Ionic Interactions are Everywhere

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Physiologists know about ionic solutions. We all know that, without ions, cells swell, proteins denature and life is impossible. What we sometimes forget, however, is that the properties of ionic solutions are dominated by the interactions between ions [5, 51, 85, 105, 106] Interactions make Ringer solutions quite different from the ideal solutions of noninteracting solutes described in physiology and biochemistry textbooks. The enormous experimental literature documenting ionic interactions is sampled in [37, 40]

'Bio-ions'— Na^+, K^+, Ca^{2+} and Cl^- — interact so strongly that they always 'come in pairs': solid salts and ionic solutions are electrically neutral, always containing exactly (within one part in 10^{16}) equal numbers of positive and negative charges [157]. Otherwise, electric forces would tear electrons from atoms, sparks would fly as molecules disintegrate, and life would be impossible, along with most chemistry. Because of these interactions, ionic solutions are nothing like ideal. Ions interact everywhere.

An ion in a solution is accompanied by an equal and opposite amount of charge centered in an ionic atmosphere around that ion. Each ion is in the atmosphere of other ions. The charges of ions in a quite small volume (say a 2 nm cube of salt concentration 1M containing roughly 10 ions, anions and cations combined) add nearly to zero. The charges of one sign shield and screen the effects of ions of other charges.

Interactions are evident in the fundamental measure of ionic solutions, the activity of its components, its free energy per mole. Only in an ideal solution is the activity proportional to concentration. The excess free energy of an ideal solution is zero. Real ionic solutions containing a mixture of the bio-ions are not like that. Ringer's solutions have strong interactions determined (mostly) by the charge and diameter of its spherical ions [3, 4, 26, 51, 102, 105, 106, 140, 147, 172]. The excess free energy of biological solutions is large and it depends on the concentrations of all the species of ions: everything interacts with everything else.

The activity of ions in the solutions in biology is almost never proportional to the concentration of ions. Pure solutions (e.g., Na^+Cl^-) have significant excess free energy in biological concentrations, > 50 mM ionic strength. Pure solutions of divalents like $Ca^{2+}Cl_2^-$ have large excess free energy at all concentrations. Mixtures like Ringer solutions nearly always have excess free energy. The precise definitions of activity, chemical potential, excess free energy, and so on are important, tricky, and described well in texts [51, 106].

Concentrations range over an enormous scale in biology. Concentrations in biologically important places can be extremely high, far beyond the solubility of salt in bulk solutions. (Concentration in this paper simply means the number of particles in a unit volume, the number density. Concentration does not imply activity. In real solutions, number density of a species is not a good estimator of free energy of that species because the free energy depends on the concentration of ***all*** species. The free energy of an ion does not depend only on the number density of that ion. The free energy of K^+ for example depends on the concentration of Na^+.) Concentrations of ions near nucleic acids are more than 10 M; concentrations in ion channels are often much more than 10 M [15, 18, 37, 178, 179]; concentrations of ions in active sites of enzymes are also very large [95]. For reference, solid Na^+Cl^- is ~37 M. In such crowded systems ions experience very strong electrical and steric interactions. They are nothing like the noninteracting uncharged unconstrained particles of ideal solutions and simple fluids.

Specificity. Ion channels, active sites, and nucleic acids are locations where ions are highly concentrated. They are also places where different ions have very different biological roles. Different ions have specific properties [85, 105] of great biological importance in and near enzymes, binding proteins, nucleic acids, transporters and channels. A Cambridge (MRC) Nobel laureate said—with more hyperbole than British understatement—"There is only one word that matters in biology and that is specificity" [101].

'Specificity' certainly mattered when Hodgkin (Cambridge University) unraveled the action potential of nerve and muscle fibers, even if voltage dependence mattered as well. Nerves could not function, the heart could not contract, if Na^+ and K^+ were indistinguishable, i.e., if they behaved ideally. Na^+ and K^+ are different ***only*** because they make nonideal solutions. The action potential exists because Na^+ and K^+ have different diameters and so are different. In an ideal solution, Na^+ and K^+ would be the same. Ideally, Na^+ and K^+ have the same excess free energy, namely zero. Really, Na^+ and K^+ have substantial and different excess free energies and so can be told apart.

Primitive Model of ionic Solutions. The non-ideal properties of many bio-ions can be understood (in pure solutions of one type of salt) quite well by a simple model, in which hard spheres move in a dielectric with friction [13, 44, 52, 56, 67, 107, 128, 150, 155, 159, 161]. This primitive model is taught in textbooks and discussed in reviews [4, 7, 51, 53, 106, 109].

Water is treated implicitly in this primitive model of ionic solutions. Its frictional and dielectric properties are treated as parameters that depend on ionic composition [52, 147, 154-156, 159]. The values of diffusion coefficient and dielectric coefficient are measured experimentally in each solution of interest and those values are used in the appropriate simulation or theory.

The implicit solvent primitive model is widely accepted in physical chemistry as a reasonably adequate quantitative model of pure ionic solutions of bio-ions even at quite large concentrations [147, 164]. Much work is going on to extend this model to include more realistic atomic scale correlations, as can be seen by a small sampling of the literature [69, 83, 85, 96-99, 107, 112, 113, 117, 136, 160, 164, 173] and the hope is that this might help in dealing with ionic mixtures, containing Ca^{2+}, like Ringer solutions, as well. But most of these analyses are done with math that does not allow everything to interact with everything else. Most of the analyses start with properties of simple fluids. I believe ionic solutions are complex fluids [39, 40] and need to be dealt with mathematics appropriate for those systems [30, 32, 54, 108] particularly variational methods [44, 81, 89, 118, 151, 171, 174].

Computing the motion of all the atoms of proteins and surrounding solutions seems at first glance to be the obvious way to improve the primitive model. But the first glance needs to be replaced by a penetrating gaze, particularly given the financial and human resources involved in all atom calculations.

A glance overlooks the knowledge of atomic motion over centuries, since the kinetic theory of matter [20], through the development of statistical mechanics, to the identification of Brownian motion as the engine for macroscopic diffusion.

The motion of atoms resembles that of Brownian motion of mathematical theory, that goes back and forth an innumerable number of times [153]. This incredible nearly infinite fluctuating motion (that goes back and forth millions of times in times much less than a

nanosecond) has nothing obvious to do with the smooth monotonic motion of substrates or ions implied in nearly every textbook figure of channels, or enzymes. (Channels are nearly enzymes, [45]. Channel proteins are specialized to catalyze movement from one location to another in real space. Enzymatic proteins are specialized to catalyze movement in phase space.)

Textbooks and papers almost never show an ion going forward and backward. Ions in the real world go back and forth a great deal, reversing direction a staggering number of times in even 10^{-12} sec. Flows of ions on the biological time scale are fantastically averaged compilations of innumerable (i.e., more than 10^{11}) trajectories of atoms. The reality of ionic solutions became clear(er) for me [46], once I learned that ions in a solution move (more or less) at the speed of sound (nanometers per picosecond), moving in a condensed phase with almost no empty space [7, 46]. Very high speed motion of particles packed without empty space implies a nearly infinite rate of collisions. Condensed phases like ionic solutions and proteins are systems dominated by friction and interactions, as are most devices on the nanoscale [6, 139].

Smooth paths of atoms in our textbooks do not represent the path of any one atom. Only fantastically averaged trajectories might look like textbook figures. If paths of atoms in a figure are smooth, they are averaged, averaged a great deal.

Doing these averages involves much more than 10^{11} additions and subtractions of numbers of approximately equal size. Doing such averages accurately without bias or error is a formidable numerical problem as anyone who has written a computer program (and checked its results) can attest. Biological properties depend on small differences in these large numbers. Channels have different functions (and names) if their selectivities differ by a small fraction of thermal energy, i.e., a small fraction of 1 $k_B T$. The inherent problem in averaging such a staggering number of trajectories with required accuracy is very large. Bias is likely to be present and our results will not be biologically useful or even reproducible unless errors are controlled and bias removed.

Necessity of calibration and checking. The way to evaluate hard-to-avoid bias and errors in numerical calculations is to calibrate and check results against experiments. This is difficult but necessary in any simulation [121, 138]. The special problems of calibrating simulations of interacting ***mixtures*** of ions has only recently been addressed explicitly as far as I know [36] .

Biologically relevant variables must in fact be computed correctly in the simulations of molecular dynamics if one is to hope to fit experiments as they are in fact done, over a wide range of concentrations (10^{-7} to 10^{0} for Ca^{2+}) and compositions of solutions—e.g., Li^+, Na^+, K^+, Rb^+, Cs^+, Ba^{2+}, Ca^{2+} and many other divalents and anions—and a range of voltages, as necessary to understand ionic channels [1, 8-10, 74, 75, 77, 86-88].

Classical force fields of molecular dynamics have not been designed to produce correct values of activity of ions in concentrated solutions (>50 mM), or in solutions of divalents, or mixtures at all. Indeed, it is not clear that molecular dynamics—with full atomic resolution as it is performed today—can ever calculate the concentrations of signaling molecules like Ca^{2+} (10^{-7} M) let alone hormones (10^{-11} M). Just work out how many molecules of water are needed in such a simulation (answer: something greater than 10^{12} for 10^{-7} M Ca^{2+}), and remember that water molecules interact over substantial distances, because of Coulomb's law and hydrogen bonding, and so multi-body interactions must be computed among many of the 10^{12} molecules.

Much work is being done to extend simulations to realistic concentrations of ions, and to use models much better than the primitive model mentioned previously. But calibrated simulations of ***mixtures*** like solutions inside and outside cells are not close at hand, as far as I know.

Multiscale models are needed. I am skeptical that calibrated simulations of all the atoms in real biological solutions are feasible, particularly if they contain signaling molecules. Rather, I advocate a multiscale engineering approach, in which a succession of models is used with different resolution, each chosen to deal with a phenomenon that is important to life, and is determined at the level of resolution that the mathematics and physics of the model can in fact analyze.

Atomic scale calculations are used in the multiscale approach only where needed, and feasible, but not where they are dauntingly close to impossible. Attempting nearly impossible calculations creates a temptation to cut corners for anyone, certainly including me. Cutting corners means making calculations that are not accurate and so not very useful. (It is interesting to notice that the computers that perform these calculation are possible, in my view, exactly because their transistor components are understood so precisely. Computations of the movement of charges in transistors cut few corners [28, 94, 122, 162, 163]). They use multiscale analysis and do not even attempt to deal with all the atoms of the system. They can predict macroscopic properties of transistors within a few per cent with almost no adjustable parameters.

Mathematical difficulties of convergence can be successfully solved in this multiscale approach to ions in solution or charges in transistors. If problems of convergence are ignored, the results of analysis are no longer mathematics. Infinite series that are truncated arbitrarily without consideration of the accuracy of the resulting approximation produce theory that does not have the power of mathematics. The resulting scientific approximations are not unique. Different research groups may force convergence in different ways. The treatment of electrostatics in molecular dynamics usually involves such truncation of infinite series without explicit computation of error terms or direct comparison with analytical solutions of known problems. This problem is recognized and being attacked but there is a long way to go.

Atomic scale distances must be included explicitly in this multiscale approach. The effects of a single protonation event in an ion channel or enzyme can be profound. Protonation events clearly must be calculated with a spatial resolution that resolves the acid or base group involved, i.e., a handful of atoms But the time scale on which the protonation event matters is biological, milliseconds instead of the femtoseconds on which molecular dynamics is calculated. The calculations of molecular dynamics must extend reliably, in a calibrated way, to the time scales of biology, while preserving the spatial detail known to control biological function.

Engineers and physicists deal with this enormous range of scales by using an array of reduced models. One model at one scale feeds another model at another with parameters, boundary conditions, and appropriate information. The approach is general but the conclusions cannot be general. Some systems have macroscopic effects that depend on atomic detail in a specific way, and some do not.

Multiscale models and Interactions. The greatest difficulty in dealing with this multiscale array of models is interactions. The electric field is long range. On the time scale of atomic interactions the electric field is not shielded or screened, and so electric forces range ‘to infinity’, involving boundary conditions very far away. Indeed, it may be necessary to deal with Maxwell’s

equations, not Poisson, on the time scale of atomic motions, because of the large size of the polarization field and displacement current in such rapidly varying systems. Even on the long time scales of biology, when perfect screening occurs in equilibrium situations [70, 123], electric fields extend a long way in the nonequilibrium systems of life, e.g. nerve cells, as cable theory tells us [2, 47, 93, 100, 137]. That is, after all, how nerves signal: they conduct action potentials over meters, and local potentials over millimeters, in solutions in which the screening distance is less than a nanometer.

Action potentials depend on multiscale coupling. Action potentials indeed provide a most dramatic example of multiscale coupling. Conformation changes and flows in channels only a few atoms wide are controlled by voltages far away. Those voltages in fact control the conformation changes and flows in individual channels. The movement of a few atoms in a channel is controlled by far field electrical potentials, and the flow of ions modulated by those motions in turn control the far field electrical potentials. These statements are confirmed by every patch clamp recording of a Na channel during an action potential.

Thinking of this situation on the atomic scale is indeed dizzying but fortunately our ancestors were clear headed. Hodgkin and Huxley [75, 77, 87, 88] showed how a reduced model ***without atomic detail*** could quantitatively couple atomic scale conductance changes to voltage changes to produce action potentials that propagate from toe to spine, meters away. Neher and Sakmann [131, 152]—and a host of workers using bilayer reconstitution [50, 127, 130]—showed how these conductances arise from individual molecules of channel proteins. And MacKinnon showed us what those channel proteins look like in a few of their equilibrium conformations [120].

What is needed now I believe is a consistent extension of reduced models to the atomic scale. What is needed is a systematic method of coupling motions and interactions from atomic to macroscopic scale. We need this to compute properties of action potentials, properties of ionic channels, even the reversal potentials we use to name our channels.

Computation of Reversal Potentials. The simple test case of computing reversal potentials is important to us as physiologists. It is also a good test of the utility of our theories and simulations.

We all know that we cannot identify the type of channel we are studying unless we can measure its 'reversal potential' an idea introduced, I believe, by Hodgkin and Katz [76] and then Hodgkin and Huxley [74, 78]. The reversal potential is the electrical potential at which the current through the channel is zero, and is a measure of the selectivity of a channel. It is a measure of the equilibrium potential, or the gradient of chemical potential, or the ratio of activities for an ion if the channel is perfectly selective. If a channel is selective to two types of ions, the reversal potential lies between the chemical potential (difference, in electrical units) of the two ions.

We cannot determine the selectivity and thus the type and even the name of a channel, unless we know the chemical potential of the ions in our Ringer solution, unless we know the activities, and the deviations of these ions from ideality. Computing those chemical potentials is a test case, a challenging necessity if we are to perform calibrated and quantitative analysis of biological systems. Sadly, the test case is difficult because it of necessity deals with interactions of ions in mixtures like Ringer solutions, containing Ca^{2+}.

A specific challenge. So our test case is a simple challenge. We need a theory and/or simulations of Ringer solutions, and intracellular solutions that can calculate the chemical potentials of bio-ions (i.e., 'equilibrium potentials' in the classical language of electrophysiology), including divalents, hormones, and all the organics (and inorganics) which are the actually controllers of biological function.

We need to compute the activities (free energy per mole) of these different ions in the mixtures that actually occur in life. Ions interact strongly in such mixtures. Our theory and simulations must deal with these interactions realistically, in a calibrated way, in calculations of the activities of ions in Ringer and intracellular solutions that actually occur in life.

Sadly, no theory or simulation is able to fulfill this challenge today, although work is beginning. Scientists have "adopt[ed] a counsel of despair, confining their interest to concentrations below about 0.02 M, ..." as stated in the classical treatise of Robinson and Stokes [143], a book otherwise devoid of emotion, as far as I can tell.

These feelings of despair are stated vividly in present day publications: "It is still a fact that over the last decades, it was easier to fly to the moon than to describe the free energy of even the simplest salt solutions beyond a concentration of 0.1M or so."[105] This, in a recent summary of knowledge of specific ion effects in bulk solutions.

The psychological reason for despair seems clearly to be frustration. The simplest properties of salt solutions could not be calculated in 1959, and cannot be calculated today. The logical reason for this failure is not known. It will not be known until the calculation can be done, and the despair and frustration are removed.

Most workers feel that more detailed knowledge of ions, their physical and chemical interactions with each other, their interactions with water, and the properties of water itself will resolve this problem. And a great deal of work of high quality is focused in this direction, only some of which I know about [69, 83, 85, 96-99, 107, 112, 113, 117, 136, 160, 164, 173].

I myself do not think that detailed knowledge ***itself*** will solve the problem. I think we must ***also*** use mathematics designed to deal with interactions. Detailed knowledge interpreted with a theory of ideal gases seems unlikely to allow understanding of a system in which everything interacts with everything else, at least in my view.

The reader should note that the reversal potential calculation is given here only as an example illustrating the importance of nonideal properties in bulk solutions. The implications of nonideal properties for the functioning of channels is much greater, more important, and unsettled. My collaborators and I have been able to deal with the selectivity of three important types of channel with a simple approach introduced by Nonner and Eisenberg that depends on the calculation of nonideal properties of ions.

Selectivity of Ion Channels. In a series of some 40 papers (reviewed in [37]), a model and approach introduced by Nonner and Eisenberg [132, 134] has been shown to describe the selectivity properties of three distinct ion channels of considerable importance, the Ca_V channels that control the heartbeat, the Na_V channels that produce signaling in the nervous system, and the RyR channel that is the final common pathway controlling calcium signaling in nearly all cells, including cardiac and skeletal muscle. In the calcium and sodium channels a single model with three parameters, never adjusted in value, can account for the quite different selectivity properties in solutions of many monovalent and divalent cations over some 6 orders of

magnitude of concentration, including mixtures. Crystal radii of ions are used and are never changed. The same set of parameter describe the quite different properties of sodium channels with selectivity sequence of amino acids DEKA = Asp Glu Lys Ala and calcium channels with selectivity sequence of amino acids EEEE = Glu Glu Glu Glu.

The sodium vs. potassium selectivity of the DEKA channel arises automatically without adjustment of anything in this model, as the result of the depletion of potassium ions in the selectivity filter of the channel. The sodium vs. potassium selectivity is in fact set by orthogonal control variables in this model: the diameter of the channel controls the selectivity ***only***. (Diameter has no effect on the total ionic content of the channel.) The dielectric coefficient of the protein controls the total ionic content of the channel. (The dielectric coefficient has no effect on the selectivity of the channel.)

One could hardly imagine a simpler or more robust model. The model fits the selectivity properties of both types of channels over an enormous range of conditions and so it seems that it captures the physics used by evolution (i.e., the adaptation) to create the selectivity of these important channels.

The RyR channel has been shown mostly by Gillespie et al [22-25, 27, 55, 57-60, 62, 64, 65, 166, 170] to be described by a closely related model. In this case current voltage relations have been fit in more than 120 solutions of many types of ions, including mixtures of two and three types of ions, and the results of drastic mutations involving removal of all (~17 molar) permanent charge have been predicted successfully, in fact before the experiments were done. The subtle anomalous mole fraction effect was predicted in a range of solutions by Gillespie's analysis before the experiments were done. The measurements confirmed the predictions within a few per cent. Single filing [71, 72]was not involved.

Recently, the model introduced by Nonner and Eisenberg has been analyzed [44, 90, 92, 110, 116, 129] with the powerful methods of variational analysis using the energy variational approach introduced by Chun Liu [81, 89, 114, 115, 118, 151, 171], more than anyone else. These methods provide a mathematical foundation for the computational extension of the Nonner/Eisenberg model and allow easy computation of the full range of current vs. voltage vs. time phenomena observed in ion channels [48, 90, 110], without the complexities and ambiguities of the more intuitive PNP-DFT [21, 49, 55, 59-61, 68, 111, 144-146, 148, 149, 169] theory used earlier.

Mathematics of Interactions. Classical physical chemistry was built on the foundation of the theory of ideal gases without interactions. It had difficulty dealing with systems dominated by interactions because mathematicians had not provided the tools and engineers had not provided the computers to use those tools. I do not believe ionic solutions can be understood or activities of mixtures calculated successfully by adding perturbations to a theory of ideal solutions.

I think we must recognize that ionic solutions are complex fluids, and use the mathematics of complex fluids to deal with ionic solutions, the liquids of life.

The theory of complex fluids deals with systems dominated by interactions over a wide range of scales, from nanometers to millimeters. Think of the liquid crystals of our watches, television screens, and everyday life. These are soap films as thin as biological membranes with atomic scale properties that control their macroscopic function. Liquid crystals can be computed,

without atomic scale simulations for the most part, by an hierarchy of reduced models, that can be written in a page or two of partial differential equations [30-32, 54, 108].

Recently, pure mathematicians have shown (in derivations, theorems and proofs of existence and uniqueness) that the partial differential equations of the theory of complex fluids [31, 44, 79, 81, 90, 91, 114, 115, 118] along with the Navier Stokes equations of incompressible fluids, can be derived from a compact energetic variational principle we call ***EnVarA***. This variational principle includes both (Helmholtz free) energy [158] and (Rayleigh) dissipation [11, 30, 31, 89, 135, 141, 142]. This variational principle can be applied to ionic mixtures like Ringer solutions, and it can be written so it links details of the diameter of bio-ions to macroscopic flows and fields. Derivations of ***EnVarA*** in tutorial detail are available [44] and I am obviously biased in its favor. Nonetheless, it should be clearly understood that a variety of approaches are possible and no one believes that ***EnVarA*** has been proven correct, or even optimal [44, 79, 84, 89, 90, 92, 116, 119, 129, 177]. Compare [167, 168, 175, 176] along with many other papers I do not know about, no doubt.

I do believe, however, that understanding the interactions of ions in living systems requires a mathematics like that of complex fluid theory. This mathematics must handle the steric and electrical interactions of ions consistently and uniquely (for a given physical model) with explicit boundary conditions (to avoid the convergence problems that plague classical theories of ionic solutions and prevent a proper use of mathematics and its unique results) [37, 39, 40].

The theory of complex fluids is not magic. It must include the proper physics if it is to succeed. The application of complex fluid theory to ionic solutions is just beginning. It remains to be seen which properties of the bio-ions need to be included in this description. Will the primitive model itself allow prediction of the reversal potentials of mixtures? Will details of ionic interactions with each other and with water be needed? We will not know until we try to include each in a consistent mathematical theory that represents each physical model and tells what it will do in each experimental situation [39].

Consequences for biology are profound. Since ionic solutions are assumed ideal in most textbooks of enzymology and biochemistry, and in the thoughts of most biochemists and physiologists, the consequences of their nonideality are profound. Nearly every chemical reaction in biochemistry is likely to have to be reinterpreted because all have been studied in solutions of ions concentrated enough to cause 'counsels of despair'[143]. Most chemical reactions and binding sites in biochemistry and physiology involve regions in which local concentrations are very large indeed, just where the chemical reaction occurs. Ions tend to be important where they are concentrated (to produce large flows) or depleted (to produce control).

Simple experimental checks can tell whether nonideal effects are important. Ion species and concentrations can be changed. The species of divalents can be changed and then varied in concentration, being careful to use systems that buffer calcium concentrations. If the natural function of an enzyme does not change, for example, when the ionic solution is modified, nonideal effects are likely to be unimportant. But few enzymes have rates that are unchanged when potassium ions are replaced with sodium ions, when concentrations of ions are doubled or tripled, or when calcium ion concentrations are varied in the signaling range of 10^{-8} to 10^{-6} M.

Some enzymes, binding sites, nucleic acids, transporters or channels may behave ideally. If experiments show that their function is independent of ion type and concentration, then

theories using ideal properties are reasonable. If experiments show that enzymes, binding proteins, nucleic acids, channels, or transporters are sensitive to ion type and concentration–don't forget calcium ions–then they have significant excess free energy. They behave non-ideally. Ideal theories are not appropriate because ideal theories ignore excess free energies and what those can do to the system's function. Indeed, ideal theories of nonideal systems are likely to mislead more than inform. The excess free energy that makes the systems nonideal is likely to be used by biology for its purposes. Mechanisms using excess free energy cannot be understood if that free energy is assumed to be zero. Mechanisms using excess free energy will most likely be attributed to special properties or states, or 'allosteric interactions' within the enzymes, binding proteins, nucleic acids, transporters or proteins. Once scientists start down the ideal path creating it is hard to return to the real path. A great deal of logical and emotional energy is needed to backtrack and change paths.

Excess free energy and selectivity. The successful treatment of selectivity (of three channel types) described previously in this paper (presented from different perspectives in [14-19, 22, 24, 33, 35, 37, 44, 66, 80, 90, 95, 104, 125, 126, 132-134]) depends on the excess free energy of the ions in the channel, that is to say, selectivity depends on the nonideal properties of ions. Ions (that permeate) are the substrate for this channel 'enzyme' [45] that catalyzes movement across a barrier of potential (of mean force) from one side of a membrane to another [34, 38, 40-43]. The channel enzyme like other enzymes, proteins, and nucleic acids must be able to tell different ions apart if it is to function.

If ions were ideal, sodium and potassium would be identical and could not be told apart.. Thus a classical treatment of ion channels cannot account for sodium vs. potassium selectivity. Biological function here **requires** excess free energy. Biological function depends on the nonideal properties of the substrates. The biological function of calcium, sodium, and RyR channels require them to tell ions apart.

One must expect a similar importance of nonideal properties in other enzymes, proteins and nucleic acids. One must expect that biology will use nonideal properties of substrates to provide selectivity and other crucial functions of these systems.

Unfortunately, classical treatments of channels, transporters, nucleic acids, binding proteins and enzymes all assume ideal properties of substrates and ions. They all use the law of mass action with rate constants independent of concentration.

Classical theories all use the law of mass action in a way that assumes substrates and ions are ideal. These classical treatments are thus unable to deal with biological properties and functions that arise from the excess free energy of substrates and ions. For example, classical treatments cannot deal with the difference between sodium and potassium. This is not a trivial problem. Life depends on the ability of sodium channels, potassium channels, and sodium pumps to distinguish sodium from potassium. If sodium pumps, channels, or potassium channels cannot distinguish the excess free energy of sodium from that of potassium, nerve fibers, muscle fibers, and most cells of the body swell. Nerve endings quickly swell and burst. Animals die.

Law of Mass Action. We have all been taught the law of mass action with rate constants independent of concentration [38]. We have been taught rate models of enzymes and channels of some complexity, with rate constants independent of concentration. We have been taught of the interactions of ionic substrates with proteins, called allosteric, because they change protein structure at a distance. These teachings all depend on the treatment of ionic interactions.

Interactions measured in systems containing ions, ionic substrates and proteins are all attributed to the proteins. But ions and ionic substrates must have large excess free energies at the concentrations in and near active sites and channels. These excess free energies depend on all species. The excess free energy of a substrate depends on the concentration of all types of ions in a concentrated solution like that in and near active sites and channels. Thus, large interactions must occur among the ions themselves, as well as with the side chains of the proteins that mix with ions. As a matter of experimental fact [3, 26, 51, 82, 85, 102, 105, 140, 172], the free energy of one ion in a mixture depends on the concentration of all ions in the system.. Even in concentrations of Ringer solutions ~0.2 M or seawater ~0.6 M, these effects are large. In the highly concentrated multi molar solutions near and in active sites and channels these effects are huge. Interactions must occur among the ions and side chains themselves, as in the models of the calcium, sodium and RyR channels. Allosteric interactions involving the rest of the protein may also occur, of course, but they cannot be the entire story, as has been nearly universally assumed for many decades. In fact, the models of calcium, sodium, and RyR channels (discussed previously in this paper) include no allosteric effects, no 'chemical' effects at all. They include only the effects of charge and steric interaction in a confined crowded environment. Yet these models fit an enormous range of experimental data [12, 15, 18, 19, 27, 55, 66, 103, 104], predict detailed properties before they are measured [27, 55, 58, 62-64], and allow construction of new channel types [124-126, 165].

Rate constants involving ions in crowded conditions like this must then in reality not be constants. Rather, they must depend on the concentrations of most of the ions in the system (and other variables as well, including certainly the properties of the surrounding protein).

When rate constants are assumed constant, the natural way to deal with experiments showing interactions is to assume large numbers of states [29] and special vital properties of proteins, defined by metaphors like allostery, rather than physical laws. The classical approach lives on [73] despite its unrealistic assumptions of ideal substrates in ideal solutions, despite the complexity of resulting ideal models, involving states that change with every experiment and experimenter.

Models involving numerous states and special vital properties have not proven very useful because the vital models change state, as the experimenters change and conditions change. Metaphors are not very useful when building engineering devices. Equations and numbers are needed.

It seems likely to me that the interpretation of classical experiments of biology will change dramatically when a mathematics and model of ionic solutions is used that allows everything to interact with everything else. Some allosteric interactions attributed to proteins will be found to arise in interactions of ions among themselves in the crowded environments maintained by proteins, but without changes of location of the atoms of the protein at all. The conformation of the forces in the protein may change, even if the protein is fixed in space.

Of course, many of the special properties of ions, channels and proteins imagined by classical biologists will survive this reanalysis. Not all will disappear in a consistent theory of complex ionic fluids in and near proteins. But we will not understand how biological systems work until we use a mathematics and physics that allow ions to interact with each other as they actually do in ionic mixtures. In my opinion, channels, transporters, binding proteins, and enzymes use ionic interactions to perform crucial biological functions. Biological functions will

not be understood quantitatively, often qualitatively, until ionic interactions are given their proper role. Everything interacts with (nearly) everything else in ionic solutions. Everything must be able to interact with everything else in our theories and simulations. The words and metaphors of structural biological and classical thermodynamics cannot deal with complex interactions in complex changing structures. The theory of complex fluids can.

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