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HISTORICAL PERSPECTIVE

This historical perspective of the functions of calcium in biology lays the foundation for understanding the central role of calcium binding proteins. Ten interrelated themes bring us to about 1970. The successes and frustrations of those concepts help us understand recent decades. For clarity, these themes are each treated chronologically; however, there are myriad, complex interactions among them. Much of that integration is left for subsequent chapters and future research. The themes include biomineralization, coagulation, secondary messengers, colloids, cross-linking, pumps, calcium binding proteins, mitochondria, hormones, and measurements.

1.1. BIOMINERALIZATION

The study of calcium in biology began with bone. "Examination of the red stained human bones, excavated in the cemetery of the Community of the Dead Sea Scrolls at Qumran, showed that the pigmentation was due to alizarin. Its characteristic anatomical location on the extremities and in the medullary cavities was consistent with the location of intravital staining due to a diet containing madder root" (Steckoll et al., 1971). Although we cannot say who first observed this staining by madder root, it provides a fascinating introduction to biomineralization, one of the many functions that involve calcium binding proteins.

Calcium Binding Proteins, By Eugene A. Permyakov and Robert H. Kretsinger

In 1581, Lemnius wrote in *De Miraculis Occultis Naturae*: "So some penetrate into the remotest and farthest parts, and are carried to the Nerves, as Hermodactyls, sweet Mullens, commonly called Palsey-hearb, Madder that colours the bones of the Cattle red, if they chance to eat it green, though they touch not the root that is red, which may be seen in the boyled flesh of that cattle, and in sheep that are painted red with decoction of it, as Wood dyes them blew, wild Saffron, and yellow Ocre."

Stephan Hales (1727) performed a remarkably broad range of experiments; growth and development were explored. "I took a half-grown chick, whose leg-bone was then two inches long; with a sharp-pointed iron, at half an inch distance, I pierced two small holes thro' the middle of the scaly covering of the leg and shin bone; two months after I killed the chick and upon laying the bone bare, I found on it obscure remains of the two marks I had made, at the same distance of half an inch: so that that part of the bone had not at all distended lengthwise, since that time that I marked it; notwithstanding the bone was in that time grown an inch more in length, which growth was mostly at the upper end of the bone, where a wonderful provision is made for its growth at the joining of its head to the shank, called by anatomists symphysis."

In 1736, Mr. Belchier, an English surgeon, was dining at the home of a dyer. He noted that the pork bones were red. After investigating, he wrote: "The diet with which the Hogs are fed is Bran, after it have been boiled in a Copper with printed Callicoes, in order to clean them from a dirty red Colour occasioned by an infusion of Madder Root, which was made use of to fix the Colours printed on the Cloth. . . ." "The Brass having absorbed the red Colour discharged from the Cloth is mixed with the common food of the Hogs, and produces this effect on the Bones." ". . . the Bones of several Hogs of a different Breed, changed to a deep red Colour merely Aliment. And what makes this still more surprising is that neither the fleshy nor cartilaginous parts suffer the least alteration in Colour or in Taste."

Mr. Sloane, president of the Royal Society, wrote of Belchier's observation to M. Geoffry, who communicated a letter to the Académie Royal des Sciences in Paris. This letter motivated the du Hamel's research. None of these men appear to have heard of Hales' earlier results. From 1739 through 1743, du Hamel (1739) presented seven "Memoirs on Bone," in which he pursued the analogy between ". . . the formation and the structure of the two types of living bodies, plants and animals. . . ." In the fourth (1743, p. 87): ". . . one proposes to report new evidence that establishes that bones grow in size by the addition of bony layers which originate in the periosteum as the woody parts of trees increase in size by the addition of woody layers, which are formed in the bark." "One obtains by use of madder red layers and white layers distinct from one another . . ." (1743, p. 11). In the fifth (1743, p. 111): ". . . one proposes to clarify by new experiments how the growth of bones follows their length and show that this growth operates by a mechanism very similar to that which one observes in nature for the elongation of the woody parts in the buds of trees." ". . . one takes a six week old chick, the bone of its leg is two inches long; one pierces it with

a drill a half inch from the ankle, one makes another hole a half inch higher, finally one makes a third hole yet half a inch higher & this last hole being a half inch from the knee, so that the length of the bone was divided by half inches." "At the end of the experiment the tibia of this animal was 3 inches long instead of the 2 inches at the beginning of the experiment.. . . " "The first (hole), which at the beginning of the experiment was 6 twelfths from the lower end, was at the end of the experiment at 9 twelfths; thus it was elongated 3 twelfths in this region. The second hole was at the end of the experiment as at the beginning 6 twelfths from the first and from the third hole; there had been no elongation between the first and the third hole. But the third hole which at the beginning of the experiment was located at the termination 15 twelfths away. . . ." "When the bones have not been well hardened, they elongate in all their parts. . . ." For all his ingenuity and eloquence, he missed the significance of the epiphysis.

"Mémoires sur les Os": ". . . la génération & la structure des deux espèces de corps vivants, les plantes & les animaux. . . ." In the fourth (1743, p. 87): ". . . on se propose de rapproter de nouvelles preuves qui établissent que les Os croissent en grosseur par l'addition de couches osseuses qui tirent leur origine du périoste, comme le corps ligneux des Arbes augmente en grosseur par l'addition de couches ligneuses qui se forment dans l'ecorce." "On obtient par le moyen de la garence des couche rouges & des couches blanches fait distinctes les unes des autres. . ." (1743, p. 11). In the fifth (1743, p. 111): ". . . on se propose d'eclaircir par de nouvelles experiences comment se fait la crûe des Os suivant leur longeur, & de prouver que cet accroissement s'opère par un mèchanisme très approchant de celui qu'observe la Nature pour l'alongement du corps ligneux dans les bourgeons des Arbes." ". . . on choisit un poulet âgé d'environ six semaines, l'os de sa jambe avait 2 pouces de longueur; on le perca avec un fout à un demi-pouce de l'articulation du pied, on fit un autre trou un demi-pouce plus haut, enfin on fit un troisième trou encore un demi-pouce plus haut, & ce dernier trou étoit éloingé de l'articulation du genou d'un demi-pouce, de sorte que toute la longeur de l'os étoit divisée par demi-pouces." "A la fin de l'expérience" (treize semaines) "l'os tibia de cet animal avoit 3 pouces de longeur au lieu de 2 pouces qu'il avoit au commencement de l'expérience. . . ." "Le premier, qui au commencement de l'éxperience étoit à 6 lignes de l'extrémité infèrieure, étoit à la fin de l'expérience à 9 lignes; ainsi il s'étoit alongé de 3 lignes en cet endroit. Le deuxième trou étoit à la fin de l'expérience comme au commencement, à 6 lignes du premier & du troisième trou; il n'y avoit donce eu aucun alongement entre le premier & le troisième trou. Mais ce troisième trou qui au commencement de l'expérience étroit éloigné de 6 lignes de l'extrèmité supérieure du tibia en étoit éloigné à la fin de 15 lignes. . . ." "Que quand les os ne sont pas bien endurcis, ils s'alongent dans toutes leurs parties. . . ."

The "Experiments and Observations on the Growth of Bones" of the famous surgeon John Hunter were read some years after his death in 1798 by Mr. Horne (J. Hunter's Works, 1837). "The colouring principle of the Rubia tincorum

[madder] has strong affinity to phosphate of lime, which earth, if artificially precipitated from a solution of madder, carries down with it the colouring matter in a state of combination, which water does not disturb." Two pigs were fed madder for a fortnight. One was killed; "... the exterior part was found to be principally coloured, and the interior was much less tinged. The other pig was allowed to live a fortnight longer, but had no madder in its food; it was then killed, and the exterior part of the bone was found of the natural colour, but the interior was red." "Accordingly, although an inference may be safely drawn with respect to the part of a growing bone which receives the accessions of osseous substance, by observing the part which is coloured with madder, yet we cannot too certainly conclude that a superficial colourless layer, in an animal killed after remission of the madder, is a new deposit, since it may be the old, from which the madder has been removed, after having been redissolved in the serum." "To ascertain that the cylindrical bones are not elongated, by new matter being interposed in the interstices of the old he made the following experiment: he bored two holes in the tibia of a pig, one near the upper end, and the other near the lower: the space between the holes was exactly two inches: a small leaden shot was inserted into each hole. When the bone had been increased in its length by the growth of the animal, the pig was killed, and the space within the two shot was also exactly two inches." "Bones, according to Mr. Hunter's doctrine grow by two processes going on at the same time, and assisting each other; the arteries bring the supplies to the bone for its increase; the absorbents at the same time are employed in removing portions of the old bone, so as to give the new proper form." He contrasted Hunter's explanation with that of du Hamel, who "... placed a ring of silver wire round the middle of the shaft of the thigh-bone of a young pigeon; and found at a subsequent period the ring in the medullary cavity of the bone. ..." Hunter's interpretation would be "... that the arteries of the periosteum had deposited new bone on the external surface of the ring, while the absorbents had removed the old bone in contact with the internal surface of the ring. ..." "Du Hamel explains the facts on mechanical principles; assuming that the bony layers of the shaft of the thigh bone were expanded by the interposition of additional osseous matter, and that the layers were cut through in this process of expansion by the unyielding wire which had been placed around them."

In these days of nearly miraculous genetic engineering, it gives one pause to contemplate that we still do not understand the basic mechanisms of biomineralization. Other aspects of calcium physiology have fared better.

1.2. COAGULATION

During the latter decades of the nineteenth century, the effects of calcium on various organisms, organs, and extracts were tested. Analogies were sought among these systems; not all were apt.

In 1873, Hammarsten examined the effects of various heat and salt treatments on milk coagulation and subsequent cheese formation. "We have thus found that

calcium salts were the necessary component for cheese formation in solution no. 1; the proteins previously existing in the same solution were without any significance and thus it is generally proven, that the chemical course of casein coagulation by ferment does not consist of the fact, that there are found two proteins in milk; a caseinogen and caseinoplastic substance, which by the action of a ferment can join themselves to a new protein body, cheese."

"Vi hafva sålunde funnit att kalksalterna voro une för ostbildningen nödvändiga beständsdelen af lösningen no 1; den i samma lösning förhandenvarande ägghvitan var utan någon betydelse och härmedär dat sålunda bevisadt, att det chemiska förloppet vid caseinets coagulation med löpe ej bestor deri, att I mjölken finnas tvenna ägghvitekropper, en caseogen och en caseoplastisk substans, som genom löpets inverkan förena sig till en ny ägghvitekropp, osten."

In 1887, Green noted that "there is considerable similarity between the coagulation of milk and that of blood." He also presented preliminary experiments showing that the addition of $CaSO_4$ solution to diluted plasma increases its coagulation rate markedly. Apparently unaware of Green's work, but following the earlier suggestion of Hammarsten, Arthus and Pagès in 1890 described the digestion of milk by "labferment," gastic mucosa of calf.

First they distinguished the "coagulation," as it was then called, caused by acid, from that caused by the enzyme rennin, or chymosin. By complexing the calcium with oxalate, they showed that lab ferment contains a calcium-dependent enzyme, one that cleaves caseine. Most perceptively, they distinguished two effects: first, the transformation caused by lab ferment from the subsequent "precipitation" of the casein fragments by calcium. In the second (1890), Arthus and Pagès, working from the physiology laboratory in the Sorbonne, discussed the analogy between the ". . . caseinification of milk and the spontaneous coagulation of blood . . ." and summarized this result: "Oxalates, fluorides, and alkaline soaps make the blood spontaneously coagulatable."

". . . caséification du lait et la coagulation spontenée du sang. . . ."

"Les oxalates, fluoruses et savons alcalin rendent le sang non spontanément coagulable."

Again they distinguished among calcium activation of the enzyme(s), fibrin ferment, and the involvement of calcium in fibrin precipitaion or clot.

As discussed in Chapter 13, many extracellular enzymes bind calcium and can be inactivated by removal of free Ca^{2+} ions usually found in the extracellular environment at about 10^{-3} M. Further, this extracellular calcium is ideally suited to cross-linking protein, carbohydrates, and phospholipids, either as molecules free in solution or as attached to cell surfaces. These early studies of Arthus and Pagès addressed two themes that reappear as functions of extracellular calcium: enzyme activation and cross-linking of molecules.

1.3. SECONDARY MESSENGERS (ANTICIPATED)

During the same period, even more important observations were being made by Ringer and by Locke. In 1881, Ringer described the optimal concentrations of sodium, potassium, and ammonia to maintain eel and frog heart contractility. The next year he observed: "After the publication (just mentioned) I discovered that the saline solution which I had used had not been prepared with distilled water, but with pipe water supplied by the New River Water Company of London. Gratis they furnished 38 mg calcium per liter of water." "I conclude, therefore, that a lime salt is necessary for the maintenance of muscular contractility of both eel heart and frog skeletal muscle."

Locke (1894) found that he could cause the dissected frog sartorius muscle to contract by both direct electrical stimulation and via stimulation of the attached motor nerve. The latter indirect response depended critically on the presence of calcium in the bathing medium. Only today are the mechanisms underlying Ringer's and Locke's observations becoming understood. They had the good fortune to use systems in which contraction and secretion could be controlled by altering the concentrations of extracellular calcium. We now know that these cytosolic processes are regulated or modulated by the concentrations of the free Ca^{2+} ion within the cytosol.

Ringer fully appreciated the significance of his observations. Yet, like most of his contemporaries, he attempted to explain the effect of calcium on muscle by a very reasonable but false analogy. Earlier, M. Foster had noted the similarities between muscle contraction and coagulation. In the fourth edition of *Text-book of Physiology* (1883, p. 66) he stated a theme to be explored for 60 years: "We may in fact speak of rigor mortis as characterized by a coagulation of blood plasma but differing from it in as much as the product is not fibrin but myosin." Subsequent results of Ringer (Ringer and Buxton, 1887) and of Arthus and Pagés (1890) seemed to confirm Foster's suggestion. Ringer himself turned to the study of calcium in blood coagulation.

1.4. COLLOIDS

Heilbrunn championed the view held by many physiologists of his day: "Living substance is colloidal—it is universally so. The activity of living things is almost certainly bound up with some sort of colloidal change" (1927). In one of his first papers (1915), he asked: "Is the egg [of the sea urchin, *Arbacia*] essentially fluid or is it a more or less rigid jelly?" He argued that the cell membrane is not lipoid, but later came to accept its lipoid nature. He made no mention of calcium but concluded: "It is also possible to believe that the primary effect of all the parthenogenic reagents is a coagulation effect." Pursuing this idea, he wrote in 1920: "In my work on artificial parthenogenesis, I showed that all substances which incite the sea-urchin egg to divide mitotically produce a marked increase in the viscosity of the cytoplasm." "Accordingly I held the view that some sort

of 'solidification' was the essential factor which initiated mitosis." By 1923 he was led to consider the effects of ions. "Perhaps the most important characteristic of a colloid is its electrical charge." He discussed experiments with sea urchin eggs and with the paramecium, *Stentor*, that ". . . show clearly that the bivalent cations, calcium and magnesium, do not exert as powerful a coagulative action as sodium, potassium and ammonium." First at the University of Michigan and subsequently at Penn, he and his students found system after system in which calcium plays an important role. For the next three decades he was the voice of calcium.

In his 1928 *The Colloid Chemistry of Protoplasm*, he argued that the particles or molecules within protoplasm carry a net positive charge while the surface membrane has a net negative charge. "It is thus apparent that the greater the amount of cation absorbed, the more fluid is the protoplasm." "The effect in decreasing the viscosity of the protoplasmic suspension . . ." ($Ca > Mg > Na > K > NH_4$) is equal to ". . . the order of decreasing absorbability."

He also argued that "the experimental study of the surface precipitation reaction has shown that in various cells, the presence of calcium is a necessary prerequisite." The fact that the viscosity of protoplasm decreases briefly following fertilization and his observation on vacuole formation led him to speculate: "Perhaps the calcium within the egg is not in an available form." He went on to suggest: "If we make the assumption that the calcium within the egg is for the most part bound with some fatty or lipoid substance, then we can offer an explanation which will fit practically all of the known facts." He suggested that free calcium might be increased by (1) cell rupture, (2) decrease in cell volume, (3) long exposure to isotonic calcium solutions, or (4) dissolution of protoplasmic lipoids by heat or fat solvents.

In his proposed mechanism, "(1) Calcium set free in cell interior (2) Calcium reacts with the pigment granule of some constituent of it to produce a substance which we have called ovothrombin (3) Ovothrombin reacts with a substance in the protoplasm, presumably a protein, to cause vesicle formation."

Heilbrunn and his colleagues studied the action of ultraviolet rays on *Arbacia* eggs (1930) and on amoeba (1933). They argued a general model in which ultraviolet ". . . stimulation of amoeba protoplasm consists first of the breakdown of a calcium gel in the cortex, and second in the entry of this calcium into the interior, where it produces a preliminary liquefaction followed by a gelation."

Mazia, a student of Heilbrunn, investigated ". . . free calcium in the action of stimulating agents on *Elodea* cells . . ." (1936) and ". . . the release of calcium in *Arbacia* eggs on fertilization" (1937). The Penn group focused on the release of calcium: "The results are shown to be consistent with the idea that when stimulants act on these cells, they cause a release of Ca from combinations located in the periphery of the cells" (1936). "It is intended that a primary effect of a stimulating agent on a cell is to cause a release of calcium from organic combinations in the cortex of the cell into the main body of protoplasm" (1937).

In Heilbrunn's *An Outline of General Physiology* (third edition, 1952), calcium played a crucial role. "There is a large amount of corrobative evidence to show

that when cells are stimulated, calcium is set free" (1952, p. 606). The cell ". . . cortex contains, as an essential part, calcium proteinate. . . ." ". . . the clotting action of the calcium ion is important both for the cell exterior and for the interior." These ideas were expressed in his 1940 paper "The Action of Calcium on Muscle Protoplasm." "On stimulation calcium is released from the cortex and enters the cell interior. There it produces gelation as clotting. This clotting reaction is essentially the same type of reaction as that which occurs when naked protoplasm is exposed by teasing or cutting a cell (surface precipitation reaction)."

At Woods Hole, Heilbrunn was well known for his comment: "Yes, calcium is everything!" His 1940 paper contains another characteristic passage: "The muscle physiologist, adept as he is at chemical analysis and various intricate types of physical recording, has hardly ever considered the fact that the protoplasm of the muscle cell is not markedly different from the protoplasm of less differentiated cells. . . ." Heilbrunn's reluctance to accept modern biochemistry and the remarkable variety and specificity of protein reactions prevented him from anticipating the mechanisms whereby calcium affects the protoplasm. Cell division, growth, and adhesion have now been examined in many systems; many of the calcium-modulated proteins have been identified.

1.5. CROSS-LINKING AND CELL SURFACES

Starting with men like Loeb and continuing today, scientists have searched for general laws or formulas relating cell response to the ionic composition of the bathing medium. There were many attempts to explain calcium effects in terms of general mass law characteristics. Concepts of cross-linking, like those of colloids, held sway in the first half of the twentieth century. Only gradually have two important generalizations emerged. The $[Ca^{2+}]_{cyt}$ within the cytosol under resting conditions is almost 100,000 times lower than it is in the bathing plasma, $\sim 10^{-7.5}$ vs. $\sim 10^{-2.7}$ M. Elaborate mechanisms have evolved to establish and control such concentration gradients. No one anticipated the incredible specificity of protein–addend interactions.

Hamburger, in 1910, assayed "The Influence of Small Amounts of Calcium on the Motion of Phagocytes" by placing a small capillary-filled *Bacillus coli* under the skin of a rabbit and after a fixed time measuring the length of leucocytes in the column. He concluded that a slight amount of calcium caused an ". . . acceleration of the amoeboid motion." His following discussion should have been heeded by many of his colleagues over the next 30 years. "We might be inclined to think of a modification in the agglomeration of the colloid protoplasm particles as a consequence of the electric charge, caused by the entering of a number of bivalent calcium ions. This explanation however can hardly be the correct one. For the experiment teaches that other bi-valent cations namely barium, strontium, magnesium do not cause an acceleration of the amoeboid motion. It must be assumed then, that the action of calcium in this case, is based upon a specific, hitherto unknown, biochemical property."

In the same year (1910), Osterhout pointed out that the Overton theory of a lipoid outer layer (membrane) demanded that the inorganic salts not penetrate the membrane. He grew seeds of *Dianthus barbatus* on the surface of distilled water. These cells normally produce a great deal of oxalate. Following addition of 5.0 mM calcium to the external medium, crystals of calcium oxalate appeared in the cytosol. At least in these cells some calcium can cross the cell membrane. Until the discovery of the photoprotein, aequorin, some 50 years later, there existed no method of measuring directly the concentration of the free Ca^{2+} ion within the cytosol.

Galstoff (1925) studied the adhesion of sponge cells following dissociation by squeezing them through bolting cloth. "According to their effect on amoeboid movement and coalescence, the cations can be put in the following order. . . : $Ca > NH_3 > Li > K > Mg > Na$."

In addition, calcium might alter the properties of membranes by interacting with either proteins or phopholipids. Wilbrant (1940) studied the increase in osmotic resistance of the erythrocyte to various salts and found that "$CaC1_2$ actually acts more strongly and between NaCl and $CaC1_2$ concentrations of the same effectiveness the relationship $Na/Ca^{1/2} = const.$ also holds approximately." In 1948, he and Koller extended these comparative studies to the frog heart.

"$CaC1_2$ wirkt tatsächlich viel stärker and zwischen Konzentration gleicherchen Wirksamkeit von NaCl and $CaCl_2$ besteht auch hier annähernd die Beziehung $Na/Ca^{1/2} = const.$"

In 1944, Carruthers and Suntzeff reported that "estimations of the calcium content of the mouse epidermis during the process of experimental carcinogenesis reveal two distinct phases: an immediate reduction in the calcium content which persists at a fairly constant level for many weeks and a further reduction when the epithelial cells have been transformed into cancer cells."

Coman (1944) found that "malignant neoplastic squamous cells from carcinomas of the lip and from carcinomas of the cervix showed mean values of adhesiveness far below that of the normal cells." Although he had not yet done any calcium measurements, from the literature he suggested ". . . that decreased mutual adhesiveness in cells of squamous cell carcinoma may be related to a lowered calcium content of these cells." De Long et al. (1950) studied rapidly dividing, nonneoplastic cells. "As compared with normal rat liver, the calcium and sodium contents [of regenerating rat liver] were not significantly altered, but potassium was increased by an average of 11 per cent. It is concluded that the increased potassium content of cancers is at least in part an expression of cellular multiplication, but that the diminution in calcium is peculiar to cancer and is partially responsible for decreased mutual adhesiveness of cancer cells. . . ." Coman (1954) observed tissues in the electron microscope. "In liver perfused with versenate (EDTA) cells were no longer closely opposed but were separated to great or lesser degree." "These findings suggest a molecular bond of the calcium to the

carboxyl groups of the proteins and to the phosphate groups of lipoids, as the basis of cellular adhesiveness." These interpretations are still controversial.

Steinberg (1962) extended these ideas to embryology. In the amphibian gastrulae, "calcium is shown not to be functioning as a desolvating agent by experiments in which other desolvating agents, substituted for Ca^{++}, fail to duplicate its effects." "The supposition that Ca^{++} functions by countering the negative charge on cell surfaces, thereby reducing mutual electrostatic repulsion, is demonstrated to be incorrect by the observation that suppression of these negative charges by a reduction in pH prevents, rather than encourages, cell adhesion, in consonance with the prevention of Ca^{++} binding. These observations are shown to be compatible with theories of Ca^{++} bridging between cells and with those invoking 'extracellular' cementing material. . . ."

In 1962, Whitfield and Dixon described postirradiation mitotic delay in cultures of L cells: "Not only does calcium prevent radiation-inducted mitotic delay, its chromatin condensing effect is also in opposition to a radiation-induced elongation of prophase chromosomes." Balk in 1971 described another system that was to become very significant: "Whereas multiplication of normal chicken fibroblasts is limited and controlled by the concentration and access of ionic calcium, cells transformed by the Rous sarcoma virus appear to have gained independence from these environmentally imposed controls."

Alford (1970) reported on the metal cation requirements for phytohemagglutinin-induced transformation of human peripheral blood lymphocytes: "Citrate inhibition of PHA-induced lymphocyte transformation as induced by diminished ^{3}H-thymidine uptake was due to chelation of ionized calcium and could be reversed by added calcium ion." At 20 mM, magnesium is 75% as effective as calcium.

1.6. SECONDARY MESSENGERS (UPDATED)

Steinhardt and Epel (1974) confirmed and extended Mazia's observations. "Micromolar amounts of the divalent ionophore A23187 can activate echinoderm eggs." ". . . eggs preloaded with ^{45}Ca show a 20-fold increase in ^{45}Ca efflux when activated by inonophore A23187 or fertilization." "We propose that both normal fertilization and ionophore activations affect the metabolism of the egg by releasing Ca sequestered in intercellular stores."

Brachet et al. (1975) studied the induction of maturation (meiosis) in *Xenopus laevis* oocytes: "Organomercurial- and progesterone induced maturation have many features in common: they do not occur when the inducer is injected into the oocyte, they require the presence of Ca^{++} in the medium, they are inhibited by cycloheximide but not by actinomycin D."

Since the studies of Ringer, contractile systems have provided many insights into the biological functions of calcium. The gel nature of protoplasm is far more complex than Heilbrunn anticipated. In 1939, Engelhardt and Ljubimowa in Moscow described an ATPase function for myosin: "Thus the mineralization

of adenosintriphosphate, often regarded as the primary exothermic reaction in muscle contraction, proceeds under the influence and with the direct participation of the proteins considered to form the main basis of the contractile mechanism of the muscle fiber." Bailey (1942) explored the ATPase activity of myosin activated by calcium: "We suggest that the essential feature of excitation and contraction—we cannot at present dissociate the two phases—is the liberation of the Ca ion in the neighbourhood of the ATPase grouping, which can thus by the almost instantaneous catalysis of ATP breakdown make available a large amount of energy." Bailey was right, but because of the complexity of the muscle system it was some years before the role of calcium was generally accepted.

In 1949, Hill found that frog muscle at 0°C reaches maximum tension within 40 ms of stimulus to the cell surface. "... it is impossible, therefore to assume that only a fraction of the cross-section of each fibre is involved in it" [the twitch]. He calculated that nearly a half second would be required for a small molecule to diffuse from the surface to the center of a 100-μm-diameter muscle cell. "We must look for some physical or physico-chemical process which is released by excitation at the surface then propagated inwards."

One of the keys to understanding the function of calcium in muscle was to separate the calcium-sequestering system from the calcium-activated system. In 1948, Kielly and Meyerhof described "a new magnesium-activated adenosine triphosphatase in a particulate fraction free of actin and of myosin." Marsh (1951, 1952) found "a factor modifying muscle fiber synaeresis." "Thus there appears to be a substance or substances in muscle, easily removable by dilute potassium chloride solution, the presence or absence of which determines the effect adensoine triphosphate will produce." If ATP is added to muscle brei in the presence of the "Marsh factor" or "relaxing factor" there is a low level of ATPase activity and the volume of the actomyosin gel increases slowly and reversibly by 40%. With the relaxing factor removed, ATP causes a rapid, irreversible contraction of 30%. Bendell (1952), a colleague of Marsh at Cambridge, found stabilizing conditions for the relaxing factor and noted that calcium causes contraction and synaeresis; similar supporting results were published by Hasselbach and Weber (1953) in Tübingen. Bozler (1954) used Albert Szent-Györgi's glycerinated muscle preparation to demonstrate that physiological levels of Mg-ATP can make such muscle fibers "extensible and plastic"; they then "... give strong contraction on addition of small amounts of $CaC1_2$...." "The results indicate that relaxation is caused by the inactivation of bound calcium... by either the relaxing factor or by EDTA." "Previous evidence that the relaxed state is due to the formation of an enzymatically inactive ATP–protein complex was confirmed."

In 1952, Sandow reviewed the evidence that the action potential, per se, does not initiate contraction. He focused on the link between excitation and contraction and suggested "... that, in the living muscle, activation of the contractile material may be attributed to the enzymatic activation of the myosin-ATPase system by Ca^{++}." Two years later, A. F. Huxley and Niedergerke and H. E. Huxley and Hanson presented their sliding filament model.

1.7. PUMPS, CHANNELS, AND IONOPHORES

During this period, morphologists were using new OsO_4 fixation procedures and methacrylate embedding to obtain higher-contrast electron micrographs. Porter and Palade (1957) summarized their and others' work on the sarcoplasmic reticulum, its proximity to the Z band, and the "triad" appearing to connect it to the sarcolemma: "... it is proposed that the membrane limiting the system [the sarcoplasmic reticulum] is polarized like the sarcolemma and that the corresponding potential difference is utilized in the intercellular distribution of the excitatory impulse."

Anne Maria Weber (1959), the daughter of H. H. Weber, suggested that "... it might be worthwhile to investigate whether the Marsh–Bendall factor, which is particulate and presumably does not interact directly with the myofibrils, acts by binding Ca^{++}." In retelling such a story one easily imparts the false impression of a straightforward, linear progression. In the muscle field especially, many distinguished scientists have pursued the logic of reasonable experiments to incorrect conclusions. For example, Nagai et al. (1960) found that the inhibition of myosin ATPase ceased when relaxing granules were removed. "Thus the granules do not withdraw any functionally essential substances from the contractile proteins, nor do they release any stable contraction inhibiting substance to the actomyosin or to the solution."

In 1961, Ebashi, still working at the Rockefeller Institute (now Rockefeller University), before returning to Tokyo "... demonstrated that a purified preparation of the relaxing factor of skeletal muscle, shown by electron microscopy to be a vesicular fraction, probably the endoplasmic reticulum, is able to strongly bind calcium and furthermore that this binding of calcium by the fraction depends on the presence of ATP. We have supposed that the calcium binding represents the physiological action, or the mechanism of the relaxing factor. The results demonstrated in the present paper support this concept, and suggest that the calcium ion is the main controlling factor in muscle contraction."

By 1964, Franzini-Armstrong and Porter, now using glutaraldehyde as a fixative, could better reconstruct the sarcoplasmic reticulum (SR) as well as the connecting T-tubule system. "... the T system is a sarcolemmal derivative that retains its continuity with the sarcolemma and limits a space that is in direct communication with the extracellular environment. These structural features favour the conclusion that the T system plays a prominent role in the fast intracellular conduction of the excitatory pulse." The membranes of the SR enclose a volume topologically distinct from the sarcosol, which bathes the thick and thin filaments. When the SR is homogenized, it reseals to form closed vesicles. Better preparations of such vesicles were becoming available. Hasselbach and Makinose (1963) showed that two Ca^{2+} ions were pumped per ATP and that isolated SR vesicles could lower $[Ca^{2+}]$ to 10^{-8} M or pCa 8 (Chapter 12).

1.8. CALCIUM BINDING PROTEINS

If calcium does, in fact, couple excitation to contraction, what does it activate? What is its target? We now know that calcium relieves the effects of an inhibition; under appropriate conditions, myosin ATPase is active with no calcium present. Ebashi, by 1963 in Tokyo, described a "third component participating in the superprecipitation of 'natural Actomyosin.'" He noted that "synthetic" actomyosin as prepared by Perry and Grey (1956) had ATPase activity without the addition of calcium, in contrast to "natural" actomyosin, which requires calcium for superprecipitation. The factor imparting "calcium sensitivity" is in the tropomyosin fraction described by Bailly, but tropomyosin would not restore calcium sensitivity. Finally, in 1967, Ebashi et al. characterized "troponin as the Ca^{++} receptive protein in the contractile system." "It is conceivable that binding and detaching of Ca^{++} to and from troponin might be of primary importance in regulation of muscle contraction, i.e. some conformational change of the troponin molecule induced by the removal of Ca^{++} might inhibit interaction of adjoining actin molecule with myosin and this inhibition might be cancelled by Ca^{++}." They also anticipated an as yet unanswered problem: "However, before accepting this explanation, we must answer the question as to how the troponin molecules, which are distributed along the thin filaments at a 400 Å periodicity, can exert their influence on those actin molecules which are located at some distance from adjacent two troponin molecules."

So, finally, in one system we approach a molecular interpretation for one intracellular function of calcium. Its concentration is very low in the resting muscle cell. Stimulation causes calcium inflow from a storage vesicle or from the extracellular space. This calcium interacts with a target, a calcium-modulated protein, which, in turn, "activates" an enzyme. The cycle is complete when the calcium is pumped out and the enzyme is no longer active.

While biochemical studies on troponin progressed, Kretsinger et al. determined the crystal structure of parvalbumin found in the cytosols of many vertebrate cells. In 1972, Kretsinger published "Gene Triplication Deduced from the Tertiary Structure of a Muscle Calcium Binding Protein" and suggested that "both troponin and MCBP are acidic, pI about 4.5; both have high phenylalanine contents and high calcium affinities. The molecular weight of TNC, however, is 19,000 while that of MCBP is 11,500. I consider it possible that MCBP exists in mammals in some reduplicated form, considering its tendency to duplication." The next year Collins et al. published the article "The Amino Acid Sequence of Rabbit Skeletal Muscle Troponin C: Gene Duplication and Homology with Calcium-Binding Protein from Carp and Hake Muscle." "The correspondence of α-helices and hydrophobic residues suggests that each of the four regions of TNC has a three-dimensional structure very similar to the CD and EF regions of MCBP." The basic structural and evolutionary domain, about 30 amino acids long, consists of two turns of α-helix, a calcium binding loop, and two more turns of α-helix: the EF-hand. In 1975, Kretsinger published "Hypothesis: Calcium Modulated

Proteins Contain EF Hands." Chapter 11 treats this homolog family, which now includes calmodulin and over 70 other distinct subfamilies.

1.9. SECONDARY MESSENGERS (YET AGAIN)

Calcium controls motility in systems other than those based on actomyosin. The ciliate *Paramecium caudatum* normally swims forward. By 1900 it had been established that a variety of chemical, mechanical, and electrical stimuli cause it to reverse its direction of ciliary beat and swim backwards. In 1926, Mast and Nadler measured the duration of reversal as a function of the cations in the medium. "The amount of $CaC1_2$ required to neutralize KCl is not proportional to the concentration of the KCl. Weber's law does not hold. The results seem to indicate that ciliary reversal is associated with differential absorption of the cations and subsequent changes in electrical potential, but that there are also other factors involved." Since the early 1930s, Kamada, in Tokyo, had studied the effects of electrical currents. In 1938 he published "Intracellular Calcium and Ciliary Reversal in Paramecium." "If a paramecium is immersed in a medium relatively low in [Ca]/[K] ratio, the direction of ciliary beat can be reversed. However, since the cilium itself is not provided with the mechanism to reverse its effective stroke in a direct response to stimulation, some intercellular change must be involved in this reaction." His intracellular injections were very damaging. "It may however be concluded that the diminution of the intercellular calcium ions causes ciliary reversal."

Naitoh (1968) extended the observations on the effects of extracellular cations and hypothesized: "Externally applied cations bind to the anionic sites on *Paramecium* in exchange for bound calcium in a manner consistent with the law of mass action. The calcium ions which are thus liberated are effective in activating, directly or indirectly, a contractile system which is energized by ATP." "It is not known why only those calcium ions liberated from the cellular binding sites activate the reversal system. The reason may be a difference in the effective diameter of liberated and free calcium ions resulting from a difference in the degree of hydration." A reasonable speculation, but wrong; the mechanism of calcium release and its mode of action are discussed in Chapter 2.

The study of secretion developed in parallel to that of contraction. Douglas and Rubins' "stimulus–secretion coupling" is analogous to the "excitation–contraction coupling" of Sandow. Calcium is the common coupler. As we shall see, many of the mechanisms are homologous. The act of secretion, or more specifically, exocytosis, is not observed as easily as is muscle contraction. In 1893, Locke published "Die Wirkung der Physiologischen Kochsalzlö sung auf quergestreifte Muskel." His nerve, muscle (frog sartorius) preparation in NaCl responded to "direct" stimulation over 15 hours; however, it lost its response to "indirect" stimulation (via the nerve) within an hour. If calcium was then added to 5 mM, the indirect response was regained. Calcium is not required for neurotransmission along the axon but is required for the nerve–muscle coupling

and/or the actual contractile event. Over the next 40 years this observation was repeated and the calcium contents of nervous tissues were determined. However, it was only after acetylcholine (ACh) was established as (one of) the chemicals involved in nerve transmission that the function of calcium could be established. Harvey and MacIntosh in 1940 declared that "calcium ions are necessary for the transmission of the excitatory state at the neuromuscular junction." They continued: "Our experiments show that, when calcium is absent, there is no release of ACh from the preganglionic nerve endings, either during stimulation of the sympathetic trunk or following the injection of potassium salts."

Recalling Ringer's observations of the frog heart, one might ask whether these effects are exerted on the cell surface and/or in the cell interior. The studies of muscle that led to the characterization of troponin showed that calcium has a direct effect on the contractile proteins. However, it also has significant and less well understood interactions with cell membranes. First, as discussed for the sarcoplasmic reticulum and the mitochondrion, membranes can pump calcium either directly using ATP as an energy source, as in the sarcoplasmic reticulum or via preformed gradients of Na^+, as in the axon, or of H^+ ions, in mitochondria and bacteria.

An understanding of calcium's role must await other discoveries. In 1952, Fatt and Katz wrote that "... the end-plates of resting muscle fibres are the seat of spontaneous electrical discharges which have the character of miniature end-plate potentials." "The results point to the conclusion that some terminal spots of the motor nerve endings are spontaneously active and release ACh in the same impulsive manner as they do after the arrival of a normal motor nerve impulse." In their 1957 review, Birks and MacIntosh noted that some ACh is "free" in the protoplasm and some is "packaged" for release, after Katz's idea of quantal release. Calcium does not affect spontaneous release but does determine the amount of ACh released under stimulation. "The problem of what determines an effective collision [of a package with the surface membrane] is unsolved: the calcium concentration of the presynaptic axoplasm may be an important factor."

Douglas made the generalization; he coined the phrase. In 1961, he and Rubin wrote: "Our experiments show that the excitant action of Ach on the adrenal medulla is dependent on the presence of calcium, and suggest that Ach evokes secretion by causing calcium ions to penetrate the adrenal medullary cortex." This was later confirmed. They argued the analogy with muscle. It is interesting that both the release of ACh and the subsequent secretion of catechol amines by the adrenal medulla involve exocytosis. In "A Possible Mechanism of neurosecretion: Release of Vasopressin by Depolarization and Its Dependence on Calcium," Douglas (1963) argued that in "... neurones and endocrine glands of nervous origin ... calcium acts as a crucial link in the process of stimulation–secretion coupling ..." and that "... the appearance of free calcium ions somewhere in the endings then causes the release of the stored hormone." This generalization would prove valid for exocytosis by all cells.

In hindsight, so much is obvious. By 1970 the role of calcium in muscle excitation and contraction was generally accepted. That calcium is the link in

stimulus–secretion coupling had been inferred in ten systems. Calcium was suspected of playing a control function in metabolic processes such as glycogenolysis. Yet much of the creative thinking in cellular control processes was focused on cyclic adensine monophosphate (cAMP). In 1958, Rall and Sutherland identified cAMP as the heat-stable factor synthesized in particulate fractions of liver, heart, skeletal muscle, and brain following addition of ATP and stimulation by epinephrine and glucagon. They cautioned: "However, the only biochemical event which is at present known to be influenced by cyclic 3,5-AMP is the phosphorylation of dephosphorylase [now called phosphorylase] and, as yet, it is difficult to understand the multitude of physiological effects of epinephrine in the light of this one reaction." By 1965, Sutherland and his colleagues had extended his studies of cAMP to other hormone-sensitive systems. "In brief summary, the hormone (the first messenger) interacts with a component of the cell membrane to initiate increased accumulation of a mediator (the second messenger), which then acts upon components of the effector cell." They anticipated the extension of this concept. "To date cyclic 3′, 5′-AMP is the only second messenger which has been identified. It is proposed other such messengers may exist, for example, to mediate the action of insulin, and that these may, or may not be other cyclic 3′, 5′-nucleotides."

In 1970, Rasmussen published "Cell Communication, Calcium Ion, and Cyclic Adenosine Monophosphate," one of the most frequently cited papers in the literature of biology. He summarized his extensive review and analysis as follows: "The basic elements of this system are two interrelated intercellular messengers, 3′-5′-AMP and Ca^{++}. Activation or excitation of the cell leads to an increase in both."

1.10. MITOCHONDRIA

In 1970, Lehninger addressed the problem of mitochondria and calcium ion transport and suggested two possible functions, which although they remain unproven, have not yet been replaced by anything better. He emphasized the high affinity and specificity of calcium transport and "its primacy over oxidative phosphorylation in most tissues." He first considered a possible role in muscle activation and/or relaxation but conceded that "Very little is known about the mechanism of release of Ca^{2+} from mitochondria, which must be a very fast process if it is to serve a primary physiological role in the excitation process." "One possible mechanism for the release of Ca^{2+} from the mitochondria in red muscle is a sudden depolarization or collapse of the electrochemical gradient across the membrane, triggered by depolarization of the T-system and the sarcoplasmic reticulum, with which mitochondria appear to make contact in some red muscles." Such functional contact has not been confirmed and remains speculative.

Then Lehninger considered a more promising alternative: applicable, however, only to mineralizing tissue. "We have adopted the working hypothesis that what living cells 'do' to Ca^{2+} and phosphate is to bring about their accumulation in

the mitochondria to such concentrations as to exceed the solubility product of tricalcium phosphate . . . .” “The end products of this stage are suggested to be ‘micro-packets’ of insoluble amorphous tricalcium phosphate in the mitochondrial matrix, which we regard as the essential precursors of extracellular hydroxyapatite.” As with his first suggestion of muscle activation, we still face the problem of communication with the plasma membrane. “We postulate that micro-packets of amorphous calcium phosphate, which are colloidal in dimensions, may dissociate from the larger aggregates in the matrix and then depart in essentially intact form from the mitochondria to the cytoplasm, each micro-packet being stabilized by one or more molecules of inhibitor.” The micropacket is extruded by reverese phagocytosis and “. . . converted to crystalline hydroxyapatite. . . .” The mitochondrion probably plays a role in intracellular calcium buffering and possibly is involved in mineralization. The mechanisms remain unknown.

Forty years ago no one anticipated that the mitochondrion plays a key role in calcium metabolism; today, no one seems to understand what that role is. Research on cytochrome and respiratory enzymes began with Keilin (1929). Twelve years later, Claude (1941) could prepare by differential centrifugation “. . . formed elements [that] might represent well known cellular constituents, i.e. mitochondria or fragments of mitochondria.” Another decade passed before functionally active mitochondria were prepared and their enzymatic characteristics defined. Lehninger and Kennedy (1948) wrote: “We have found that all of the demonstrable fatty acid oxidase activity of rat liver can be recovered in that fraction of 30 per cent sucrose homogenates of rat liver stated by Hogeboom et al. (1948) to consist of morphologically intact mitochondria, free of extraneous intercellular elements.” The initial observations on calcium uptake by mitochondria indicated that calcium might inhibit normal respiration by displaying a metal ion prosthetic group (Slater and Cleland, 1953). “All this calcium in the heart is found in the sarcosomal (mitochondrial) preparations.” “The destruction of DPN, accelerated by calcium prevents the formation of oxaloacetate.” “In the presence of EDTA and the reaction mixture, sacrcosomes oxidized α-ketoglutarate at practically a uniform rate for an hour or more at 25°.” “. . . sarcosomes took up large amounts of calcium from solutions. The localization of the calcium in the isolated sarcosomes does not; therefore, necessarily reflect the position in the intact heart.” This admonition is still valid. The report of DeLuca and Engstrom (1961) suggested a more physiological process: “This communication indicates that kidney mitochondria take up large quantities of calcium by a process which requires adenosine triphosphate (ATP), and oxidizable substrate, and magnesium ions, but which is not directly dependent upon oxidative phosphorylation.” Vasington and Murphy (1962) wrote: “The evidence presented in this paper shows that rather large quantities of Ca^{++} may be actively bound by rat kidney mitochondria in a process that is directly dependent on respiration and that requires adenosine triphosphate, Mg^{++}, and inorganic phosphate in the medium.” “Ca^{++} itself uncouples oxidative phosphorylation in concentrations in which it is actively bound.” Rossi and Lehninger (1964) extended these findings: “From the observations it appears that activation of the energy-conserving site by Ca^{++} is the

first stage in ion accumulation. This does not require phosphate." If Mg-ATP is added to the reaction suspension, 1.0 phosphate and nearly 2.0 calcium ions are accumulated for each pair of electrons at each of three energy-converting sites.

In parallel with these biochemical studies, histological work indicated the involvement of mitochondria in bone formation and/or resorption. B. L. Scott and Pease (1956) examined epiphyses and concluded: "There is a fibrous preosseous zone between osteoblasts and calcified matrix. This is thought to indicate a lag between the deposition of the organic matrix and subsequent calcification." "Calcification of both the cartilagenous and preosseous matrices is a progressive accretion and aggregation of inorganic crystals." The "... abundance of mitochondria in the osteoclast, and local changes in the bone in contact with the ruffled border suggest that a 'collagen dissolving substance' is probably secreted through the ruffled border." Gonzalez and Karnovsky (1961) described osteoclasts in healing fractures. "Mitochondria containing clusters of fine granules, were abundant." "... osteoclasts phagocytize crystals of bone salts and these crystals are very probably free of collagen."

Recalling Ringer's observations on the frog heart, one might ask whether the effects are exerted on the cell surface and/or in the cell interior. The studies of muscle that led to the characterization of troponin showed that calcium has a direct effect on the contractile proteins. However, it also has "significant and less well understood interactions with cell membranes." First, as discussed for the sarcoplasmic reticulum and the mitochodrion, membranes can pump calcium either directly using ATP as an energy source, as in the endoplasmic reticulum or via preformed gradients of Na^+, as in the axon, or of H^+, as in mitochondria and bacteria. In addition, calcium can alter the properties of membranes by interacting with either proteins or phospholipids.

1.11. PUMPS, CHANNELS, AND IONOPHORES

Brink (1954) cast his review "The Role of Calcium Ions in Neural Processes" in terms of more specific interactions instead of mass colloidal effects (Wilbrant, 1940). He noted that sodium and potassium ions function primarily; "in contrast, calcium ions seem to affect primarily the constraints imposed upon these ionic movements." From 1955 through 1957, Keynes and his co-workers published a series of important papers on the content of calcium and its function in squid giant axon. Hodgkin and Keynes (1957) injected ^{45}Ca into axons and observed little diffusion with or without stimulus, "... suggesting that most of the calcium is in a relatively immobile form." "... it is attractive to suppose that the calcium entry during the spike may not be an accidental accompaniment of activity, but is somehow connected with the development of the state of increased sodium permeability." Niedergerke (1963) extended these studies and reported that "both Ca uptake and release were increased during activity" or "... on application of the contracture fluids." Quite reasonably, but incorrectly, he argued: "According to this hypothesis a compound CaR is present which in some way activates

tension whereas the compound Na_2R is inactive." He anticipated separate pools of calcium: "The results have been interpreted by assuming (1) that entry of Ca occurs after combination of this ion at the cell surface with the carrier molecule whose presence is suggested by the Ca-Na antagonism of the heart, (2) that intracellular exchangeable Ca is present in two interacting forms, the activator-Ca which induces contraction and a larger store of inactive Ca." The problem was well posed. Does the Ca^{2+} ion that binds to troponin come directly from the bathing medium immediately before that contraction event? Does the Ca^{2+} ion have other effects within the cytosol or on membranes?

These questions have not yet been fully answered, but additional insights have come from other systems. Reuter and Seitz (1968) recalled that "Ca influx in cardiac muscle is greatly increased during excitation." Also, "quantitatively, Ca efflux from auricles has been shown to depend to a large extent on the ratio $[Ca^{2+}]^{1/2}/[Na^{+}]$. The affinity for Na of the activation site for Ca efflux (carrier) is much less than for Ca." The distinction between calcium pores and calcium pumps and between calcium effects on sodium and/or potassium pumps and pores is difficult. In 1961, Dunham and Glynn wrote: "The ATP-ase activity of human red cell ghosts has been shown to consist of two components. The first component requires the presence of magnesium ions but occurs in the absence of alkali metal ions and is not inhibited by cardiac glycosides. In the presence of magnesium, activity is greatly increased by small amounts of calcium but inhibited by larger amounts. The second component requires the presence of magnesium and also of both sodium and potassium ions. It is completely inhibited by cardiac glycosides in concentrations sufficient to inhibit ion transport in intact red cells. Ca^{2+} ions inhibit at both low and high concentrations." Schatzman (1966) confirmed these findings and suggested that "... red cells are able to maintain low intracellular Ca^{++} concentrations ...." Whitham in 1968 found that extracellular "calcium markedly raised passive potassium efflux but did not affect potassium influx." "Another view is that an increase in concentration of ionic calcium in cells raised permeability of the potassium."

The squid axon has served neurobiology well. Baker et al. (1969) found that "the rise in Na efflux resulting from partial replacement of NaCl by dextrose or choline chloride consisted of two components one of which was oubain-insensitive and calcium-dependent and the other was inhibited by oubain but calcium insensitive." Blaustein and Hodgkin (1969) continued: "After injecting ^{45}Ca along the axis, the efflux of calcium reached its maximum much more rapidly in a cyanide treated axon than in an unpoisoned axon." "A possible explanation of the cyanide effect is that, after poisoning, calcium ions are released from a store and can then exchange at a higher rate with external sodium or calcium." "The experiments suggest that part of the calcium efflux may be coupled to sodium entry." In 1972, Meech published "Intracellular Calcium Injection Causes Increased Potassium Conductance in *Aplysia* Nerve Cells," in which he stated: "Furthermore, when a muscle fibre is stimulated electrically the threshold of contraction is close to the membrane potential at which the increase in potassium conductance called 'delayed rectification' begins." Krnjević and Lisiewicz

(1972) injected calcium into spinal motor neurons and "... it was concluded that the fall in membrane resistance caused by intracellular Ca^{2+} is mainly due to an increase in gK" (potassium conductance).

The dark current of the retina is a well-studied system in which the sodium channel appears to be under calcium control. In 1926, Feenstra studied the retinal current of frogs: "By lowering the calcium in the Ringer solution and thereby removing as much as possible this element from the tissues, one can cause the resting current as well as the action current to disappear."

"En suppriment le sel de calcium dans le solution de Ringer et en enlevant ainsi autant que possible cet element aux tissus, on put faire disparâitre aussi bien le courant de repos que le courant d'action."

Both Fulpius and Baumann (1969), working with honeybees, and Millecchia and Mauro (1969), working with the horseshoe crab, *Limulus*, found that low external calcium increases the dark current, while low bathing sodium decreases it. H. M. Brown et al. (1970) studied another arthropod, the barnacle: "The results indicate that illumination increases the membrane permeability mainly to Na^+ ions and that the primary effect of Ca^{2+} ions is suppression of the permeability increase; Ca^{2+} permeability may increase slightly during illumination." Lisman and Brown (1972) gave a different interpretation of their results from *Limulus*: "We propose that an increase in $[Na^+]_{in}$ leads to an increase in $[Ca^{++}]_{in}$ and that an increase in $[Ca^{++}]_{in}$ by any means leads to a reduction in responsiveness to light." Hagins (1972) presented a different hypothesis for the vertebrate rods and cones. "(I) The activity of Ca^{++} ion is maintained by pumps at a much lower level in the cytoplasm of the outer segments than in the intradisk spaces as in the external fluid. (II) The Na^+ conductance of the rod envelope membrane decreases as the cytoplasmic Ca^{++} activity increases, possibly because internal Ca^{++} reacts reversibly with sites at which the dark current enters, and blocks their Na^+ permeability. (III) Light transiently increases the permeability of the disk membranes and the envelope membrane to Ca^{++}, allowing many more than one calcium ion to enter the cytoplasmic space per photon absorbed."

In 1966, Lowenstein reported his initial studies on the junctional membranes of the epithelial cells of the *Chironomus* salivary gland. "A primary factor in controlling junctional membrane permeability is Ca^{++}." "When its concentration is raised above 10^{-4} M, the junctional membranes become as impermeable as the non-junctional ones; each cell seals itself off as a unit." The mechanism(s) whereby calcium controls these various gates, pores, or pumps remain the topic of many grant requests. It is, though, a safe guess that the basic concept will differ little from that involved in excitation–contraction or stimulus–secretion coupling.

It became ever more apparent that one had to determine the concentration of the free Ca^{2+} ion within the cytosol. As early as 1934, Tipton reported the total content of frog sciatic nerve as 7.3 milliequivalents per kilogram net weight, but he lacked techniques to determine its cellular distribution. Hodgkin and Katz

(1949) wrote that "... extruded axoplasm was dispersed by millimolar calcium." "... but it apparently contrasts with the coagulation which calcium is known to produce in other colloidal materials." In 1957, Gilbert and Fenn reported a thorough study of calcium equilibrium in frog muscle. "The percentages of total calcium in the whole muscle immersed in Ringer's solution [for five hours] was as follows: 10 percent in the surface phase; 12 percent in the extracellular water space; 17 percent in the dry connective tissue; 24 percent in the extracellular water space; and 37 percent as nonexchangeable calcium." "It appears that there is a calcium pump pushing calcium out of the cell against an electrochemical gradient of about 4 cal/mM of calcium." Portzehl et al. (1964) wrote: "When buffers containing calcium and EGTA were injected [into crab muscle fibers] it was found that contraction was not obtained if the ionized calcium in the buffer was below a certain level" (pCa $\sim$ 6.2). Similar experiments in barnacle by Hagiwara and Nakajima (1966) yielded similar results. "The threshold concentration for contraction was about 8×10^{-7} M." At rest the concentration is lower. A more direct determination of $[Ca^{2+}]$ would have to await the availability of aequorin.

Most bacteria, when they form spores, synthesize dipicolinic acid (DPA) to about 10% of their total dry weight. The molar ratio of calcium to DPA is usually in the range 1.5 to 0.7. Although compositional analyses of bacteria had been performed at the beginning of the twentieth century, Curran et al. (1943) were the first to apply the newly available spectrograph. "Spores were materially higher in Ca and lower in K than the vegetative cells from which they were derived." "While there was apparently no direct correlation between elemental inorganic composition and degree of resistance to heat, in general, Slepecky and Foster (1959) found that high concentrations of Ca were associated with enhanced heat tolerance and resistance." "The results showed that the content of individual metals in spores is flexible within a very wide range and is dependent on the relative concentration of the particular metal in the growth medium." The thermosensitivity studies were somewhat inconclusive but tended to suggest "... that calcium is essential for highest thermal resistance of spores."

In 1971, Bronner et al. established that there is an active uptake system for calcium in sporulating *Bacillus megaterium*. Its cellular localization remains unknown. Subsequently two groups, in 1975, Tsuchiya and Rosen and Silver et al., demonstrated that under normal growth conditions, bacteria actively extrude calcium by a mechanism resembling that used in mitochondria. Although there are yet only a few examples from bacteria and lower plants, it appears that the maintenance of a low cytosolic Ca^{2+} ion concentration is a characteristic of all cells, prokaryote or eukaryotic.

Ordal (1977) suggested that the direction of flagellar rotation is controlled by cytosolic calcium concentration, with the switch occurring about pCa 7.5. A report on actin- and myosinlike proteins in the bacteria (Nakamura and Watanabe, 1978) promised more messenger functions for calcium in prokaryotes.

Quite obviously, an experiment in which one adds or removes an ion to the extracellular medium and then observes a cellular response gives, at best, only an indirect suggestion as to the effect of the test ion in the cytosol. Nature has

provided another approach to the problem: antibiotics that function as ionophores. An ionophore facilitates the diffusion of an ion, usually a metal monovalent or divalent cation (Me^+ or Me^{2+}) from an aqueous phase into a low-dielectric environment or through a membrane. Most ionophores (I or I^-) are highly lipid soluble. At the lipid–water interface, they complex the cation, displacing all or most of its water of hydration. The ionophore–cation complex then diffuses across the membrane as the cation complex (I^*M^+) or as the neutral complex (I^-M^+ or $I^{2-}M^{2+}$). On the other side of the membrane, the metal cation is hydrated and the ionophore diffuses back to the original side, either with an alternative cation (electroneutral exchange, diffusion, usually involving I^-) or without (electrogenic exchange usually involving I). Other natural antibiotics, and related synthetics, form channels or pores in membranes. These pores permit the selective diffusion of specific cations, wholly or partially dehydrated.

Valinomycin was the first antibiotic to be recognized to function as an ionophore (Moore and Pressman, l964). This neutral, cyclic decapeptide has a K^+/Na^+ preference of 10^4 : 1. In 1951, Berger et al. isolated three antimicrobially active molecules, one each from three previously unidentified strains of *Steptomyces*: X-206, X-464 (product, later called nigericin), and X-537A. Johnson et al. (1970) determined the crystal structure of X-537A as $Ba(C_{34}H_{53}O_8)_2 \cdot H_2O$ and suggested that it is a general ionophore for divalent cations. In 1972, Reed and Hardy characterized A23187, from *Streptomyces chartreusensis*, as a rather specific Ca^{2+} or Mg^{2+} ionophore.

Experimentalists can use such ionophores to equilibrate the ion content of the intracellular medium with the concentration that they set in the external medium. Initially, it was not appreciated that these ionophores will also "transport" ions to or from any intracellular stores, such as mitochondria and endoplasmic reticulum. The challenge for chemists is to understand the nature of the ion selectivity and to synthesize more specific ionophores and ion channels to be used in ion-selective electrodes as well as in biological experiments.

It is certainly true that the longer one works with any biological system, the more one is impressed with the significance of membranes and their abilities to establish and maintain selective compartmentalization. Calcium has specific interactions with membrane-embedded channels, ionophores, enzymes, and pumps. There also exists the possibility that calcium may affect the general properties of membranes by interacting directly with the phospholipid component. The 1966 study of Bangham and Papahadjopoulos was one of the first to explore this possibility: "... phosphatidylserine interacts with Ca^{2+} in the presence of physiological concentrations of univalent ions ...." "... a structural modification of the Ca^{2+} phosphatidylserine complex occurs at approx. 1 mM Ca^{2+} when approx. 1 equiv. Ca^{2+} is bound on the surface for each phosphotidylserine molecule." Such calcium synthetic phospholipid bilayer interactions have now been explored in great detail. It is still not known what the physiological implications of these effects are, either on cytosolic surfaces, where $[Ca^{2+}]$ seldom exceeds 10^{-5} M, or on external surfaces, where the bathing $[Ca^{2+}]$ exceeds 10^{-3} M.

1.12. HORMONES

Given these diverse functions of calcium, one is hardly surprised to find that the body has developed elaborate mechanisms for controlling serum levels of calcium. Although anatomists and embryologists such as Remak and Owen had previously referred to "accessory thyroids," Sandström (1880) was the first to describe their anatomy and histology in humans and other mammals. "... I suggest therefore employing for these the designation glandulae parathyreodiea; a name in which the relationship of the accessory gland to the thyroid is expressed, in the same way as by analogy parovium, paradidymis leads the thoughts to images of an embryonic nature." In fact, both the development and function of the parathyroid are quite different from those of the thyroid.

"... föreslår jag derföre att för dessa använda benämningen Glandualæ parathyreoideæ; ett namn, hvari deras egenskap af bikörtlar till sköldörteln fått sitt uttryck, på samma gång som det genom analogierna parovarium, paradidymis leder tanken på bildningar af en embryonal natur."

Gley (1891) rediscovered the small glands and argued that it was their unnoticed removal that was responsible for the "incessant muscular twitches and clonic convulsions" that usually brought death within a day of total thyroidectomy. Incorrectly, he, too, speculated that "the structure of this organ is analogous to the body of the thyroide in an embryonic state."

"secousses musculaires incessantes et convulsions cloniques"

La structure de cet organe est analogue à celle du corps thyroide à l'état embryónnair.''

Moussu (1898) first claimed an active glycerine extract to relieve postoperative tetany in dogs: "I have indicated the facts and experiments that have forced me to acknowledge the existence of distinct functions of the thyroid and of the parathyroid." A real demonstration of the parathyroid hormone would await Collip's work in 1925. Vassole and Generali (1900) described the effects of parathyroidectomy in much greater detail. "In the dog and in the cat, the abolition of the function of the parathyroid (complete parathyroidectomy) gives rise to a mortal tetany ...." The characteristics included fibrillary contractions and muscular spasms, psychial depression, rigid and uncertain gait, anorexia, tachycardia, rapid emaciation, and fall in body temperature. About this time, Loeb (1901) reported "on an apparently new form of muscular irritability by anions liable to form insoluble calcium compounds." This and other bits of evidence inspired the experiments of MacCallum and Voegtlin (1909) "on the relation of tetany to the parathyroid glands and to calcium metabolism." Parathyroidectomized animals have lowered calcium contents in their tissues and elevated calcium in their urine; their symptoms can be relieved by injection of calcium. Collip in 1925 succeeded in "the extraction of a parathyroid hormone which

will prevent or control parathyroid tetany and which regulates the level of blood calcium." He summarized his findings as follows: "1. An extract has been made from the parathyroid glands of oxen by the use of which parathyroid tetany in dogs can be prevented or controlled. 2. The active principle in this extract produces its effect by causing the calcium content of the blood serum to be restored within normal limits." "A rise in the level of blood calcium in the normal dog has been observed following the injection of parathyroid extract." What controls the synthesis and release of this protein, and subsequently how it mobilizes calcium and phosphate from bone, are still under investigation. Additional homeostatic systems are involved.

Many urban children in temperate zones suffered from rickets through the first quarter of the twentieth century. McCollum et al. at John Hopkins wrote in 1920: "During the past 15 years studies made on the metabolism of children suffering from rickets has made it reasonably certain that the administration of cod liver oil alters the calcium balance in such a manner that calcium will be retained by the body. He suggested that ". . . some substance or substances in the oil cause calcium to be deposited in the same fashion in which deposition occurs in spontaneous healing of rachitis in man."

In London, Mellanby (1921) was reaching similar conclusions: ". . . it will be generally admitted that experimental results on animals and clinical experience are opposed to calcium deficiency as being the main cause of rickets." "The following conditions tend to prevent rickets in puppies: . . . (2) Something associated with certain fats probably identical with the fat soluble vitamine." Fresh air and sunlight seemed to help. Hume and Smith (1923) examined "the effect of air, which has been exposed to the radiations of the mercury-vapour quartz lamp, in promoting the growth of rats, fed on a diet deficient in fat-soluble vitamins." They concluded that ". . . it is the air and not some property impressed on the glass jars which is active." Their general idea was right, but the controls and conclusions were wrong. By 1924, two groups, apparently unaware of one another's work, had a better grasp of the effect. Steenbock and Black wrote: "By irradiation with the quartz mercury vapour lamp, rat rations can be activated, making them growth-promoting and bone-calcifiying, to the same degree as when the rats are irradiated directly." "It suggests itself that, in ultimate analysis, both light and the antirachitic vitamin may represent the same antirachitic agent—possibly a form of radiant energy." Hess and Weinstock came a bit closer: "It was found that cottonseed oil and linseed oil [and green vegetables] could be rendered specifically active by this means. . . ." ". . . An antirachitic factor therefore had been produced in vitro and outside the living organism." Work then proceeded on the characterization of and conversion of vitamin D as well as its general site of action. In 1937, Nicolaysen separated the phosphorus from the calcium effects: ". . . the action of vitamin D, in the gut is confined to a direct action on the absorption of Ca. The well-known reduced absorption of P in vitamin D deficiency is due to a precipitation by the increased amount of Ca in the bowel." The effects of 1,25-dihydroxycholecalciferol on protein synthesis and calcium transport are discussed in Chapter 12.

For some years, PTH and vitamin D seemed to account for calcium metabolism. Quite reasonably, McLean (1957) suggested a negative feedback control: "The setting of the regulator of the parathyroid glands is fixed under physiological conditions; it is raised in hyperparathyroidism and lowered in hypoparathyroidism." P. H. Sanderson et al. (1960) examined "calcium and phosphorus homeostasis in the parathyroidectomized dog" and concluded that "... the surviving dogs became adapted to existence without any parathyroid tissue whatsoever." Their results, which remain unexplained, implied "... that the parathyroid glands play an important part in dealing with acute disturbances of the serum calcium level. However, a secondary mechanism, capable of more sluggish regulation, seems to be revealed when the parathyroid glands are removed." Rasmussen (1961) agreed with McLean that "... on the basis of the present evidence, it appears that the primary factor regulating the secretory activity of the parathyroid glands is the calcium ion activity of the plasma." However, "if, as McLean postulates, the bone were the only means of regulating the Ca^{++} of plasma in conjuction with the parathyroids, the resulting feedback system would lead to wide oscillations in the level of Ca^{++} in the plasma." He then reasonably, but mistakedly, suggested that "the kidney is an organ admirably suited, both anatomically and physiologically, to the task of responding rapidly to minor fluctuations of parathyroid concentrations." Copp (1964) reviewed his "... evidence that hypercalcemia stimulates release from the glands of a fast-acting hormone that lowers plasma calcium. This hormone, would account for the lack of oscillation in the feedback mechanism and for the control of hypercalcemia by Sanderson et al." It remained for G. B. Foster et al. (1964) to establish the "thyroid origin of calcitonin": "Perfusion of the parathyroid glands in isolation from thyroid tissue is not possible in the dog, but this can be accomplished in the goat." "The results establish the thyroid as the source of this new hormone." Now, finally, when we thought that we had all of the components of calcium homeostasis, the most intriguing of all is just being explored. DiSaia (1966) and Kerber et al. (1968) reported fetal anomalies possibly correlated with warfarin administration during pregnancy. Postmenopausal osteoporosis is a pathological condition of bone characterized by excessive loss of calcium due to resorptive processes; vitamin K treatment has significantly reduced the negative calcium balance (Tomita, 1971). In 1975, Pettifor and Benson reported "... three additional cases of nasal hypoplasia, associated in two with skeletal abnormalities not previously described; it may be that these anomalies are related to anticoagulant [of the coumarin group] therapy during pregnancy." Independently, Warkany (1975) reviewed various clinical reports and answered yes to his title "A Warfarin Embryopathy?"

In 1974, three groups reported γ-carboxyglutamic acid (Gla) as a component of prothrombin (Chapter 13). It was soon found that all the vitamin K–dependent clotting factors contain Gla as a component essential to their calcium binding and subsequent function. Hauschka et al. (1975) and Price et al. (1976) characterized a bone protein, osteoclastin, that contains Gla. It is the seventh most abundant protein in vertebrates. At a calcium/osteoclastin molar ratio of

l 000:1, osteoclastin strongly inhibits the transition of brushite ($CaHPO_4 \cdot 2H_2O$) to hydroxyapatite [$Ca_5(PO_4)_3OH$]. Vitamin K is somehow involved in the control of mineralization in vertebrates and thereby affects calcium balance.

1.13. MEASUREMENT

As is so often the case in science, advanced understanding depends on improved physical chemical concepts and techniques—and, of course, the biological questions drive the physical research. The addition of two protons and two neutrons to argon ($^2 1s^2 2s^6 2p^2 3s^6 3p$) does not make its chemistry all that much more interesting. There is no $3d$ character to any of its orbitals. It has no visible or ultraviolet absorption spectrum. The interactions of the cation are purely ionic. The two positive charges are within a volume defined by the crystal radius of 0.99 Å. Strangely enough, those very characteristics that make the Ca^{2+} ion so dull for the chemist probably account for its unique role in biology. Most of our understanding of calcium chemistry, which really means calcium coordination (Chapter 3), has come from general studies of inorganic chemistry. Historically, most of the chemistry devoted specifically to calcium has been concerned with calcium analysis or calcium buffering. Many calcium salts are insoluble. The quantity of calcium present as CaO can be determined in such precipitates by ashing and weighing if the precipitating anion is volatile and if no other nonvolatile cations are present. Příbram (1871) first exploited the insolubility of calcium oxalate in an article translated as "A New Method for Determination of Calcium and Phosphoric Acid in Blood Serum." After centifuging out the cells and adding ammonium: "Since no precipitation of calcium phosphate occurred, which as such cannot easily exist in alkaline serum, ammonium oxalate was added whereupon clouding follows immediately." For over half a century, precipation by oxalate was the first step in calcium analysis. Richards et al. (1901) removed coprecipitating magnesium oxalate by washing the oxalate precipitate with ammonium oxalate. The method of Kramer and Tisdall (1921), as optimized by Clark and Collip (1925), was to be the standard for 30 years. The washed and acid-dissolved calcium oxalate precipitate was titrated with potassium permangenate; oxidation of the oxalate was followed by clearing of the blue permangenate.

"Eine neue Methode zur Bestimmung des Kalkes and der Phosphorsäure in Blutserum"

"Da sich keine Fallung von Calcium phosphat ergab, das als solches wohl Überhaupt in alkalischen Serum nicht leicht vorhanden sein kann, so wurde Ammoniumoxalat zugesetzt, worauf sogleich Trübung erfolgt."

There were numerous, less satisfactory variations on this basic theme of oxalate precipitation and permanganate titration:

Ligand	Reference
sulfate (EtOH ppt).	Aron, 1907
ferric thiocyanate	Marriot and Howland, 1916
alizarinate	Laidlaw and Payne, 1922
picrolonate (indicator: methylene blue)	Bolliger, 1935

Calcium-specific dyes were also developed:

stearate (turbidity)	Lyman, 1917
alizarinate	Laidlaw and Payne, 1922
sulforicinicum (turbidity)	Rona and Kleinmann, 1923
8-hydroxypinoline	Yoshimatsu, 1929
murexide	Schwarzenbach and Gysling, 1949
eriochrome black T	Gilbert and Fenn, 1957
glyoxal bis(*t*-hydroxyanil)	Goldstein and Stark-Mayer, 1958
"calcichrome"	Close and West, 1960
phthalein complexan	Herrero-Lancina and West, 1963
kalcion urea	Bezdekova and Budesinsky, 1965
antipyrylazo III	Budesinsky, 1974

These methods all exhibit disadvantages. Vines (1921) tried to use the blood coagulation reaction. "The principle of the method is the recalcification of oxalate blood by the addition of the material to be tested, and comparison of the action of the latter with that of calcium chloride solutions of known strength." Their results were not reproducible. Two valuable bioassays were developed to measure the free, as opposed to total, calcium ion. McLean and Hastings (1934) turned to the problem of a direct method, or "a biological method for the estimation of calcium ion concentration." They measured the amplitude of contraction of frog heart ventricle as a function of calcium ion, or test solution, in the bathing solution. One of the early successes was recorded in 1939 by Joseph, who described a $PbHg/PbCaO_4–CaC_2O_4$ electrode sensitive to free Ca^{2+} ions. "It has been found to come to equilibrium in 1 hour or less, and to yield stable, reproducible potentials."

By far the most significant bioassay was introduced by Shimomura et al. (1962) in the paper "Extraction, Purification and Properties of Aequorin, a Bioluminescent Protein from the Luminous Hydromedusan, *Aequorea*." This coelenterase, 28,000 Da, emits light when exposed to $[Ca^{2+}]$ over 10^{-7} M. It is quite stable, calcium specific, and nontoxic. The physical techniques now available for measuring total and free calcium and for evaluating the effect of calcium on ligands are discussed in Chapter 6.

Even though the oxalate–permanganate technique is tedious, it is accurate and it certainly yields the total calcium content of a fluid such as serum. The initial ammonium precipitation releases all or most of the protein-bound calcium. The

distinction between free and total calcium was appreciated early. Příbram (1871) anticipated protein involvement: "Physiological consequences of the deposition of calcium in the bones, the passage of calcium and phosphoric acid in the urine, etc. can be deduced only if it is established that the calcium is bound to protein." Sabbatani (1904) argued the interdependence of the various body stores of calcium and suggested that citrate prevents coagulation by forming a soluble complex with calcium. Rona and Takahashi (1911) first determined the nondiffusable, or protein-bound, calcium: "From the general result of this investigation it undoubtedly appears that a significant amount of calcium (about 25 to 35%) exists in serum in a nondiffusable form."

"Physiologishe Folgerungen über die Ablagerung des Kalkes in den Knocken, den Übergang des Kalkes and des Phosphorsäure in den Harn etc. werden sich erst ableiten lassen, wenn es feststeht, dass der Kalk an Eiweiss gebunden ist"

"Aus der Gesamtheit dieser Versuche geht unzweifelhaft hervor dass eine nennenswerte Menge des Calciums (etwa 25 bis 35%) in Serum in nicht diffusible Form vorhanden ist."

In 1915, Van Slyke and Bosworth used a Pasteur–Chamberland filtering tube to demonstrate that "calcium caseinate (casein Ca_4) and dicalcium phosphate ($CaHPO_4$) are not in combination." In 1921, von Meysenburg reported a careful study of dialysis of serum through colloidin membranes. He concluded that there is "... no alteration in the percentage of diffusible calcium of the serum..." between pH 7.0 and 7.6 or between rachitic or tetanic dogs and normal. "The diffusible calcium of the serum of normal men and dogs was found to comprise from 60 to 70 per cent of the total serum calcium."

Of course, it was appreciated that the concept of "nondiffusable" calcium was valid only for very slowly exchanging calcium. Greenberg and Gunther (1930) found that half of the serum calcium was retained by a colloiden membrane. They admitted that their results "... were not completely theoretically interpretable" but nonetheless, were consistent and reasonable.

In 1926, Greenwald offered an incorrect but stimulating and testable idea. "It is suggested that the calcium content of the plasma is, normally, maintained at a constant level by equilibration between inorganic calcium and an organic compound of calcium. Resemblances between this organic compound and calcium citrate are indicated, but the substances are not identical. It is suggested that the parathyroid hormone is necessary to the preparation of this organic constituent."

McLean and Hastings (1935) applied their frog heart assay to a study of calcium metabolism. They demonstrated by graded additions that "The frog's heart is insensitive to CaCit and Cit_3." They refuted Greenwald's suggestion that "agreement of the values for the mass law constant obtained from observations upon purified serum proteins and protein containing fluids indicates that it is unnecessary to postulate any appreciable amount of other calcium-binding substances in human fluids." Total serum calcium corresponded to values published by other workers: 9.0 to 11.5 mg per 100 mL of serum in normal and

rachitic adults, 12.0 to 16.0 in hypoparathyroidism and multimyeloma, and 4.0 to 8.5 in hypoparathyroidism and infantile tetany. Most important, the level of free calcium ion could be expressed in terms of a nomograph varying total serum calcium and total serum protein. The average protein dissociation constant $K_{\text{dis}} = [\text{Pr}] \times [\text{Ca}]/[\text{PrCa}]$. They were justified in referring to the serum content of $[Ca^{2+}]$ as "... one of nature's physiological constants."

To distinguish free from total calcium within the cell was a greater challenge. Pollack (1928) injected alizarin sulfonate into amoebae. Unfortunately, alizarin sulfonate is moderately toxic; the resulting red crystals form slowly, are difficult to see, and do not represent a quantitative response. His failure defined the three criteria of a good cytosolic indicator. The microinjection of arsenazo III, antipyrylazo III, and aequorin have proven reasonably satisfactory. The development of calcium-specific microelectrodes is progressing well (Chapter 6).

It is also valuable to determine the cellular or organelle distribution of total calcium. G. H. Scott's microincineration method (1932) gave an accurate spatial distribution of the total metal distribution within the cell. His principle (should have) guided many subsequent refinements. "It is necessary to use a fixative which neither removes nor increases the inorganic elements of the tissue." The limits of spatial resolution of electroprobe analysis now approach 100 μm.

1.14. BIOMINERALIZATION: REDUX

Having left bone a century ago, we might now ask what progress has been made. Much of this research can be considered in terms of physiological and histological studies on the dynamics of bone deposition and resorption and the compositions and solubilities of the bone salts. Papillon (1870) examined the dynamics and "... the limits and the variations of physiological determinism." These "... demonstrate that one can substitute a certain quantity of strontium, of magnesium, of aluminium for the calcium normally contained in bone."

"... les limites et les variations du déterminitisme physiologique." His experiments "... démontrent que l'on peut substituer une certaine quantité de stroniane, de magnésie, d'alumine á la chaux normalement contemu dans les os."

"En résumé, l'ostéoclaste, cellule géante á noyau unique et géante à noyaux multiple, ressortissant à la classe des cellules de nature connective, a un protoplasma commun rempli d'un nombre incroyable de mitochondries, sans préjudice des vacuoles à lipoïdes et des vacuoles colorables par le rouge neuter."

Dubreuil (1910) was one of the first workers to examine bone histology and to implicate the mitochondrion in bone metabolism. "In summary, the osteoclast, giant cell with a single giant nucleus or multiple nuclei, arose from the class of cells of connective nature, and has a protoplasm completely filled with an incredible number of mitochondria, without damage to the lipoidic vacuoles and the vacuole that can be coloured by neutral red."

In his 1911 Harvey Lecture, Wells emphasized an important distinction. "In calcification we have deposited in dead tissues, or in tissues of low vitality, a considerable quantity of inorganic calcium salts, which appear at first in granular form, although later there may be more or less fusion and resulting areas of homogeneity." "In normal ossification, however, the homogeneous calcium deposits are closely related to living cells, which not only determine the form of the deposits, but which also are able to dissolve the insoluble salts or to cause their deposition as may be needed, thus rendering the inorganic salts of bone the reserve supply of a tissue of active metabolism.. . ."

Of course, for centuries it had been realized that calcium and phosphate were the primary inorganic constituents. What is the nature of this mineral? In 1931, Bogert and Hastings suggested that "the chief inorganic consituent of bone is probably a crystalline salt, $CaCO_3 \cdot nCa_3(PO_4)_2$, where n approximates the value 2 in untreated bone." In 1937, Huggins determined the Ca/P ratio as 1 : 0.6, with variation for diet, bone type, and stage of development. He argued that $CaCO_3$ is of little significance. McConnell (1938) surveyed the wide range of compositions and unit cell dimensions available for apatite-type minerals and concluded that they were all approximately isomorphous with the fluorapatite ($Ca_{10}P_6O_{24}F_2$) structure proposed in 1930 independently by Náray-Szabó and by Mehmel. The cations Mg^{2+}, Mn^{2+}, Sr^{2+} K^+, and Na^+ could replace Ca^{2+} to varying extents; AlO_4^{3-}, YO_4^{3-}, SO_4^{2-}, and CO_3^{2-} could partially replace PO_4^{3-}; and Cl^- and OH^- could replace F^-. Neuman and Neuman (1953) summarized the evidence supporting an apatite structure, $Ca_5(PO_4)_3$(OH or F), in bone. They explored the seemingly innocent, yet still not understood subject of calcium phosphate solubility. "It has been shown that the K_{sp} of $CaHPO_4$ must be exceeded for precipitation to occur, yet calcification occurs in individuals whose blood levels of calcium and phosphate are well below this critical product."

Levinskas and Neuman (1955) continued their solubility studies of synthetic hydroxyapatite. "This crystalline solid exhibited incongruent solubility, i.e. the solutions gave a higher Ca : P ratio than that present in the solid phase in most instances." "The most significant finding was that solubility varied with the amount of solid phase added to a given volume of solution. This often observed phenomenon, in the present instance, is real evidence that the hydroxylapatite lattice exhibits incongruent solubility; it does not conform to a solubility product. This is not surprising since the solid phase is not of constant composition. The common ion effect supports this conclusion. There was no simple relation between calcium and phosphate concentration after equilibrium. In contradiction to the conclusions of Neuman and Neuman, McLean and Budy (1959) concluded that "the body fluids are supersaturated with respect to the final product hydroxyapatite. It is certain that the crystal domains are so small, the surface to volume ratio is so enormous, the crystalline disorder so great, and the contact with organic components so extensive that our traditional concepts of crystallinity and solubility are inadequate." Various polypeptides, in particular the vitamin K–dependent osteocalcin (Hauschka et al., 1975; Price et al., 1976), which stabilize supersaturated solutions of calcium phosphate, might also alter the apparent equilibrium.

Taves (1963) determined the crystal structure $Ca_4H(PO_4)_3$ and noted its similarity to that of $Ca_5(PO_4)_3OH$. "This view of the two crystals suggests an explanation for the observation that octacalcium phosphate grows more rapidly than hydroxyapatite, even though the latter is more stable" (Chapters 3 and 4). Apatite is well named, α'πατα'ω, "I deceive."

Even when we do understand the solubility of calcium phosphate, we will still be ignorant of the cell's contribution. The abundance of phosphatase in ossifying cartilage led Robison in 1923 to suggest that the hydrolysis of "hexosephosphoric esters" resulted in increased local concentrations of phosphate and resulting calcium phosphate precipitation. There is little supporting evidence for the many variations of this postulate. The preformed precipitate and/or packet idea of Lehninger (1970) still motivates much research; yet confirmation is still lacking. A popular idea is that some organic component, collagen being the leading candidate, provides a nucleation site. Why all collagen in contact with serum does not nucleate remains a mystery. Yet no matter whether one argues selective solubilization or selective nucleation, the problem remains: How does the cell cheat equilibrium thermodynamics in the extracellular environment?

Ossification, after Well's usage, is certainly not limited to the vertebrate skeleton. For example, filtrates of cultures of *Leptothrix buccalis*, an organism always present in dental tartar, does not induce precipitation of saturated solutions of calcium phosphate, whereas filtrates from macerated cells do. In 1925, Bulleid concluded that "it is therefore probable that the precipitating action of the *Leptothrix* is due to some property in the actual organism, not in any way to the production of an extotoxin." In 1967, Ennever and Creamer summarized their own (Ennever, 1960) and others' work supporting the interpretation that calcum phosphate crystals are formed intracellularly in *Bacterionema matruchottii*. This implies that if, in fact, bacteria do extrude calcium from the cytosol, some bacteria have special vacuoles for calcium acccumulation.

Algae appear to have such organelles. Watabe (1967) described his electron microscopic observations. "Coccoliths are simple or complex aggregates of microcrystals of calcite [$CaCO_3$] covering the cells of algae belonging to the Coccolithineae." "... the coccoliths of *Hymenomonas* (*Cricosphacra*) are found in vacuoles [within the cytoplasm] the outlines of which do not differ greatly from the coccolith-forming region of *Coccolithus* (*huxleyi*)." Pienaar (1971) continued: "It was found that the Golgi body was directly involved in the production of the baseplate scale, the organic matrix membrane and the deposition of calcium carbonate to produce the coccolith." Such ossification by primitive organisms had already provided new insights to students of evolution (Margulis, 1975). Biochemists will find them equally rewarding.

It is natural that early investigators should seek reasons for the unique distributions and functions of the metal ions of biological fluids. Quinton (1900) first noted a correspondence that has since intrigued physiologists: "Osmosis establishes, from the point of view of minerals, a remarkable communication between the internal milieu of the marine invertebrate and the external milieu." "... in

the natural states a great number of animals have as their internal milieus for minerals that same milieu as the ocean itself."

"L'osmose établit donc, au point da vue minéral, une communication remarquable entre le milieu intérieur de l'Invertébré marin et le milieu extérier." He then suggested: "... a l'état de nature, le plus grand nombre des organismes animaux a pour milieu interieur, ou point de vue minéral, le milieu marin lui-même."

Independently MacCallum (1910) observed: "The inorganic composition of these Medusæe, on this view, reflects to a certain extent the composition of the ocean water, not only of today but also of past, perhaps very remote, geological periods." Our bones not only provide unique challenges to contemporary cell biologists, but also bear witness to our evolution.

Lowenstam (1981) summarized "minerals formed by organisms": "four calcium carbonates, four calcium phosphates, two calcium oxalates, three calcium sulphates, calcium fluorite (all 14 of these calcium minerals with amorphous forms), as well as silica ($SiO_2 \cdot nH_2O$), five ferrous/ferric oxides, two pyrites, and manganese oxide. We have yet to understand how cells convert ions to stones. However, one can safely postulate that the mechanisms involved in regulating biomineralization and in avoiding intracellular precipitation of calcium salts preceded the evolution of the use of calcium for information transduction."