Preface

Calcium plays an important role in a wide variety of biological processes. This divalent metal ion can bind to a large number of proteins; by doing so it modifies their biological activity or their stability. Because of its distinct chemical properties calcium is uniquely suited to act as an on–off switch or as a light dimmer of biological activities. The two books entitled *Calcium-Binding Protein Protocols* (Volumes I and II) focus on modern experimental analyses and methodologies for the study of calcium-binding proteins. Both extracellular and intracellular calcium-binding proteins are discussed in detail. However, proteins involved in calcium handling (e.g., calcium pumps and calcium channels), fall outside of the scope of these two volumes. Also, calcium-binding proteins involved in bone deposition will not be discussed, as this specific topic has been addressed previously. The focus of these two books is on studies of the calcium-binding proteins and their behavior in vitro and in vivo. The primary emphasis is on protein chemistry and biophysical methods. Many of the methods described will also be applicable to proteins that do not bind calcium.

Calcium-Binding Protein Protocols is divided into three main sections. The section entitled *Introduction and Reviews* provides information on the role of calcium in intracellular secondary messenger activation mechanisms. Moreover, unique aspects of calcium chemistry and the utilization of calcium in dairy proteins, as well as calcium-binding proteins involved in blood clotting, are addressed. The second section entitled *Calcium-Binding Proteins: Case Studies* provides a wealth of information about protein purification and characterization strategies, X-ray crystallography and other studies that are focused on specific calcium-binding proteins. Together, these two sections comprise Volume I of this series. By introducing the various classes of intra- and extracellular calcium-binding proteins and their modes of action, these two sections set the stage and provide the necessary background for the third section. The final section entitled *Methods and Techniques to Study Calcium-Binding Proteins* makes up Volume II of *Calcium-Binding Protein Protocols*. Here the focus is on the use of a range of modern experimental techniques that can be employed to study the solution structure, stability, dynamics, calcium-binding properties, and biological activity of calcium-binding proteins in general. As well, studies of their ligand-binding properties and their distribution in cells are included. In addition to enzymatic assays and more routine spectroscopic and protein chemistry techniques, particular attention has been paid in the second volume to modern NMR approaches, thermodynamic analyses,

kinetic measurements such as surface plasmon resonance, strategies for amino acid sequence alignments, as well as fluorescence methods to study the distribution of calcium and calcium-binding proteins in cells. In preparing their chapters, all the authors have attempted to share the little secrets that are required to successfully apply these methods to related proteins. Together the two volumes of *Calcium-Binding Protein Protocols* provide the reader with a host of experimental methods that can be applied either to uncover new aspects of earlier characterized calcium-binding proteins or to study newly discovered proteins.

As more and more calcium-binding proteins are being uncovered through genome sequencing efforts and protein interaction studies (e.g., affinity chromatography, crosslinking or yeast two-hybrid systems) the time seemed right to collect all the methods used to characterize these proteins in a book. The methods detailed here should provide the reader with the essential tools for their analysis in terms of structure, dynamics, and function. The hope is that these two volumes will contribute to our understanding of the part of the proteome, which relies on interactions with calcium to carry out its functions.

In closing, I would like to thank Margaret Tew for her invaluable assistance with the editing and organization of these two books. Finally, I would like to thank the authors of the individual chapters, who are all experts in this field, for their cooperation in producing these two volumes in a timely fashion.

Hans J. Vogel, PhD

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Calcium

Robert J. P. Williams

1. Introduction

When one considers the chemistry of any element in the context of biological organisms, it is exceedingly important to observe it in relationship to the chemistries of all the other elements in the environment that are used, or even not used, by cells. One major concern is the limitation of the availability of the element concerned, in part because of the presence of other elements, which may have changed during the evolution of Earth and its organisms. This is a geochemical problem, but even when the element has an easily available form in a compound, the accessibility to biology may be restricted through this very combination. One or two simple points then need stressing. Elements such as H, C, O, and, to lesser extent, N, S, and P, the major elements of bioorganic chemistry, are all abundant in the universe and are geochemically available. However, unfortunately, several of these elements are locked up in compounds so their accessibility for transformation into manipulatable atomic elements in cells is very restricted — consider H in H_2O, C in CO_2, N in N_2, and S in SO_4^{2-}, where in each case the respective elements, H, C, N, and S are difficult for organisms to obtain. Only O and P as O_2 today and HPO_4^{2-} are genuinely available and in a suitable form for immediate use in a cell. By way of comparison, metal ions such as calcium and a few nonmetals such as chlorine (as chloride) are quite abundant and are relatively freely available as ions in the forms in which organisms use them. Note that calcium concentrations are restricted by the presence of carbonate. However, for the simple purpose of the essential cellular organic synthesis of H, C, N, O, S, and P compounds, these two elements, calcium (Ca^{2+}) and chlorine (Cl^-) together with sodium (Na^+) are of little value and, in fact, are deleterious to the general stability of cell life in the condition in which they are available in the sea. That is, 10 m*M* Ca^{2+} and 500 m*M*

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Table 1
The Essential Elements of Life[a]

		H								
–	–	–	C	N	O					
Na	Mg	–	(Si)	P	S	Cl				
K	Ca	–	–	(V)	–	Mn	Fe	Co	Ni	Cu Zn
				(As)	Se	(Br)				
(Sr)					Mo	(I)				
(Ba)					(W)					

[a]Elements in brackets are essential in some organizms.

Table 2
Physical Properties of Calcium and Other Divalent Ions

Ion	Ca^{2+}	Mg^{2+}	Sr^{2+}	Ba^{2+}	Mn^{2+}	Fe^{2+}	Zn^{2+}	Cd^{2+}
Radius Å	1.00	0.65	1.13	1.35	0.75	0.70	0.65	0.90
Electron Affinity (eV)	18.00	22.7	16.7	15.2	23.1	24.1	27.4	25.9

Na^+ and Cl^-. Ca^{2+} at this level tends to precipitate many vital anions, whereas Na^+ and Cl^- at the levels in the sea would cause osmotic problems if allowed to enter cells freely. Even Mg^{2+} 30 m*M* and SO_4^{2-} 20 m*M* in sea water are too concentrated to be allowed free access to cells. On the other hand, in fresh water, almost all 20 essential elements of life (*see* **Table 1**) have to be taken up into cells because their concentrations are so low. In particular, note that in soil water the retention of calcium by soil silicates can be strong. The problems of handling calcium in organisms therefore parallel the handling of many other elements because each element of some 20 is required in a certain amount and both deficiency and excess cause problems for the organism. We turn now directly to problems concerning calcium.

The sea was the original source of life, so we first describe sea water conditions here. If the sea was initially somewhat more acidic than it is today (pH = 8.0), then calcium could have been even more readily available and greater protection against it was necessary for cells. Before going further into the biological chemical problems, we need to look at calcium and its basic chemistry.

2. The Character of the Calcium Ion

This chapter is a combination of earlier literature on calcium biochemistry as given in somewhat more detail in two books ***(1,2)*** and several recent review chapters ***(3–5)***. This chapter attempts to give an overall view of calcium bio-

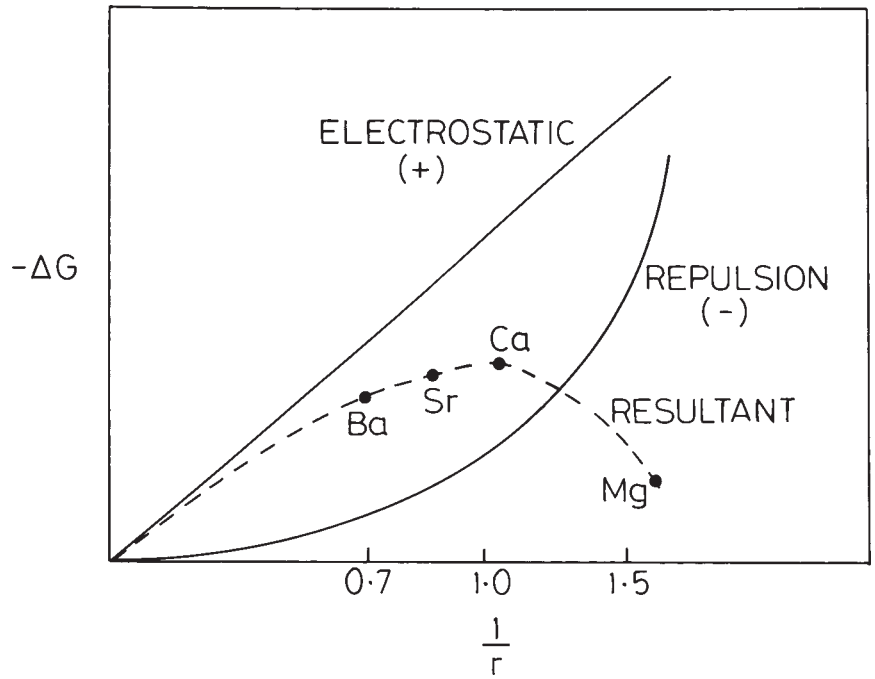


Fig. 1. The changes in attractive electrostatic energy ($1/r$) and repulsive energy ($1/r^n$) as a large number of anions are brought closer to a central cation. The resultant energy has a maximum at a certain cation size for a given anion size.

logical activity as seen by a chemist and more detailed and sophisticated views of particular features must be found in later chapters in this volume.

Calcium exists in nature in one form only — the Ca^{2+} ion. Its character relative to other ions of its own charge type is shown in **Table 2** and **Fig. 1**. It is different from all other ions, except Sr^{2+}, by a considerable margin. We must note, however, that it is the same size as Na^+. The other readily available ions, which are somewhat similar in size and/or charge, are Mg^{2+} and Mn^{2+}. There are interesting parallels and differences in the resulting chemistries of these elements.

3. The Chemistry of the Calcium Ion: Principles and Precipitation

We shall consider that all calcium–ion interactions are electrostatic with no covalent contribution and that, although this is also true for Mg^{2+}, Na^+, and Sr^{2+}, for example, it is less and less the case in the series of divalent ions which show increasingly covalent bonding

$$Ca^{2+}\ (Mg^{2+}) < Mn^{2+} < Fe^{2+} < Co^{2+} < Ni^{2+} < Cu^{2+} < Zn^{2+}.$$

The upshot of this ionic character is that as far as biochemical relevance is concerned Ca^{2+}, Mg^{2+}, Sr^{2+}, K^+, and Na^+ only bind to ligating oxygen (O) donors such as H_2O, RCO_2^-, R_2CO, R_2O, and RCH_2OH and not to nitrogen (N) or sulphur (S) donors. Two major factors in cells then affect the binding ability of the calcium ion in solids or solution in competition with other ions: 1) the ability of N- and S-donors to remove competing ions from Mn^{2+} along the above series to Zn^{2+}; and 2) the ability of Ca^{2+} to bind preferentially relative to Mg^{2+} to particular O-donor ligands because of its size and ligating capability, which is less restricted sterically than is Mg^{2+} binding.

Calcium ion binding must then be seen in the context of its size, and the possible oxygen atom donor arrangements around it. Turning first to solids where oxygen anions or atoms are close-packed as in say, oxide lattices, there are generated octahedral holes. The holes of radius 0.6 Å fit a magnesium ion excellently, but calcium is too large and forms an 8-coordinate oxide lattice. This is the same distinction as applies between NaCl (6-coordination) and CsCl (8-coordination) lattices. Hence, with the small anions, such as OH^- and F^-, which can pack tightly around small cations, we find that $Mg(OH)_2$ and MgF_2 are more insoluble than $Ca(OH)_2$ and CaF_2. However, as anions become larger, their packing gives rise to larger and larger holes and the balance in the equation

$$M(H_2O)_n^{2+} + X^2 \rightleftarrows MX + nH_2O \qquad (1)$$

changes. Because Mg^{2+} interacts extremely well with six (small) water molecules in $[Mg(H_2O)_6]^{2-}$, larger anions do not replace the water readily to give precipitates. The larger Ca^{2+} ion binds more strongly to those anions large relative to water and, hence, these anions remove Ca^{2+} readily, but not Mg^{2+} to give precipitates (*see* **Table 3**). Typically large anions, CO_3^{2-}, and PO_4^{2-}, precipitate with calcium at lower metal ion concentrations than with magnesium. As a consequence, Ca^{2+} has a lower concentration in sea water and we find deposits, both geochemical and biochemical, of $CaCO_3$, $CaSO_4$, and $Ca_3(PO_4)_2$, but not of the corresponding magnesium salts except mixed in the calcium salts. Sulphate is so large in fact that the order of precipitation is

$$Ba^{2+} > Sr^{2+} > Ca^{2+} >> Mg^{2+}.$$

The common form of Ba^{2+} and Sr^{2+} in geological or biological hard structures is as $BaSO_4$ and $SrSO_4$, respectively (*see* **Fig. 1**).

The logic extends to organic anions, which by their nature, are generally large, e.g., organic carboxylates and phosphates. For example, calcium has an insoluble oxalate, often found in plant tissue, but magnesium does not. Moreover, calcium tends to precipitate many polyanions, such as DNA, RNA, and some acidic proteins. There is difficulty then in keeping calcium and accompanying anions in solution inside cells and as a protection calcium is rejected. We

Table 3
Solubility-Product Constants (SP)[a]

Substance	Formula	Solubility product
Aluminum hydroxide	$Al(OH)_3$	2×10^{-32}
Barium carbonate	$BaCO_3$	5.1×10^{-9}
Barium oxalate	BaC_2O_4	2.3×10^{-8}
Barium sulfate	$BaSO_4$	1.3×10^{-10}
Cadmium hydroxide	$Cd(OH)_2$	5.9×10^{-15}
Cadmium oxalate	CdC_2O_4	9×10^{-8}
Cadmium sulphide	CdS	2×10^{-28}
Calcium carbonate	$CaCO_3$	4.8×10^{-9}
Calcium fluoride	CaF_2	4.9×10^{-11}
Calcium oxalate	CaC_2O_4	2.3×10^{-9}
Calcium sulfate	$CaSO_4$	2.6×10^{-5}
Magnesium ammonium phosphate	$MgNH_4PO_4$	3×10^{-15}
Magnesium carbonate	$MgCO_3$	1×10^{-5}
Magnesium hydroxide	$Mg(OH)_2$	1.8×10^{-11}
Magnesium oxalate	MgC_2O_4	8.6×10^{-5}
Manganese(II) hydroxide	$Mn(OH)_2$	1.9×10^{-15}
Manganese(II) sulphide	MnS	3×10^{-15}
Strontium oxalate	SrC_2O_4	5.6×10^{-8}
Strontium sulfate	$SrSO_4$	3.2×10^{-7}
Zinc hydroxide	$Zn(OH)_2$	1.2×10^{-17}
Zinc oxalate	ZnC_2O_4	7.5×10^{-9}
Zinc sulphide	ZnS	4.5×10^{-24}

[a]Data from *Stability Constants* (1964), *Spec. Pub. No. 7*. The Chemical Society, London. Note the preferred use of Roman labels of oxidation states.

can, therefore, ascribe the selective use of calcium in biology both in minerals and in the crosslinking of many extracellular matrices to the size of this doubly charged cation.

It is also of importance to compare Ca^{2+} precipitation with that of one important trivalent ion, Al^{3+}. Al^{3+}, a small cation, like Mg^{2+}, but more so, has an insoluble hydroxide and a somewhat insoluble phosphate. It is often found in Ca^{2+} phosphate precipitates, e.g., bone and with silica in what may be aluminosilicates outside cells. Silicic acid does not precipitate with Ca^{2+} at pH = 7.0. Because of its propensity to precipitate organic anions as well as inorganic anions Al^{3+}, like *all* trivalent ions and Ca^{2+}, is prevented from entering all cells. To the best of the author's knowledge this includes not only Al^{3+} and Sc^{3+}, but

also Fe^{3+}. Although none of these ions as free ions in solution exceeds 10^{-11} *M* at pH = 7.0, they could compete for certain external anionic sites, which can also bind Ca^{2+}, *see* **Subheading 11.**

4. Calcium Salts Precipitated in Organisms

Table 3 shows the solubility products of calcium salts. It also shows the effective solubility products of some salts of magnesium and aluminium at the biological pH = 7.5 of most intracellular fluids indicating why ions such as Ca^{2+} must be excluded. The sea is at a high pH of 8.4 and it is close to a saturated $CaCO_3$ solution so that many unicellular and multicellular rather simple organisms in the sea make $CaCO_3$ external shells quite easily. Notice that the precipitation of phosphates in the sea is less probable than carbonates because of the low level of phosphate. Now some fresh water organisms can also make $CaCO_3$ shells, which means that the precipitation has to be carried out in an environment of $Ca^{2+} + HCO_3^-$, which is made by the living system. Vertebrates have an extracellular pH = 7.0 and precipitate calcium phosphate $Ca(HPO_4)_2$ or $Ca_3(PO_4)_2$ before it is transformed into the more insoluble $Ca_2(OH)(PO_4)$, an approximate formula for hydroxyapatite. Note that in circulating liquids of these organisms, HPO_4^{2-} and free Ca^{2+} concentrations are not far from 1.0 m*M* so that phosphate, and not carbonate, is precipitated. As stated, the free $[Ca^{2+}]$ in the sea is much higher approaching 10 m*M*, but phosphate is much lower.

It is easy to see how a biological mineral, such as bone, which is a composite of polymer and crystals, can redissolve by making the extracellular fluid of pH << 7.0 locally. In the body of higher animals there are special cells, osteoblasts, for this dissolution. The cells bind to the bone trapping a small aqueous volume between the bone and themselves (*see* **Fig. 2**). They then release into this volume acid to solubilize the phosphate plus enzymes for destroying the bone polymers.

There are other factors that affect solubility, because a compound such as apatite readily incorporates other ions than Ca^{2+}, HPO_4^{2-}, (PO_4^{3-}), and OH^-. Most notable are F^-, Mg^{2+}, CO_3^{2-}, and Al^{3+}. The effect of fluoride is to harden apatite, whereas if anything, Mg^{2+} and CO_3^{2-} weaken it. The uptake of Al^{3+} into bone needs special comment (*see later*). Of course, both $CaCO_3$ and $Ca_2(OH)PO_4$ are more soluble in acid so that acid-producing bacteria decay teeth.

One insoluble calcium salt not affected by acidity until below pH = 5.0 is calcium oxalate. Plant extracellular fluids are at about pH = 5.5 and this pH prevents carbonate or phosphate precipitation. Several plant species then generate calcium oxalates, e.g., rhubarb leaves, which are poisonous to humans as they redissolve. The formation of oxalates appears to be both protective, as is shell and bone, but also may be a way of eliminating excess calcium. A particu-

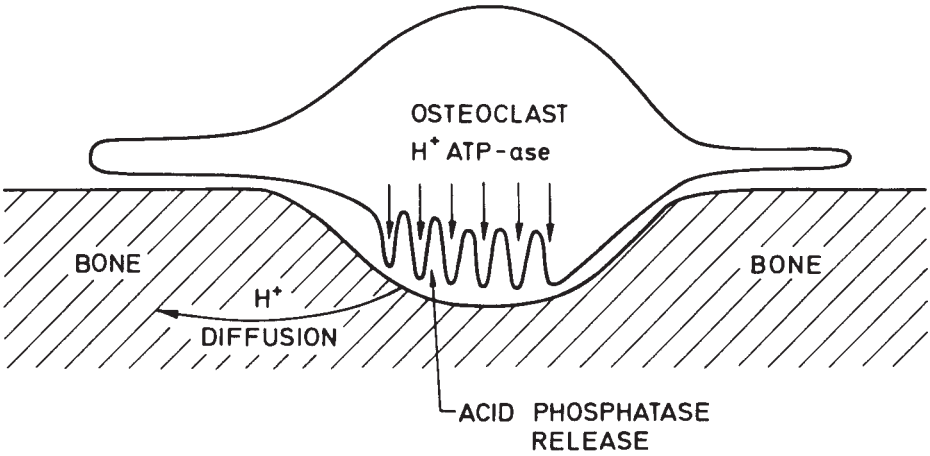


Fig. 2. The interaction of an osteoclast cell with a bone surface. The cavity is extended as acid and enzymes are ejected into the small local space shown trapped between cell and bone surface. NB: protons diffuse readily in bone.

larly fascinating example of oxalate precipitation is found in the hairs of the stinging nettle.

5. Solubility and Precipitation Mechanisms

Solubility depends upon the size of a crystal and it is the case that smaller crystals are more soluble. Two factors affect solubility relative to size:

1. The internal free energy of the salt excluding any surface effects, $-\Delta G_i$ per mole, is approximately independent of crystal size.
2. The surface free energy, $-\Delta G_s$, which is a larger contribution per mole the smaller the crystal, and is less than $-\Delta G$(solution).

Now $-\Delta G_s < -\Delta G_i$ (NB for $-\Delta G$ the more negative means the more stable) so that the weighted sum for a given crystal size per mole is less negative for small crystals and the smaller the crystal the greater the solubility. It takes a degree of supersaturation to cause precipitation initially. The picture changes in the presence of an organic polymer if the polymer binds to the crystal surface when there is a new term stabilizing the surface $-\Delta G_p$. When $-(\Delta G_s + \Delta G_p)$ approaches $-\Delta G_i$ crystallization can occur without any supersaturation. Furthermore, if $-(\Delta G_s + \Delta G_p) > -\Delta G_i$, then the insolubility of the small crystallites exceeds that of the large crystals. An obvious way of stabilizing a small crystal is then to build a small cavity to stabilize all surfaces. The polymers of the cavity then

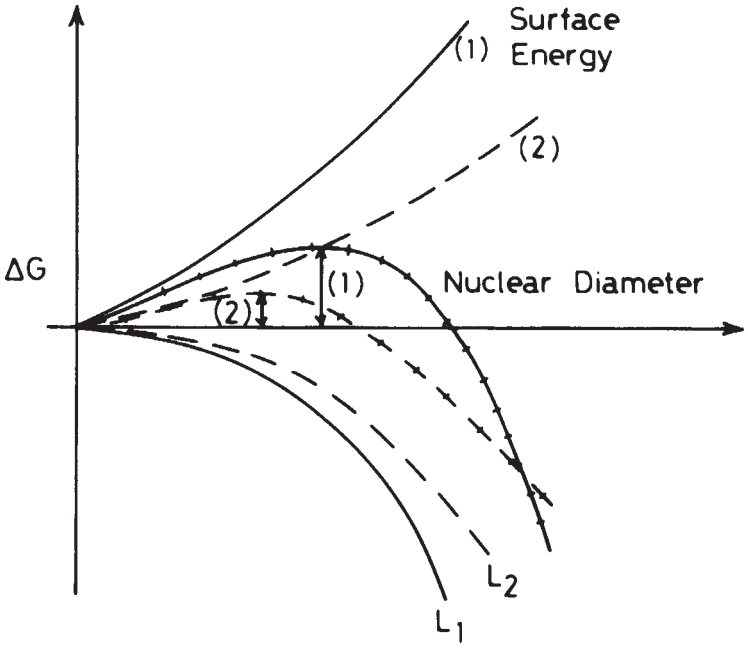


Fig. 3. The free energy per mole relative to the hydrated ions on forming the interior of a crystal, L_1 and L_2, or a pure surface layer (1) and (2). The second is unstable and crystals can only grow when (L_n – (n)) exceeds the points indicated by the double arrows ↕ (n). The figure shows that despite a lower internal energy (lattice energy) and ultimately a lower total stability and hence a higher solubility the crystals L_2 will crystallize more rapidly because the nucleation barrier ↕ (n) for the weaker and more soluble crystals is lower.

protect the crystal from dissolution both in a thermodynamic and a kinetic sense (*see* **Fig. 3)**. This is undoubtedly the case in the making of shells and bones.

Now opposing crystallization are small molecule inhibitors. Consider a newly formed nucleus of a crystal that has exposed surfaces. $-\Delta G_i - \Delta G_s - \Delta G_p$ is not favorable to growth until the crystal reaches a given size. Before that size is reached, dissolution can occur. A small molecule (inhibitor) that blocks growth points on the surface will favor rate of dissolution relative to growth rate and hence prevents crystal growth. Some possible inhibitors are listed in **Table 4**. Cells undoubtedly use both in the control of precipitate formation.

6. Complex Ion Formation

The same principles of binding strengths relative to ion sizes apply to equilibrium in solution. The simplest equilibrium reaction is

Table 4
Inhibitors of Crystal Growth

Mineral	Inhibitor (examples)
Calcium carbonate	F^-, HPO, oxalate
Calcium phosphate	F^-, organic phosphonates, malonate

Table 5
Some Stability Constants, log*K*, of Magnesium, Calcium, and Manganese Complexes at Zero Ionic Strength and T = 25°C

Ligand	Log*K* Mg^{2+}	Ca^{2+}	Mn^{2+}
Acetate	0.8	0.8	approx 1.0
Glycine[a]	approx 3.0	1.5	3.5
Iminodiacetate[a]	3.7	3.4	
EDTA[a]	9.1	11.0	14.1
EGTA[a]	5.5	11.2	12.5
4,5 Dihydroxybenzene 1,3 disulphonate[a]	5.9	6.0	8.6

[a]Small donor centers, N or O, assist Mg^{2+} binding relative to Ca^{2+}, e.g., unsaturated amines and phenols. Data for pH = 7.0 are $>10^3$-fold lower.

$$M^{2+} + X^- \rightleftarrows MX^+ \quad (2)$$

We write in place of a solubility product an associative stability constant *K*.

$$K = |MX| / |M||X| \quad (3)$$

The stability constant is dependent upon the type and charge of the donor groups of *X*, but also on the steric constraints that impose themselves as several groups crowd around the Ca^{2+} ion, **Fig. 1**. Steric hindrance can be in the coordination sphere or arise as interference generated internally in a large unit such as ethylene glycol-bis *N*,*N*,*N*',*N*'-tetraacetic acid (EGTA), **Fig. 4**, as it folds. Notice how calcium ions cause the organic moiety to fold in a particular way so that obviously the outside of the complex has more selective recognition features than the bare ion. Some examples of steric factors influencing stability constants are given in **Table 5**. The coordination in all these complexes remains as dominantly through O-atom donors. Notice that Mg^{2+} does not bind strongly to these particular centers, but preferential reagents for Mg^{2+} (and Al^{3+}) can be developed using small RO^- centers such as phenolates. RO^- here is a small anion in the sense that only $-O^-$ presents itself in the coordination sphere.

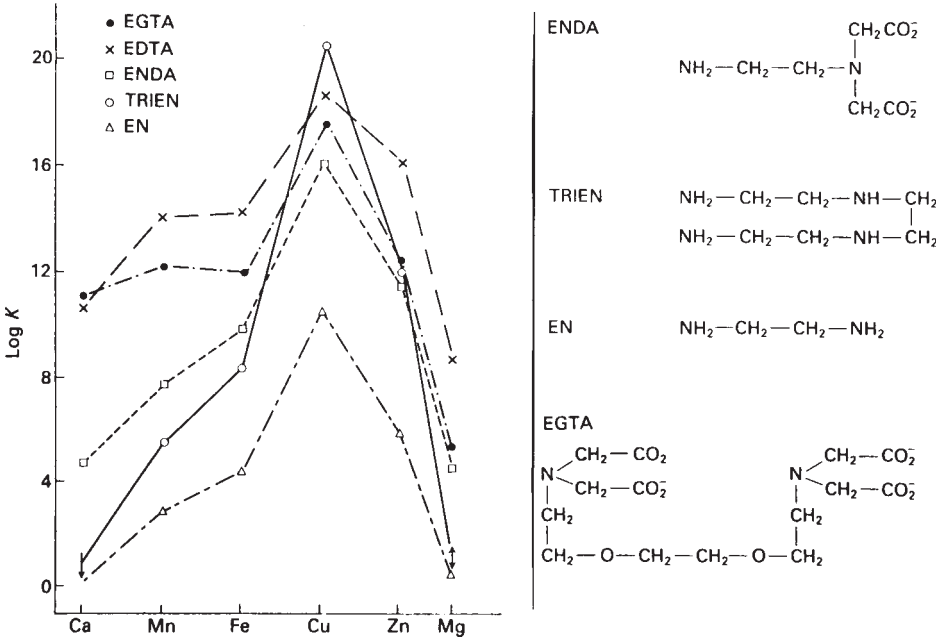


Fig. 4. The stability constants of some complexes of the divalent cations. EDTA has a similar formula to EGTA but with the central -O.CH_2.CH_2.O- unit missing. Values for Mg^{2+} are EDTA 10.5 and EGTA 6.5. Note how the larger ligands which are anions stabilize Ca^{2+} and Mn^{2+}.

Now complex ion formation may not be in thermodynamic equilibrium between free Ca^{2+} ions and bound species. We can illustrate this problem best in the case of Mg^{2+} binding to chlorophyll in proteins. The Mg^{2+} ion is found to be held by 5 N-donor atoms, four from the chlorin and one from the histidine of the protein, **Fig. 5**. This is a very unusual site to be occupied by Mg^{2+} especially when it is remembered that in biological cells there are competing ions such as Fe^{2+}, Co^{2+}, and Ni^{2+} all of which bind ring chelates related to chlorin extremely strongly. We know that in this case Mg^{2+} is forced in chlorin by an insertion reaction and that the hydrophobic nature of the chlorophyll forces this molecule into a protein leaving the Mg^{2+} adjacent to a histidine N-donor. We do not know of any parallel example in calcium chemistry as yet, but the assumption that equilibrium holds may not be universally true.

In **Eq. 3**, we have written the free concentrations of both Ca^{2+} and X^- as determining factors in forming a complex. Hence, we must discover the levels of free Ca^{2+} and of other cations and of the ligands, X^-, in order to see how selectivity of association is managed. In cells, the free-calcium concentrations

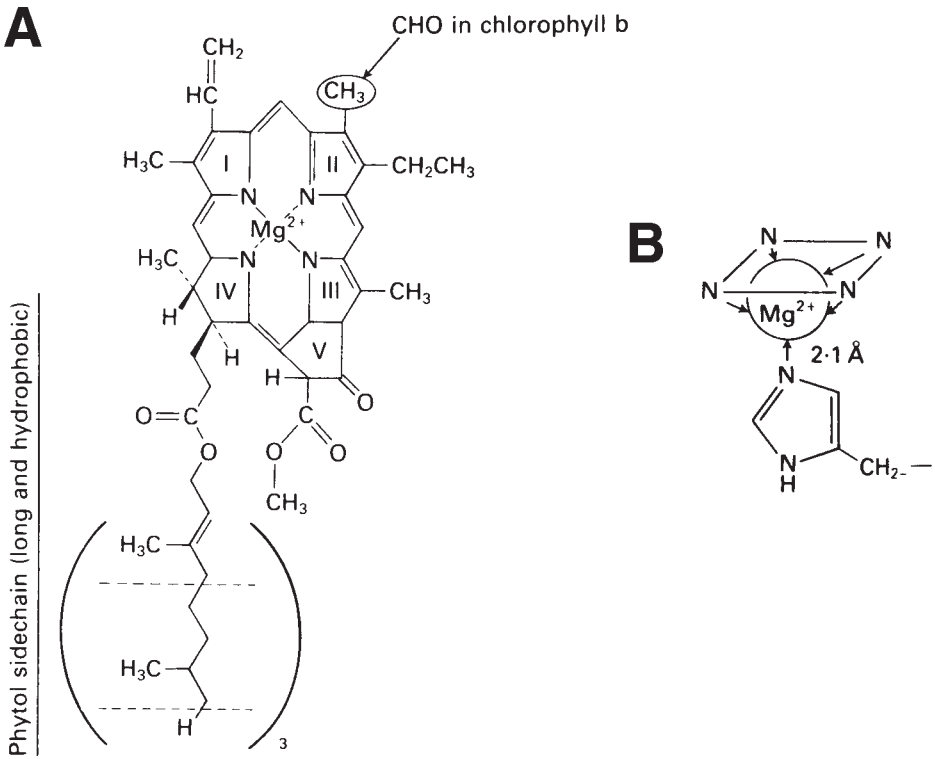


Fig. 5. The formula of chlorophyll and an illustration of how Mg^{2+} nearly fits the central cavity. N- and 5-coordination is forced upon the Mg^{2+} ion.

are manipulated by pumps in membranes and the free concentrations of binding ligands, particularly proteins, are also controlled by pumping across membranes in addition to the controls over their syntheses. This pumping reduces the Ca^{2+} ion concentration to 10^{-8} *M* in the cytoplasm of resting cells. It is the reaction of such low Ca^{2+} concentrations that have to be protected from competition from the ions in the Mn^{2+} to Zn^{2+} series. Ca^{2+} concentration in vesicles or in extracellular fluids is usually close to 10^{-3} *M*. Ligands to which Ca^{2+}, rather than other cations, can bind depends on the competition from other cations and ligands *in the same compartment*. Outside the cell, the design of binding agents has to allow Ca^{2+}, but not Al^{3+} binding and complex ion formation. The competition between cations for a given ligand is then dependent on the condition of the solution, which includes all other anions, pH, and cations. The constant for binding is then called a conditional binding constant.

It is now worth stressing that the Mg^{2+} concentration in the cell cytoplasm is 10^{-3} *M* so that without giving precipitates it can bind to many organic anions,

especially phosphates, e.g., adenosine triphosphate (ATP). It is not the difference in stability constant that prevents Ca^{2+} binding, because its absolute constants are very closely similar to those of Mg^{2+}, but the difference in the permitted concentration, 10^5, between these two divalent ions in the cytoplasmic solution.

7. Ca^{2+} and Transition Metal Binding

All simple ligands bind metal ions in the order

$$(Ca^{2+}<)\ Mn^{2+} < Fe^{2+} < Co^{2+} < Ni^{2+} < Cu^{2+} < Zn^{2+},$$

and all these metal ions bind all types of donor more strongly than Ca^{2+}. Without constraints on the free metal ion concentrations in cells and on the steric demands of complicated ligands Ca^{2+} would not be able to bind to any organic molecule in cells. In the first instance the concentrations of competing metal ions in the cytoplasm is reduced by binding to stronger donors so that the free ion levels are approximately

	Mn^{2+}	Fe^{2+}	Co^{2+}	Ni^{2+}	Cu^{2+}	Zn^{2+}	Cd^{2+}	Ca^{2+}
$\log[M^{2+}_{free}]$	–7	–8	–9	–11	–15	–12	–15	–8

At these concentration levels, none of these ions can bind to the calcium-binding sites because the structures generated by the protein folds that hold calcium do not allow a collapse of ligand donor groups to give a smaller hole size than a radius of 1.0 Å and so chelation of the resultant O-donors is relatively weak. The radius of Ca^{2+} is 1.0 Å, whereas the other ions have the following average radii (Å)

Mn^{2+}	Fe^{2+}	Co^{2+}	Ni^{2+}	Cu^{2+}	Zn^{2+}	Cd^{2+}
0.75	0.70	0.68	0.65	0.60	0.65	0.90

We see the effect of size very clearly when we compare ethylenediaminetetracetic acid (EDTA) with EGTA binding, **Fig. 4**. Note that Cd^{2+} is removed overwhelmingly by its preferential binding to strong RS^--containing proteins such as metallothionein because otherwise it would be a serious competitor. Using selective chelation we can see how a cell can devise stratagems for even leaving a good calcium conditional-binding site, $K = 10^7$, open and unoccupied in the presence of other metal ions at the concentrations of free ions found in cells.

Now, whereas we have described the simplest equilibria

$$Ca^{2+} + X^- \rightleftarrows CaX^+,$$

there are many examples of more complex reactions of the kind

$$nCa^{2+} + X^{2-} \rightleftarrows Ca_nX^{(2n-2)+} \qquad (4)$$

and

$$Ca^{2+} + X^{-} + Y^{-} \rightleftarrows CaXY, \quad (5)$$

or even

$$nCa^{2+} + mX^{-} + pY^{-} \rightleftarrows Ca_nX_mY_p \quad (6)$$

These cases can result in cooperative or anticooperative combinations where the ultimate extent of cooperation is precipitation. You will find throughout this volume many examples of cooperative binding of several calcium ions to one protein, e.g., in calmodulin, and of extensive crosslinking by Ca^{2+} to give CaXY chains as in fibrillin. The exact distribution of species in these cases is often extremely difficult to evaluate.

8. Condensation Equilibria

Now there is an intermediate condition between simple-complex ion formation, **Eq. 1**, and precipitation. Consider a large polymer such as DNA with a distribution of negative charge all along its backbone. There is association with cations, but it is observed that although the DNA remains in solution, it can collapse and condense into a very small volume at a critical concentration of the cation. It is not necessary for the cation to be bound directly to the polymer, but it is held by the high-surface potential generated by the fold. We believe that this is the mode of retention of Ca^{2+} by sequestrin (also, *see* Chapter 18, in this volume. Here, the protein surface has a high concentration of carboxylate groups. Segments of it collapse, or rather now condense, around some 40 Ca^{2+} ions so that effectively they all have the same binding constant. Because the Ca^{2+} is in equilibrium with free ions, it is readily released on exposure to solutions of low free Ca^{2+}.

9. Calcium, Membranes, and Walls

The membranes of all cells are negatively charged and as such attract Ca^{2+} ions. Now there is no requirement for either an even distribution of Ca^{2+} across membranes or along membranes so that Ca^{2+} together with other cations may form a pattern of cations lying under the membrane. This binding can also cause a membrane to collapse. In fact, membranes are supported by an underlying structure of proteins that prevent condensation as described. They are likely to be localized differences both along a membrane and across it of its negatively charged headgroups. Together, with localized membrane curvature, these differences will lead to corresponding variations in associated calcium-ion concentration. A recent atomic force microscopy study shows the way Ca^{2+} induces coalescence of lipids into domains (*see* **ref. *9***). However, because the calcium ions have pumps and channels forcing or allowing Ca^{2+} ion flow, the

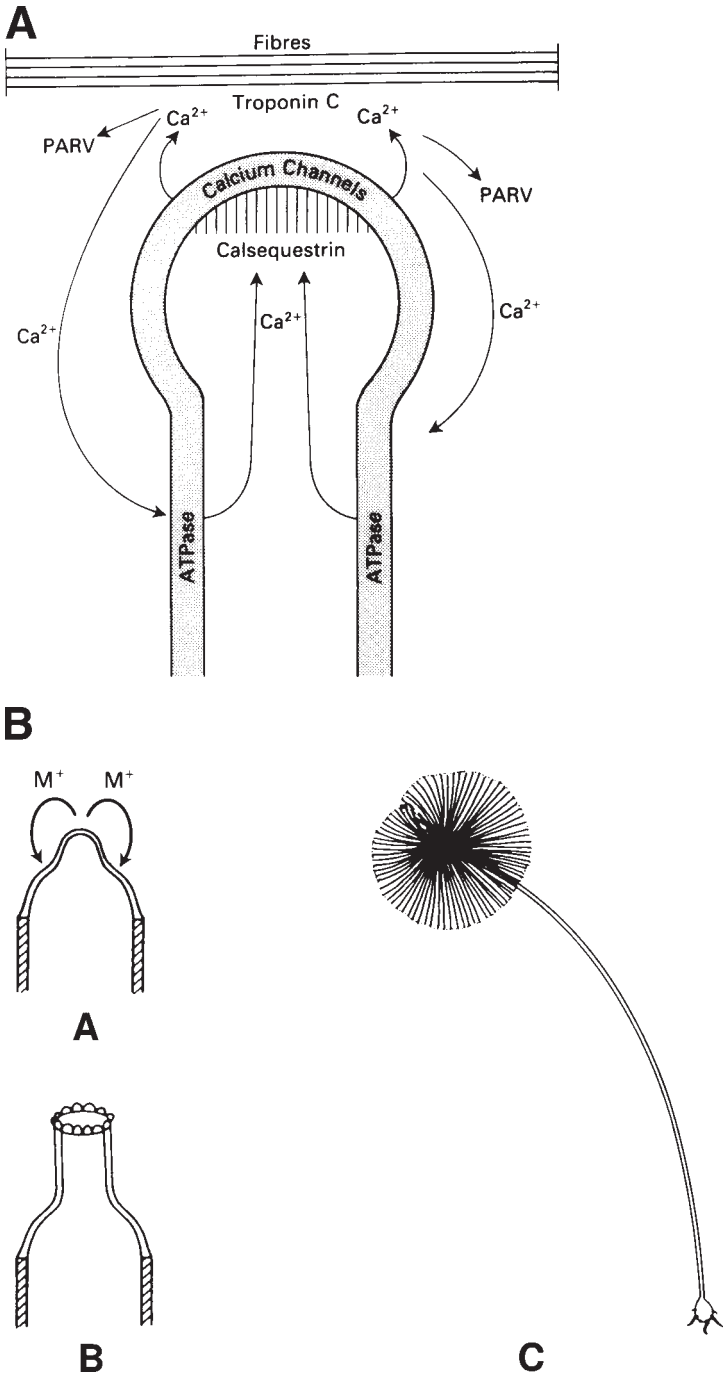


Fig. 6. The calcium currents around the terminal of a T-tubule of the sarcoplasmic reticulum.

Table 6
Variety of Ca^{2+}-Binding Proteins

Class	Examples	Strength (log*K*)
Classical EF-Hand	Calmodulin, Parvalbumin Troponin-C	7
Nonclassical EF-Hand	S-100, Calbindins	7
Others	Annexins (+ Lipids)	3–4(7)
	C_2 Domains (+ Lipids)	3–4(7)
	Phospholipases (+ Lipids)	3–4(7)

Table 7
Extracellular Binding Proteins

Protein Class	Binding Constant
Digestion	Proteases, saccharases, nucleases (Ca^{2+} often activates)
Bone proteins	Osteocalcin, phosphophoryn
Storage	Calsequestrin

association of membranes with Ca^{2+} must then produce Ca^{2+} currents locally (*see* **Fig. 6A**,**B**).

10. The Fixed Binding Constants for Calcium Ions in the Cytoplasm

So far we have described proteins available for calcium binding $K = 10^7\ M^{-1}$, but most of them remain unbound in the resting state of the cell when $[Ca^{2+}]$ is less than $10^{-7}\ M$. On excitation, the Ca^{2+} rises to $10^{-6}\ M$. An amazing feature of the binding by intracytoplasmic proteins on excitation is therefore that, despite their variety, **Table 6**, the binding constants in the precise place where they are used, are virtually identical and close to $10^7\ M^{-1}$. This is a necessary feature because they must all respond to the *same input* concentration of calcium from stores. To manage this result for many different sites, for example, calmodulins and S-100, and even many combinations of sites, for example, annexins or phospholipases plus phospholipids, is a remarkable achievement of evolution. It implies that unless proteins have different time constants of action then there will be intense cooperation between many Ca^{2+} triggered events. A parallel achievement is found in the extracellular fluids where the binding constants for calcium of many different sites is close to $10^3\ M^{-1}$ compared with $10^7\ M^{-1}$ for the internal cytoplasmic sites, **Table 7**. Here, external sites include those of the extracellular fluids and those of the endoplasmic reticulum. In each case, too,

the amount of protein present in the case of mammals is controlled so that equilibrium is balanced close to the solubility product of bone, which gives a precise standing concentration of calcium around 10^{-3} *M*.

Concentration gradients of 10^4 across both the exterior cytoplasmic membrane and the interior endoplasmic reticulum membrane are maintained by ATPase pumps rejecting calcium from the cytoplasm. The pumps stop once the internal calcium is below the binding constant of the internal proteins, e.g., calmodulins. This is an example of feedback control, which is very common in cells. In animals, bone buffers the external fluids while excess calcium is rejected by the kidney and epithelial cells manage the intake. An animal then has a tight homeostasis relative to that which a plant can achieve outside cells. If the Ca^{2+} concentration rises to 10^{-5} *M* internally, the cell is killed and this may be part of the process of apoptosis.

In the discussion of Ca^{2+} binding, the general effect of physical forces as opposed to chemical binding must not be forgotten. The membranes of biological cells usually carry a potential because of the overall directional pumping of ions. The negatively charged side of the membrane concentrates all cations near it while the positively charged side repels them. When Ca^{2+} ions move through membranes, physical fields are of considerable consequence.

The implication of this vast accumulation of data concerning calcium binding to a diversity of proteins inside cells, outside cells, and in vesicles and organelles is that much, if not all, the calcium is bound in the resting state of a cell at equilibrium. The need to protect the inside cellular DNA from calcium and yet use the calcium gradient in signaling (allowing Ca^{2+} to enter the cytoplasm) has demanded a free Ca^{2+} of 10^{-8} *M* so as to allow the evolution of series of Ca^{2+} binding *systems* all with binding constants close to 10^7. At the same time, the need to protect external fluids from general damage, while allowing specified precipitation, demanded a maximum concentration of some 10^{-3} *M* Ca^{2+}. So that external signaling should be operationally effective, for example, the onset of digestion by release of hydrolases to the external calcium, the concentration in external fluids and in vesicles could not fall below 10^{-4} *M* Ca^{2+}. To secure both buffering and further value of Ca^{2+} ion properties in these external fluids, demands a second vast series of Ca^{2+}-binding proteins to which Ca^{2+} bound with log*K* approx 10^4. These features began to appear as cellular life appeared and then developed extreme degrees of sophistication (*see* **Fig. 7** and other chapters in this volume).

11. Calcium and Aluminium (Acid Rain)

We have stated most calcium salts are more soluble in acid solution and this applies also to the salts of aluminium. Of particular importance is the release of Al^{3+} from clay minerals of soil. The minerals are aluminosilicates with a vari-

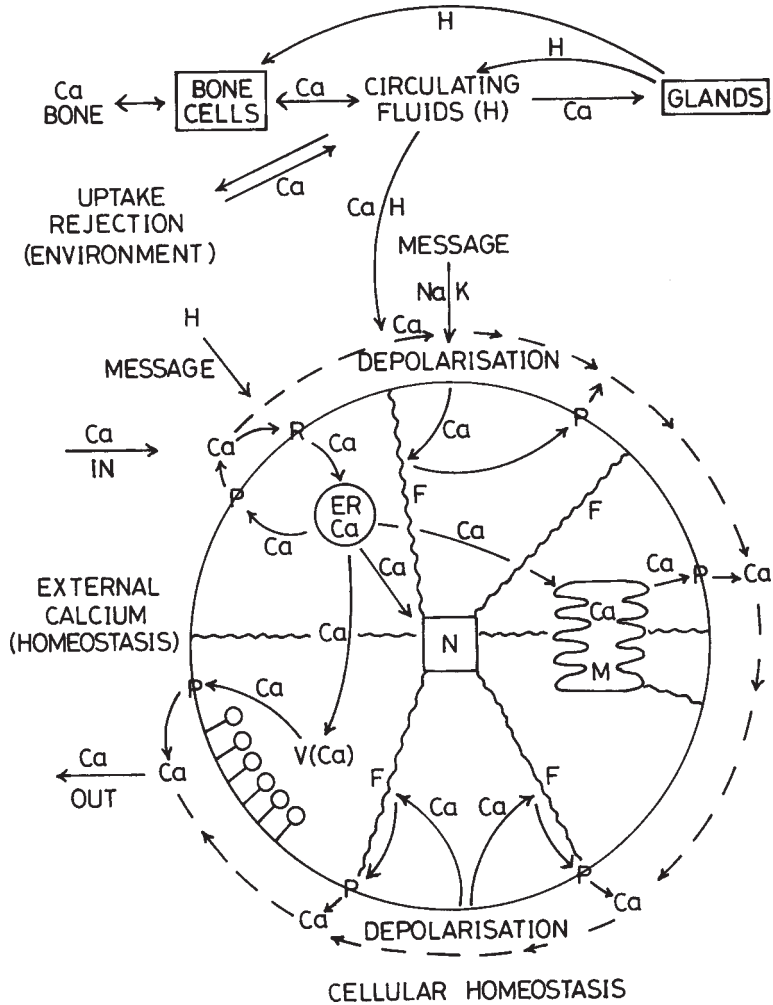


Fig. 7. **(A)** The vast complexity of calcium current flow in advanced cells (N-nuclease; ER, endoplasmic reticulum; M, mitochondria; H, hormone; F, filament), compare the simpler flow in **(B)** the tip of acetobalaria.

ety of other cations. When soil releases aluminium to waters, this metal ion damages plant life. We need to see how.

The external surfaces of most plant roots and indeed the exposed surfaces of most cells from bacteria upward are made of anionic polymers. The anions are neutralized to some extent by calcium which then crosslinks the extracellular matrix. The polymers are anionic in fair part because of the presence of carboxylate groups with a $pK_a < 5$ so that protons do not compete with Ca^{2+} until

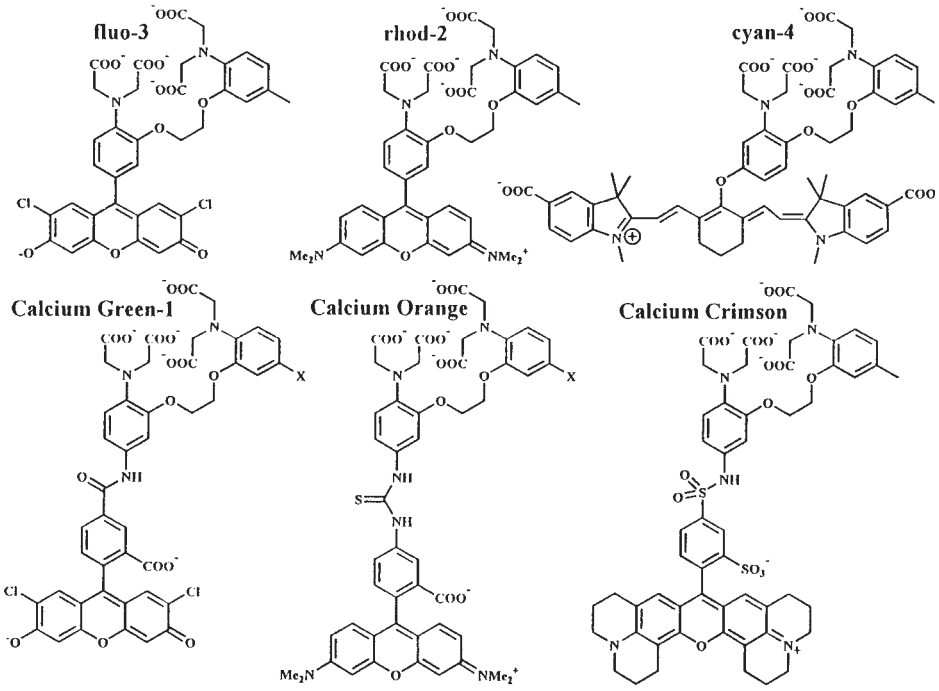


Fig. 8. The formulas of some calcium dyes and indicators.

extreme and very unusual acid conditions. Ca^{2+} binding is then pH independent from pH = 7.5 down to 5.0. Aluminum in silicates of the soil is extremely pH dependent and even the favored groups of organic matrices at pH = 7.5 for aluminum binding, phenolates, have a high pK_a (>10.0) so that they too hold Al^{3+} less and less as pH is lowered. Decrease in pH, acidity, then causes free Al^{3+} to increase and Ca^{2+} to be displaced by Al^{3+}, which, once released from soil or organic phenolates, will bind better to carboxylates.

There is in humans a disease called dialysis dementia, which is caused by Al^{3+} getting to the brain and destroying cells. It could well be that because the brain fluids are very low in calcium and there is no bone in contact with the fluid, once Al^{3+} enters these fluids it destabilizes cells by effectively destroying Ca^{2+} crosslinks. As stated earlier, Al^{3+} is also accumulated in bones.

12. Calcium Indicators

Indicators of free-calcium concentration are based upon selective calcium binding to fluorescent dyes or through the use of selective calcium electrodes. The commonly used dyes, **Fig. 8**, mimic the selective binding by EGTA. Their

effective binding constants are similar to that of EGTA at pH = 7.5 but notice that the use of an aniline-N and not an ammonia N in the construction lowers the pK_a from around 10 to about 6.5. Thus, at pH = 7.5 the binding by these dyes is pH independent. (Of course they have an absolute binding constant approx 10^2 to 10^3 lower than EGTA.)

The dyes do bind Mg^{2+} much as does EGTA, but with a binding constant of around 10^3. Hence, Mg^{2+} binding only interferes slightly with Ca^{2+} estimations. Although free Ca^{2+} variations in cells are quite dramatic, free Mg^{2+} concentrations hardly vary so that calibration of the dye-stuffs response to calcium changes should be relatively easy, though there is much controversy. Notice that interference from Mg^{2+} is avoided again by the steric constraints of a large chelation system.

With respect to interference, a better reagent would be a pure O-donor ligand. In fact, biological proteins of the required kind are available as indicators. The best known and most used is aequorin, which is, in effect, a fluorescent calmodulin. It does not suffer from Mg^{2+} or other metal ion competition in its binding with calcium.

Nothing said so far has related to the kinetics of Ca^{2+} ion reactions.

13. Diffusion Rates

The rate of diffusion of a small unit such as the calcium ion is dependent upon a constant, k_{diff}, multiplied by the ion concentration. The limiting diffusion rate in water for an ion is related to the dissociation of water molecules from around the ion. The calcium ion has an almost optimal diffusion rate of 1.0×10^9 m^2/s^{-1} and an almost optimal water exchange rate of 10^8 to $10^9/s^{-1}$. This means that at high concentration there is no difficulty for the equilibration of calcium ions in water solutions of considerable volume, e.g., a millimolar solution in a beaker of water with gentle stirring. The problem in biological solutions is very different. In cells, the $[Ca^{2+}]$ is $< 10^{-7}$ *M*. Distribution over μm distances is now restricted. It is further restricted by the many negative surfaces of membranes and proteins. In fact, space is very crowded in cells (*see* **Fig. 9**). To overcome this problem, cells have evolved carrier molecules: proteins, which being negatively charged and with a high affinity for calcium can reequilibrate calcium concentrations. They are at a concentration of 10^{-3} *M* and redistribute Ca^{2+} faster than Ca^{2+} at 10^{-8} *M* can diffuse by itself. We believe this is the function of the protein, calbindin, which is described in Chapter 10, in this volume. The Ca^{2+} effectively takes a ride on the back of the protein.

14. Time Constants and Ca^{2+} Action

Certain cellular operations must be triggered by Ca^{2+} release and then relaxed while much other cell activity is not perturbed. There are two ways of managing

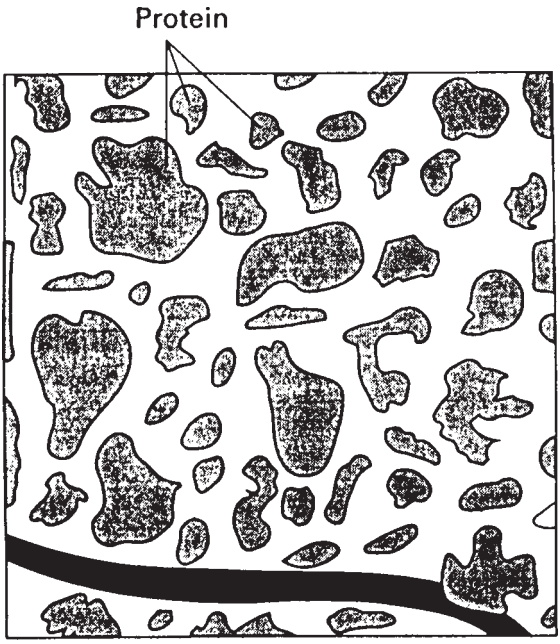


Fig. 9. The packing of molecules in cells indicating how crowded is biological space.

isolation of an activity. The first is to have some binding reactions and their relaxations much faster than others, whereas the second concerns the localized release of calcium ions within the total cell volume. Binding rates must be controlled at the on-rate step, which implies that some proteins accept Ca^{2+} more slowly than others. Two mechanisms are open. 1) The calcium site is blocked by an ion that leaves the site slowly. The obvious choice is Mg^{2+}, which, in fact, blocks access to the Ca^{2+} sites of parvalbumin. In a fast muscle, action is as follows:

$$\text{Troponin C} + Ca^{2+} \overset{\text{fast}}{\rightleftarrows} \text{Action}$$

$$\downarrow\uparrow \text{ slow}$$

$$Mg^{2+}\ \text{Parvalbumin} + Ca^{2+} \rightleftarrows Ca^{2+}\ \text{Parvalbumin} + Mg^{2+}$$

The effective binding constants are the same for the two proteins, troponin C and parvalbumin, but binding is slower to the second. A final slower step is the outward pumping of Ca^{2+} by the membrane ATPase.

The second possibility 2) relies on the fact that Ca^{2+} release into a cell at a certain point may be buffered locally so quickly that Ca^{2+} fails to reach most of

Table 8
Calcium Light Isotope Enrichment

Tissue	$^{40}Ca/Ca^{44}$ Enrichment[a]
$CaCO_3$ of foraminiferae formed directly from sea water	1.00^{+}
$CaCO_3$ of coccoliths formed by internal precipitation of calcium in vesicles	2.15^{+}
$Ca_2(OH)PO_4$ of deer bones. Note passage of calcium through many steps in plants and animals	2.50^{++}

[a]Enrichment of lighter isotope in thousandths. Data from **refs. *7*** and ***8***.

the cell. For example, at a nerve synapse, the calcium pulse does not influence the center of the cell.

15. Calcium Exchange

If our understanding of calcium binding and kinetics are correct, then calcium should exchange rapidly with its surroundings until it is trapped within a permanent structure. In such a case, the calcium binding in living organisms would equilibrate quickly with external calcium, but at a level manipulated by energy input at pumps. However, if calcium becomes trapped during the processes of metabolism, say as shell or bone, then the calcium no longer equilibrates. The study of the fractionation of isotopes of light elements such as H, C, and N indicate that the fractionation in such a thoroughly equilibrated process is likely to be small. However, in a series of rate-limited steps leading to a trapped atom, then much larger kinetic isotope fractionation can occur. It is possible today to apply this analysis to heavier elements. For example, the analysis of sulphur isotope ratios has been used to distinguish sulphide $\rightleftarrows$ sulfate exchange reactions of mineralogical as opposed to biological origin. The same approach can be used to analyze calcium fractionation.

16. Ca^{2+} Isotope Distribution and Evolution

Recently it has become possible to follow the kinetics of calcium flow using isotope fractionation. As is usual, lighter isotopes pass over barriers more easily than heavier ones. In the case of calcium, this means that the $^{40}Ca/^{44}Ca$ ratio increases on calcium passage through rate-limited chemical transfer steps. **Table 8** shows that the light isotope enrichment is greatest in those tissues that are made by passing calcium through the greatest number of steps. In evolution, the number of steps through which calcium may pass has increased with

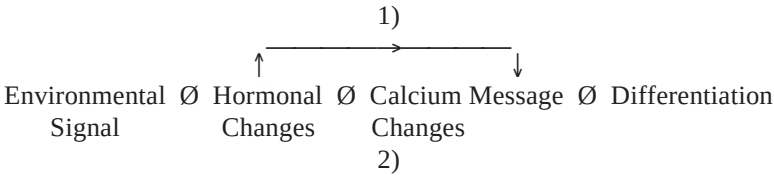
the complexity of organisms. The determination of a isotope ratio in a sediment indicates the type of organism that existed at a given time. Perhaps as expected the ratio is greatest in the order formation of external precipitates (coral) < formation of crystalline shells (coccoliths) < formation of bone (deer).

17. Calcium in Evolution

We introduce this topic here because it is necessary background to the use of Ca^{2+} isotope distribution. A major development in evolution, probably prior to the development of all, but very low levels of dioxygen, was the involvement of calcium described above, *see* **Fig. 7**. At first, calcium ions only strengthened exterior membranes or formed crudely constructed external precipitates as seen today in algal coral reefs. The next development in single-cell eukaryotes is seen in the *foraminiferas* (dating back to more than 10^9 yr ago) which have well-ordered crystalline *externally formed shells*, and in coccoliths, which now form shells from *units made in internal vesicles*, in fossil deposits. It is also notable that features common to many unicellular protozoa, depend on calcium. Examples are the cilia beating of *paramecium*, the stalk contraction of *vorticellia*, and cytoplasmic streaming in *physarium*. Again yeasts, which are fungi and separated from both bacteria and the antecedents of plants and animals around 1–2 billion years ago, have well-developed calcium control systems. In all these early examples, calcium gradients are associated with slow development, or even constant activity, so that it would appear that the stress of today's scientific investigation of fast triggering by calcium *in higher animals*, is misplaced, if applied to the whole of calcium functions. Calcium concentration changes are an essential feature of differential growth and only later do they become used in fast triggering useful for mobile animals. Growth with development is, of course, a continuous feature of all eukaryotes, while prokaryotes just divide. The final extension to the use of fast changes comes in animals, but we should see first the developing actions of calcium in plants, especially perhaps in primitive plants.

Undoubtedly, the first multicellular organisms were plants. Hence, it is to plant life we turn to appreciate how the functions of calcium developed after those in eukaryote single cells. Immediately we see that large calcium gradients were maintained across the cytoplasmic membrane, but the extracellular fluids were not particularly well controlled. The endoplasmic reticulum and the vacuole of plants quite generally were high in calcium also. The novel functional value of calcium lay now in the relationship through its concentration in the cytoplasm to the level of the newly acquired set of organic messengers, which went between cells — the plant hormones and control molecules such as phytochromes. It is these molecules which to this day control the path of differentiation during growth. Thus, calcium became a major player in linking the

outside environmental changes with the internal metabolism and differentiation into organs during the plant life cycle through a sequence of events in which the hormone 1) could or 2) could not penetrate the outer membrane. Only in case 2) was a calcium message triggered.



A very interesting example seen today is the adjustment of the steady-state cellular calcium concentration with the exposure to light and even light of different wavelengths. It is thought that the changes in calcium alter the structural organization of filamentous constructs such as actino-myosin and tubulin so allowing chloroplast reorganization and streaming and also changing development. Additionally, the new calcium levels act to adjust phosphorylation, via kinases, and then to alter gene expression as in 2) above. A very similar response in plant roots is that to Earth's gravitational field. Here, the calcium current circulating at the root tip (*see* **Fig. 8**) is apparently affected by gravity so that the internal calcium levels change and then affect the downward growth of the root.

Calcium is again not to be seen as a trigger for immediate fast response, but as the intermediary in the slow responses of development during growth and opposite a changing environment. (Of course, some fast responses such as the closing of a fly-trap plant are related to calcium also). It may be that calcium circulates constantly through the cytoplasm of all cells, maintaining homeostasis locally in different ways according to the environment, and the placings of calcium pumps and channels. Circulation changes of flow adjust the calcium input to the cytoplasm and the new stationary level of this ion then is relayed via phosphorylation to bring about cellular development. It is probable too that the unavoidable rise in basal level calcium as repeated waves of free calcium sweep cells (*see* **Fig. 10**) is, in part, responsible for differentiation. This can be seen in the development of an egg after fertilization. It is becoming apparent that calcium is involved in a vast number of steps in cells connecting the external conditions to cell development. We then must use network diagrams to illustrate the function of calcium in assisting cell homeostasis as well as triggering (*see* **Fig. 11**).

The final step in calcium circuitry in animals adds to the sophistication of the circuits of plants. Here, the external calcium experienced by cells is held constant and so is the temperature in the body's external solutions. This has allowed tight control over calcium injection into cells and has allowed rapid

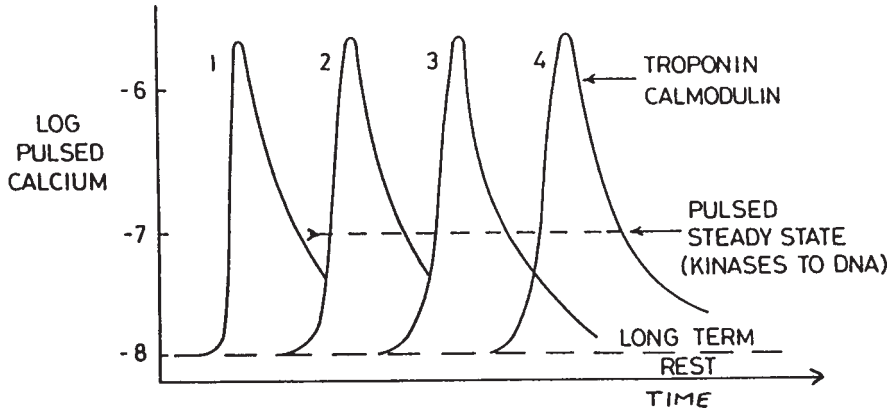


Fig. 10. The effect of a series of calcium waves is to raise the base level of free calcium. Hence, one wave triggers very fast responses, but series of waves in quick succession trigger slow responses of great sensitivity to calcium.

responses to be separate from slower activities. The best control of these external fluids is by bone as illustrated in the final paragraphs.

18. Calcium Circuits

All ions can carry currents much in the same way as electrons do but at lower speed. Hence, ions can be used to form circuits just as electrons can. A circuit needs, of course, a power supply and a conducting medium. For calcium in biological cells, the power supply is the ATPase pump, which gives the gradients across membranes. The conducting medium is water in bulk or in membrane channels. A circuit can be connected to many devices such as switches on channels, such as calmodulin for example, or condensers, such as sequestrin in the endoplasmic and sarcoplasmic reticula. Now to link the many parts of a circuit in a harmonious activity, it is necessary to introduce feedback flow of the current carrier, here calcium. This has been discussed in many publications. Given the large number of devices in a cell all connected to calcium flow it is probable that all eukaryote cells have a constant, as well as a stimulated, calcium current monitoring and keeping activity coherent. A possible view of evolution is that the change of prokaryote to eukaryote involved the development of just such a calcium circuit. Thus, although suitable organic polymers evolved in cells to make structural and functional machinery, their control circuitry appeared through the use of internal connections in the cytoplasm based initially in prokaryotes on proton, electron, and phosphate, as well as on some substrate metabolism and iron internal flows. Later in eukaryotes all these internal circuits became connected to Ca^{2+} flow across membranes that allowed

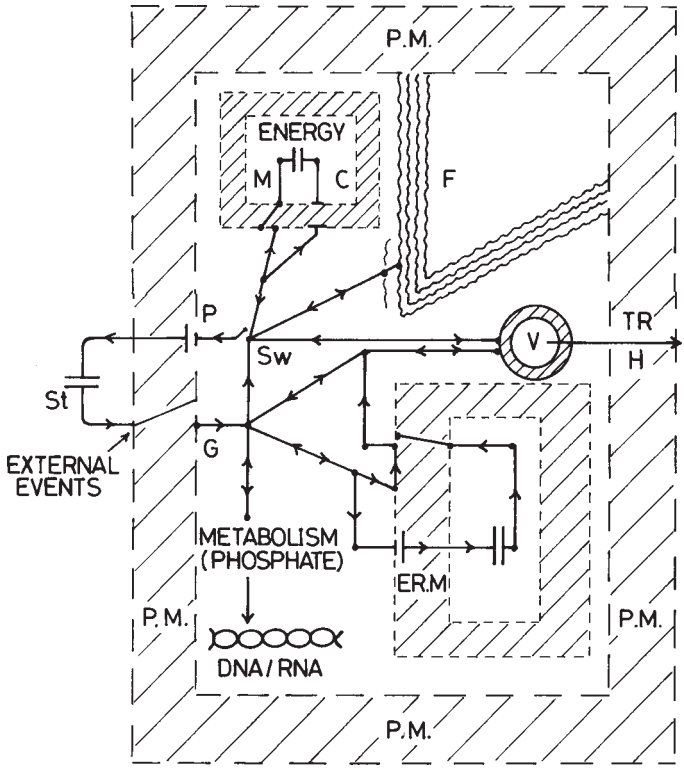


Fig. 11. A circuit diagram for calcium flow connecting a large number of internal activities to the external environment. M, mitochondria; C, chloroplast; V, vesicle or vacuole perhaps including oxalate production, as well as exocytosis; ER.M. endoplasmic reticulum membrane; P.M., plasma membrane; P, pump; G, gate; Sw, switch; F, filament; St, external store; TR, transmitter; H, hormone.

an interaction between the external environment and the cytoplasm. The further development of multicellular organisms depended upon the evolution of organic (current) carriers in circuits; the brain appeared once Na^+/K^+ circuits were developed, and evolutionary use of ion flow in nerves similar to, but simpler than, that of Ca^{2+} and based upon the production of Na^+/K^+ ion gradients for earlier and quite other reasons, namely osmotic and electrolyte balance (*see* **Table 9**). Finally, external circuits arose once man discovered how to use the electron.

19. Calcium Pumps and Buffers

To create the calcium circuits, the power system for the ion, the calcium pump, has to be selective against other ions. We know that the membrane part

Table 9
Evolution of Simple Ionic Equilibrium Signals

Primitive Organisms Prokaryotes	$Mg^{2+}/ATP^{4-}/HP^{2-}$ controls phosphorylations Fe^{2+} controls redox equilibrium $Na^{+}/K^{+}/Cl^{-}$ control osmotic pressure
Single cell Eukaryotes	Ca^{2+} controls activated states and relationship to environment Mn^{2+} controls development of plant-related organisms
Multicellular Organisms	Zn^{2+} controls hormonal responses relating to growth and development and connective tissue $Cu^{+}(Cu^{2+})$ controls connective tissue responses. Extended use of Ca^{2+} in excited states. Generation of Na^{+} (K^{+}) signaling and the evolution of the nervous system.

of the pump contains a receptor site for calcium with one carboxylate group and several carbonyls. To be useful in function, this receptor has to switch its binding strength from an uptake log$K$ = 7 (inside) to a release log$K$ = 3 (outside) as the calcium is rejected from the cell. In other words, because of the input of energy from ATP (or a coupling of a reverse gradient of Na^{+} or H^{+}) the conventional strong binding of the cytoplasm has to be weakened to that of the binding in extracellular fluids. Obviously, an energized conformational switch is necessary. Although a crystal structure of the pump will soon be available (*see* **ref. *9***), I will describe a working hypothesis here, as shown in **Fig. 12**. This pump has a feedback switch-off caused by calcium binding to an ancillary protein of the pump, calmodulin. Thus, the pump operates at a basal level of flow opposite 10^{-7} *M* Ca^{2+}, as do all the calcium-based components of the Ca^{2+} circuit. Note how on rise of Ca^{2+} through activated channels, all parts of the circuit are based on interaction of binding constant, 10^{6} to 10^{7}. This allows a rapid change of state provided relaxation is fast. However, multiple rapid pulsing or the absence of proteins for *fast* relaxation lead to a different bias in the whole circuit. This, in turn, can communicate to other circuits, e.g., that of phosphate making one communication network; that of Ca^{2+}, link to another; that of phosphate, and then to a third; for example, the proton, and so on.

20. Calcium Exchangers

Cells have always required ways of lowering calcium concentration in the cytoplasm because calcium at high concentrations $>10^{-5}$ *M* tends to coagulate many biological polymers. The primitive mechanism for reducing [Ca^{2+}] appears to be using the Ca^{2+}/H^{+} exchanger because this is found in all bacteria even where the Ca^{2+} ATPase is not observed. The importance of the ATPases

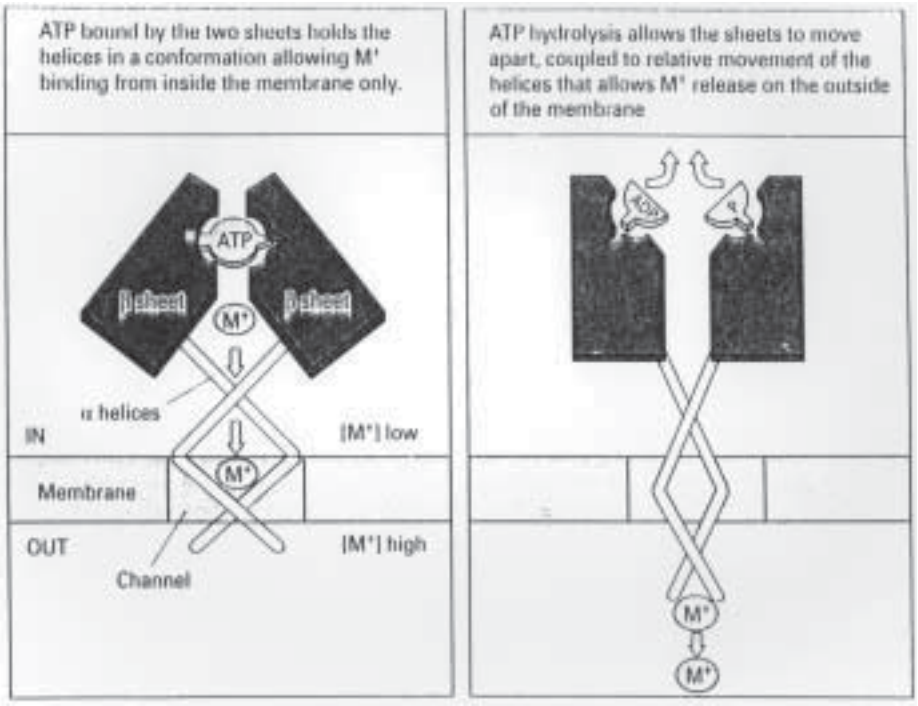


Fig. 12. An idealized version of an ion pump. The ATPase acts much like any kinase in a hinge-bending mode. The pumping is caused by cyclical transformations of the helices of the membrane causing gating of Ca^{2+} flow. The activity is different from the proton ATP-synthase and is probably more primitive being related to the pyrophosphate synthase.

develops as speedier response and relaxation are required in eukaryotes. The earliest organisms may not have needed a very low calcium concentration, that is below 10^{-5} *M*, as indicated by the level of tolerance of bacteria to high external calcium and the viable character of organelles to quite a high Ca^{2+} intake. Notice also that the process of sporulation common in lower organisms frequently requires uptake of considerable amounts of calcium without killing the organism when making a spore.

21. Calcium Networking between Compartments and Organelles

The flow of calcium into the cytoplasm from external sources is coupled to the flow from the endoplasmic reticulum. This flow raises the level of free calcium, which during relaxation, is pumped out of the cell and back into the reticulum or it is pumped into mitochondria or chloroplasts. The effect of cal-

cium in these organelles is to increase 1) the metabolism of substrates in mitochondria via activation of dehydrogenases, or 2) to generate increase photochemical activity in chloroplasts. Both these changes lead to increase in proton gradient activity and then increase in ATP production. Owing to the restricted diffusion of both Ca^{2+} and H^{+}, this upgrading of ATP occurs only locally, but of course, it aids the rapid recovery of the cell because ATP is required to pump calcium into vesicles (ER) and out of the cell. Other branches of the ER may be responsible for the delivery of proteins or lipids to mitochondria or chloroplasts. It is observed again that there are local Ca^{2+} stimulated zones for these uptakes.

22. Summary

This chapter describes the chemical and biological value of the calcium ion. In calcium *chemistry*, our main interest is in equilibria within static, nonflowing systems. Hence, we examined the way calcium formed precipitates and complex ions in solution. We observed thereafter its uses by humankind in a vast number of materials such as minerals, e.g., marble, concrete, mortars, which parallel the biological use in shells and bones. In complex formation, we noted that many combinations were of anion interaction with calcium for example in the uses of detergents and medicines. The rates of exchange of calcium from bound states were noted but they had little application. Calcium ions do not act as catalysts of organic reactions.

In biological systems, interest is in the above chemistry, but extends to the fact that Ca^{2+} ions can carry information by flowing in one solution or from one solution to another through membranes. Hence, we became interested in the details of rates of calcium exchange. The fast exchange of this divalent ion from most organic binding sites has allowed it to develop as the dominant second messenger. Now the flow can be examined in vitro as calcium binds particular isolated proteins, which it activates as seen in physical mechanical changes or chemical changes and this piece-by-piece study of cells is common. Here, however, we have chosen to stress the whole circuit of Ca^{2+} action indicating that the cell is organized both at a basal and an activated state kinetic level by the steady state flow of the ion (*see* **Fig. 11**). Different time constants of exchange utilizing very similar binding constants lead to: 1) fast responses as in the muscle of an animal; or 2) slower change as in differentiation of an egg or seed. Many other changes of state may relate to Ca^{2+} steady-state levels of flow in the circuitry and here we point to two: 1) dormancy in reptiles and animals; and 2) sporulation in both bacteria and lower plants.

In the other chapters of this volume many components of the overall circuitry will be described. The reader should try to marry these into the overall activity of the cell for on top of molecular biology there is the cooperative

system molecular biology of cells. To give an analogy, whereas much can be understood from the analysis of the properties of single-isolated water molecules, even examining their interaction in ice, this study alone cannot lead to an appreciation of the melting or boiling points of bulk water.

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