastic effect on concentration, at least in the case just cited.

This is not the place to summarize the literature on ion concentration changes (some citations are in Levis et al., 1983); nor is it the place to argue the significance of these changes for each tissue or functional phenomena of interest. Rather, the above calculation should serve as a warning that the assumption of constancy of ion concentrations is inherently implausible and must be justified for each current flow across each membrane.

The implications for interpretation of typical voltage clamp experiments are striking. Interpretations assuming constant concentration gradients are likely to be misleading, certainly quantitatively, and probably qualitatively. Considerable effort must be taken to design experiments to control and/or measure the concentration of permeant ions. Theoretical extrapolations from idealized data to realistic situations must include the possibility of concentration changes.

Functional implications of concentration changes in extracellular space. The implications for tissue function are as great as the implications for the analysis of experiments. It seems inherently unlikely that the tissue will share the view of traditional physiology that such concentration changes are an artifact, an unavoidable side effect of important channel properties. It seems far more likely that such large changes in concentration will be used by the tissue for its own purposes, to modulate or even control its natural function.

Consider, for example, the case of calcium ion in the clefts or tubules of muscle fibers. Concentration changes of the size just derived would have profound effects on the conductances of all ions of interest, since calcium concentration has been found to modulate almost all the channels studied to date. Thus, changes in calcium concentration, and the properties of the

extracellular space, would modulate the channel properties themselves.

The concentration of ions in clefts and tubules would also be modulated by 'active' transport systems, deriving energy from ATP (i.e., the Na/K pump) or from concentration gradients of ions (i.e., the Ca/Na exchange system). In tissues which are active a good fraction of the time, one must expect the fluxes mediated by active transport to be comparable to those through passive channels (otherwise the membrane could not be in steady state for the biological lifetime of the cell, tissue, or organism). Only when the duty cycle of the membrane is very low can one expect the active fluxes to be small.

If the active transport mechanisms of the tubule or cleft membrane are as important as I suspect (particularly the transport mechanisms for calcium), a number of properties of cardiac muscle and skeletal muscle can be viewed in a new perspective. For example,

(1) The steep temperature dependence of the shape of the cardiac action potential might well be a result of changes in the pump rate for calcium and thus of the calcium concentration in the extracellular space just outside the cardiac membrane.

(2) The dependence of the duration of the cardiac action potential on the frequency and pattern of stimulation might well reflect the variation in calcium (and perhaps potassium) concentration in the clefts.

(3) The dependence of the size of a contraction in heart muscle on the frequency and pattern of excitation (even when the voltage excitation is controlled to be constant in amplitude and duration) may reflect variations in the calcium concentration in the clefts and thus the calcium current through the putative

'contraction' channel linking surface membrane to the calcium induced calcium release mechanism of the sarcoplasmic reticulum.

(4) The physiological fatigue of skeletal muscle fibers (Brenda Eisenberg and Gilai, 1979) may be a consequence of changes in concentration in the tubules of skeletal muscle. (I call the response to low frequency stimulation 'physiological' fatigue to distinguish it from the response to tetanic stimulation which involves gross morphological changes.)

(5) The active transport systems of the tubular and cleft membrane may have a dominant role in determining the volume of that extracellular compartment, just as active transport mechanisms determine the volume of intracellular compartments. In that case, a long literature describing volume changes in tubules of frog skeletal muscle (and clefts of cardiac muscle as well) needs reinterpretation and re-experimentation using blockers of active transport, like low temperature.

(6) The apparent use dependence of many drugs, particularly calcium blocking agents, may reflect the variation of local calcium concentration in the clefts surrounding the calcium 'channels'. In this situation the use dependence would be expected to vary with pump activity, whether that is varied by temperature, pharmacological blockers, or other experimental or natural agents.

(7) Some of the apparent diversity in properties of calcium channels, as reported by different investigators, particularly with respect to their mechanism of inactivation, may reflect different pump rates and thus local calcium concentrations under different experimental conditions.

(8) Some of the diversity of calcium channel types, found in one laboratory under one set of conditions, may reflect the local environment of the channel. A channel sitting next to a calcium pump would have quite different properties from the same channel

isolated in a membrane, particularly if the channel and pump were under the umbrella of a calcium binding site.

(9) The variation of calcium concentration in the clefts or tubules may signal a variety of membrane bound enzymes to create or modify intracellular messengers (often indirectly through a cascade of enzymes) which in turn modify the contractile apparatus, determine the isozymes of the various contractile proteins, and control cell metabolism in general.

(10) The influence of active transport mechanisms on local calcium concentrations may complicate the interpretation of experiments in bathing solutions of reduced calcium concentration (as suggested by Miyamoto and Racker, 1982; see discussion and references in Eisenberg et al., 1983). The combination of pump activity and the existence of a calcium binding protein might make it difficult if not impossible to exhaust the supply of calcium available to a 'contraction channel', particularly if the pump and binding protein are in close conjunction to the channel, supposing such exists at all.

(11) Finally, one should point out an ironical consequence of these effects, if they are as significant as I expect. Consider cells isolated from syncytial tissues by proteolytic enzymes, for example, cardiac cells. The shape of the action potential of these cells is normally taken as an indicator of their viability; if the shape is similar to that in intact tissues, the cells are thought to be normal. If changes in cleft concentration are significant modulators of the action potential, the shape of the action potential <u>should</u> be different in isolated cells, even if the membrane properties of the cells are the same as those in the natural syncytial preparation. In fact, it seems that the action potentials could be normal only if the extracellular concentration did <u>not</u> modulate the action potential or if active transport mechanisms were potent enough to maintain the concentration constant outside the membrane. In the latter case, of course,

temperatures at which active transport is sluggish or inoperative, should have a substantial effect.

It is possible, of course, that changes in the concentration in extracellular space are not as large as one would expect. They may be buffered or controlled by negative feedback, in some way. But that would not necessarily decrease the role of extracellular spaces in the phenomena just described.

Consider, for example, the effects of a membrane active transport system on ions in narrow extracellular spaces, if the active transport system has evolved to control the extracellular concentration of ions, the concentration of ions in the cleft. This mechanism would homeostatically control the concentration of ions and the volume of an extracellular compartment, just as other active transport mechanisms homeostatically control the concentration and volume of the intracellular space. The ion concentration in the extracellular space would remain quite constant, during physiological activity, but the rate of active ion transport would vary drastically, as it produces the membrane flux needed to keep that concentration constant. Since the membrane enzyme responsible for active transport has intracellular substrates and products (as well as the extracellular product of ion flux), important changes would occur within the cell. Thus, the cell would have converted a putative extracellular concentration change into an intracellular signal. It would control the extracellular concentration of an ion by varying active transport of the ion across a membrane, just as an operational amplifier controls its input voltage (to a virtual ground) by varying its output voltage and current. The output of an operational amplifier is a useful (i.e., amplified and buffered) measure of the input, even though the input is virtual, controlled by negative feedback to a negligible size. Similarly,

the cell would have many intracellular outputs it could use as intracellular signals of the (virtual) change in extracellular concentration. The homeostatic active transport mechanisms would provide negative feedback to make the concentration change in the clefts virtual; the active transport mechanisms would do this by controlling the transmembrane fluxes of the transported ions. As a consequence, the local intracellular concentration of transported ions would change and so would the local intracellular concentration of other substrates of the membrane transport process. In this way the cell could simultaneously buffer the concentration of an important ion in the extracellular space and derive an important intracellular signal 'proportional' (or, more accurately, a monotonic function of) the integrated flux of that ion.

Experimental evaluation of extracellular concentrations. The role of extracellular cleft concentrations in physiological function can be directly evaluated with a (relatively) small extension of present day techniques. Indicator dyes developed to measure (for example) intracellular calcium concentration might be applied extracellularly and the resulting optical absorbance could be calibrated and used to measure extracellular concentration. In the case of muscle fibers, fluorescence signals might be particularly useful in easing the calibration problem, in separating signals from the tubules or clefts from signals in the bath. It might be wise to adapt the experimental set-up of Blinks (1965) in which the exciting light is applied in a plane perpendicular to the fiber axis, and the fiber is observed looking down on the plane of the exciting light. Blinks photographed the cross sectional shape of fibers held vertically below a long working distance objective. He looked down the axis of the fiber and directly observed its illuminated cross section. The light he observed was scattered at 90°. The only illumination present was

a thin plane of light at right angles to the fiber axis. This set up could be used to observe a fiber bathed in a solution containing an impermeant calcium indicator, if the thin plane of light were at the wavelength to excite fluorescence of the indicator and the observation were made at the emission wavelength. A fluorescent signal from a dye in the extracellular space within the fiber would then be visible with little contamination from dye in the bathing solution outside the fiber.

Specific experimental design can also be used to isolate signals arising within clefts or tubules from signals in the bathing medium. The experimental design can be self-calibrating and itself provide some of the necessary controls. Consider, for example, experiments using temperature to modulate the activity of an active transport system in the cleft or tubule membrane. Measurements made in the cold are likely to reflect just passive properties, because active transport systems are unlikely to function well at those temperatures. Thus, comparison of dye signals in the warm and the cold should show if the active transport systems are able to significantly modulate extracellular concentrations. The most obvious experiment would compare a putative tubule/cleft calcium signal in the warm and cold, while the fiber was bathed in a low calcium (approximatley 200 μM), high magnesium (5 mM) solution.

Measurements can also be made exploiting the greater spatial (but worse time) resolution of the electron beam microprobe. Here rapid freezing should allow direct visualization of the concentration of ions in the clefts or tubules under different conditions.

Extracellular spaces in other tissues. The discussion of the role of extracellular spaces in other tissues, particularly

epithelia, takes me too far beyond my area of competence. Suffice it to say, that the lateral intracellular spaces of epithelia are important, perhaps dominating, determinants of some of their functions, particularly those involving water flow. The problems of concentration change in these clefts are classical problems of epithelial physiology and have been a significant theme of research in this field for many years. Workers, like myself, interested originally in excitable tissues are the newcomers to these problems.

Extracellular spaces as an evolutionary adaptation. These speculations suggest that restricted extracellular spaces may have a unifying rationale. They may be a universal adaptation to allow a cell to measure, use or control the change in extracellular concentration, which is an integral part of the membrane flux and thus cell activity.

Like all speculations this certainly is well beyond the reach of knowledge. Like all speculations, it requires much thought and testing before being taken too seriously. Nonetheless, unlike some other speculations (particularly in the next part of this paper), these are directly testable. One can hope that such tests will be fruitful even if the hypotheses themselves are refuted.

PART 2: BIOLOGICAL INVESTIGATION: THE UNDERSTANDING OF EVOLUTIONARY ADAPTATIONS

Introduction

The second part of this paper tries to view biological investigation rather generally, looking at all biological systems and structures as a result of the same evolutionary mechanisms, operating at different length scales. In this view, investigation

of the functional consequences of the structure of hemoglobin must follow the same strategy and tactics as the investigation of the functional consequences of the structure of the knee joint, however different the vocabulary of chemistry is from that of gross anatomy!

Until recently the study of biology meant the study of objects larger than the wavelength of light. With that resolution, so limited as we now know, an astonishing amount was learned about principles which govern the structure, function, natural history, and evolution of living organisms. The significant questions of biology were understood. Biologists learned to ask "How are animals and plants built?", "How do they function?", "How do they live together?", and "How did they evolve?" Scientists working on each of these questions clustered into academic disciplines: anatomy, physiology, ecology, and paleontology.

Central Role of Evolution. These academic disciplines recognized the central role of evolution in each biological question. Analysis of structure reveals magnificent adaptations, as we all admire in the intricacies of the anatomy of muscles, of the nervous system, or of sense organs. But analysis of structure also illustrates missed chances, muddles, and misadaptations of evolution, evidently the result of the compulsive opportunism of evolutionary process and the constraints imposed by the environment and the raw materials for adaptation. Examples of misadaptation are numerous. The inappropriate location of retinal neurons in the light path of vertebrate eyes contrasts with their appropriate location in cephalopod eyes. In mammals, breathing in and out through the same pipe guarantees inefficient mixing of oxygen rich 'fuel' and carbon dioxide rich waste. Oxygen exchange

in fish is far more efficient, because of the morphological separation of oxygen rich and oxygen poor water.

Analysis of function reveals the same traces of its evolutionary designer: the choices open to evolution were clearly limited. No wheels or metallic wires were found in animals, and even the magnificent expediency of evolution could not overcome the resulting constraints on function. Wheels and wires would certainly improve the design, the fitness of many biological systems.

The last three decades have brought a remarkable increase in the resolution with which we study life. Molecular biologists have developed tools to study the molecules which perform so many living functions. Indeed, it seems that surprisingly many of the adaptations of evolution have occurred on the molecular scale, the familiar story of 'the chemical basis of inheritance' being the prime example. Macroscopic structures are of little importance in the mechanism of heredity in prokaryotes; they are of limited importance (as far as we now know) in eukaryotes. Along the same lines, we have learned that the intramolecular properties of enzymes and proteins (e.g., their tertiary structure) contain many of the adaptations which define life. Much of metabolism requires little macroscopic organization of enzymes: soluble enzymes reproduce complex metabolic cycles whether uniformly dispersed in test tubes, or cells.

Role of structure on different length scales. Mechanisms depending on macroscopic structure are important even in classical biochemistry. The mechanisms which produce ATP from oxygen involve evolutionary adaptations in both molecular and supramolecular structures. Once solubilized, mitochondrial enzymes cannot produce ATP from oxygen. These enzymes must be

organized into membranes across which a voltage is maintained and through which current flows in specific ionic channels. Oxidative phosphorylation was understood only when the role of the mitochondrial membrane was considered, when workers confronted the potentials across and currents through the mitochondrial membranes, as well as the enzymes, reaction pathways, and putative high energy intermediates which suffice to describe many other metabolic pathways. Structures on the molecular and atomic length scale are intimately involved in oxidative phosphorylation, but so are structures on the much larger length scale of membranes.

The study of membrane processes is now exploiting an incredible increase in the resolution of experimental technique. After all, it was only thirty years ago that the role of membranes in neurophysiology was properly perceived. Today, as this book shows better than most, questions are asked (and answered) about individual molecular channels which govern the flux of molecules and current through biological membranes. Progress in this field will undoubtedly open new vistas equivalent to those revealed to molecular biologists in the last 30 years.

But increased resolution can obscure processes, and mislead us, as well as enlighten us. It is hard to find a pass through a mountain range when one moves on foot, observing structures of meters and kilometers in size. It is easy to find the pass through the mountains looking from an airplane or satellite, observing a larger area with a lower resolution. Of course, neither approach works by itself. The pass seen from the airplane may prove to be blocked by 'invisible' boulders a few meters in diameter; and the pass over the next ridge may be hidden when walking in a steep valley. In biology, as in map making, observations are needed at all relevant resolutions, asking

questions at each scale of time and distance, if one is to find the resolution at which the relevant answer is apparent.

Resolution of experiments must match the length scale of adaptations. The need to observe and question at all resolutions is particularly important when studying the outcome of evolution. Sometimes evolutionary adaptations have occurred on the scale of atoms, as we see in the magnificent hemoglobin molecule which transports oxygen so well in the blood (Perutz, 1978; Fermi and Perutz, 1981). Sometimes adaptations occur on a much larger level, as we see in the shape of fish or sea mammals. Often adaptations occur on multiple scales of distance: the movement of limbs and animals is importantly influenced by the gross morphology and tendon insertions of whole muscles, as well as by intramolecular adaptations of the thin filaments of an individual sarcomere.

The study of membranes is one of those subjects that requires analysis on different scales of time and distance. As we have seen in such beautiful detail in this book, membrane adaptations occur on the molecular scale. It is truly exciting that we can now study these molecule by molecule, one at a time, with the molecules in their natural location, performing their natural function.

Membrane adaptations on different length scales. But adaptations also occur on the length scale of membrane architecture; they do not solely occur in and within channels. The distribution of channels in membranes are likely to be as important as the properties of channels themselves. We expect that some evolutionary problems were solved by the selective placement of channels in different membranes, as well as by adaptations within the channels themselves. Many membranes are

folded and invaginated, making the clefts and tubules which are such prominent anatomical features of cells and tissues. We must presume that this membrane architecture is functionally important, just as we must presume that the placement of channels is important, even if we do not yet understand the functional role or evolutionary heritage of these structural complexities.

The location of channels is particularly important because different membranes, or different parts of continuous membranes are functionally differentiated by their location. Thus, the same channel may have a quite different function depending on its location in a plasma membrane facing the extracellular world, in a plasma membrane facing a cleft, or in an organelle membrane, facing the cytoplasm.

Even the outer plasma membranes are nonuniform; they too are extensively differentiated to perform their function. Channels are not randomly located. They are placed where they are needed, receptors near the source of effectors (e.g., neural transmitters). Indeed, if we find a channel to be in many types of outer membranes, we should guess that it serves a universal function.

Consider, for example, the calcium activated potassium channel which is found in membranes with widely varied function. One might guess that this channel protects damaged cells from prolonged depolarization and consequent swelling and death. Damage to the cell membrane would be expected to depolarize the cell, making some cells more permeable to sodium and perhaps calcium. These ions migrate across the membrane and water flows with them, producing swelling which, if severe enough, would rupture the membrane and kill the cell. An increase in the calcium permeability of the membrane has a counteracting effect,

if the membrane also contains calcium activated potassium channels. When the internal concentration of calcium rises, these potassium channels are activated, increasing the specific conductance of the membrane, holding it close to the equilibrium potential for potassium, thus preventing the depolarization which would otherwise have resulted from the membrane damage.

The previous discussion shows that we must study all relevant length scales as we seek to understand membranes, as well as living processes. Attention to only one scale, however exciting or seductive that may be, will not give useful insight into biological problems that evolution solved on different scales of organization.

Relevant biological questions are defined by evolutionary adaptations. We face another danger resulting from the success of molecular biology and the rush to exploit that success: we can easily forget our questions.

The questions in biology have always differed from the questions of physical sciences, just as questions in historical sciences (archetypes: geology and paleontology) differ from those in experimental sciences (physics and physiology). The nature of this difference has been hard to explain or analyze, particularly to experimental physical scientists, but recent work in the most analytical of physical sciences, applied mathematics, is perhaps helpful. A class of equations has been discovered, simple equations at that, which have remarkable properties, giving chaotic solutions which appear random, and yet arise from strictly deterministic systems (May, 1976; May and Oster, 1976; Gurel and Rossler, 1979; Ruelle, 1980; Feigenbaum, 1980; Guckenheimer, 1982). These chaotic systems are extraordinarily sensitive to the way the system starts, that is, to the initial conditions. One

can presume that chaotic systems are just as sensitive to external interventions, to environmental perturbations, as they are to their initial conditions. External interventions are a form of re-set, in effect a restart of the system with new initial conditions.

Along with many others, I cannot resist the speculation that such chaotic equations govern many strictly physical processes which have traditionally been considered random and analyzed by the theory of stochastic processes. Statistical mechanics, at least classical statistical mechanics governed by strictly deterministic laws, is one candidate. Einstein might also have tried to cast quantum mechanics itself in such a form, if he had preferred a chaotic to a random universe. Indeed, I suspect important insights will emerge as chaotic systems are analyzed with the traditional tools of stochastic analysis. The insights are likely to be important to both the random and the deterministic theories, just as the interaction of potential theory and theory of stochastic processes has been so mutually beneficial.

Limitations in the analysis of chaotic systems. Imagine that historical and evolutionary processes are governed by equations of such a chaotic class, as I believe they are. Then I would argue that the nature of human understanding is inherently restricted, the class of meaningful questions is proscribed, when we study the outcome of a chaotic historical or evolutionary process.

Inherent limitations in human understanding should not be surprising. Since Gödel, mathematicians have become familiar with unprovable but true propositions, even in strictly deductive systems. Mathematicians recently have realized that proofs of some true and provable theorems may exceed human capacities, if,

for example, they take more than a lifetime to read! Physicists are familiar with systems which can only be probed in limited ways -- knowledge of atomic and subatomic processes (and certain macroscopic systems as well, such as superfluidity or superconductivity) is limited by the inherently probabilistic laws of quantum mechanics. Deterministic questioning of quantum processes has been proven fruitless -- it is not possible, and thus is wasteful of scientists' time and effort as well as unsuccessful in providing new information.

Similarly, I think that chaotic processes can be meaningfully studied in only limited ways, essentially by probing the actual outcome of the (chaotic) historical process, asking the existential question "What is?" instead of the metaphysical, nearly theological, "What might have been?" People, and certainly governments, do not, and never will, have the energy or resources to probe every possible outcome of a chaotic process, even if that were a finite set. They confine themselves to a narrow range of questions around the central questions: "What is there?", "What does it do?", "How does it work?", "How did it happen?". We must refrain from asking too many of the unanswerable questions: "What might be there?", "How could it work?", "How might it have happened?". Of course, it is fun to ask these unanswerable questions. But scarcity of resources must eventually limit the integral of pleasure, and most of us are constrained by practical considerations not to spend too much time working on interesting but inherently unanswerable questions. When studying life, we should be restricted in what we can ask, at least if we view organisms and biological systems as the outcome of a chaotic process. We must avoid questions, far from the natural function or history of the system, if we are to use our energies efficiently, proceeding in our task of understanding the systems

before us. Our task is to analyze a particular outcome of a chaotic evolutionary process.

Biology and a grand inverse problem. The relevant questions listed previously in this article fall into two classes. The questions "How is it built", "What does it do?", and "How does it work?" are quite different from the question "How did it get that way?". The first two questions are the classical questions of experimental science and are amenable to the usual approaches. The historical question is much more difficult because it requires the reconstruction of the actual outcome of a chaotic historical evolution. Perhaps that is why experimental biologists often identify the legitimate historical question "How did it evolve?". with the teleological question "Why did it evolve"

The evolutionary history of a biological system is hard to reconstruct for two reasons. The first is the paucity of historical data and the fact that most of the data we have is from one time point, namely the present. The second is the essentially chaotic nature of evolution. Chaotic processes are so sensitive to external disturbances that one needs an incredible density of experimental information before one can understand the cause of a particular event. The smallest unobserved or unknown perturbation can deflect the entire subsequent historical development.

Both of these difficulties in reconstructing an evolutionary process are reminiscent of the difficulties mathematicians face when solving 'inverse' problems even of well defined non-chaotic systems. The difficulties in solving inverse problems can best be understood by contrasting a classical inverse and forward problem. A classical forward problem is the solution of a differential equation and boundary condition for, say, the temperature distribution in the earth as a result of a certain distribution of

heat sources. The corresponding inverse problem would be the determination of the distribution of heat sources from a limited set of experimental information, e.g., the temperature on the surface of the earth. Inverse problems have a certain fascination because, like most human thought but unlike traditional mathematics, they rarely have unique solutions. Even in those cases where the solution is theoretically unique, it may not be practically unique. The unique solution of an inverse problem may be computationally unstable, sensitive to tiny perturbations or errors in the data or numerical analysis. The art and essence of solving an inverse problem, like most of experimental science, is in the casting and recasting of the problem until a prescription is found which tells what data should be measured, and how it should be processed, to give a reliable result. Sometimes the only possible reliable results may not relate directly to the original question. There may not be any possible reliable answer to the original question, using the data available. In this case, a well-determined solution of the original problem may require a new type of measurement, or it may be simply impossible, as, for example, in many chaotic systems.

Given these difficulties in solving even simple inverse problems, I think we can see why reconstruction of the history of a biological system is forbidding. The experimental biologist is instinctively correct in avoiding the teleological question, "Why did it evolve that way?"

Relevant questions in molecular biology. What I have written will hardly be surprising to the evolutionary biologist and will be trite to the mathematical analyst of chaotic systems. But the molecular biologist may not be so aware of these constraints on our questioning of biological systems. He will be tempted to study all he can of the magnificent proteins and channels we have

before us. He may not realize that the analysis of all the properties of these molecular machines is one of the forbidden questions. No doubt all the properties of these molecules are fascinating, and there certainly are enough properties to keep molecular biologists in work for many years. But why study all these properties? Shouldn't we restrict ourselves to those relevant to the biological function for which the molecule was evolved? Cannot we expect these relevant questions to be specific, and thus easier to answer than the more numerous questions, mostly concerning chemical and physical properties irrelevant to biological function or evolution?

In my opinion, the relevant biological questions for molecules are the same as those for cells and tissues. The questions are the same on the molecular and macroscopic scales because the process that built the molecules is the same on both scales. How is the molecule built? What biological function does it perform? How does it do it? How did it get that way? These questions are as central to molecular biology as they are to the biology of the last 150 years. They differ only in the length scale at which they are asked and the vocabulary in which they are answered.

Productive questions in molecular biology. Constraining his questions to the biologically relevant, the molecular biologist is likely to reap a welcome reward. He may find it easier to get answers. Investigation of natural protein structure is much easier than the investigation of arbitrary polypeptides because proteins are a tiny subset of the topologically possibly polypeptide structures. Proteins have evolved as unbranched polypeptides rarely (if ever) tied into knots. Similarly, we can expect that other properties of proteins relevant to the function for which they were evolved are a small, even tiny, subset of all

the chemical properties of such complex molecules. Perhaps by confining his attention to the physiological properties of his molecules, the molecular biologist will find his work much easier, as well as biologically more relevant.

Evolutionary processes proceed always blindly, always opportunistically, usually bluntly, sometimes stupidly, sometimes subtlely, on a macroscopic scale. It is hard to believe they proceed differently on the molecular scale. Thus, we should find microscopically what has been found macroscopically. The biologically appropriate questions, those probing the natural function for which the molecule or system has evolved, are much easier to answer than questions concerning the general properties of the molecule or system. Properties with little adaptive value are unlikely to have been selected and simplified by the processes of evolution.

Relevant and productive questions in membrane biology. We then have a set of questions to apply to the biological systems, of whatever size. How are they built? What do they do? How do they do it? How did they evolve? These questions are the questions of the classical biological disciplines of anatomy, physiology, and paleontology. They also seem to me to be the relevant questions of molecular biology and its nascent subdisciplines.

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