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Mitochondria and Calcium Ion Transport

THE FIFTH JUBILEE LECTURE

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In the last few years there has been a growing crescendo of research on the respiration-linked transport of ions by isolated mitochondria. Most of this work has been focused on the molecular mechanism of energy-coupling during electron transport, especially the burning dilemma posed by the chemical-coupling and chemiosmotic hypotheses for oxidative phosphorylation. But there is another side to mitochondrial ion transport, namely its role in cell biology. In this Jubilee Lecture I propose to discuss possible biological functions of Ca^{2+} transport by mitochondria. I shall first describe those properties of the Ca^{2+}-transport system of mitochondria that endow it with considerable specificity, high affinity and its primacy over oxidative phosphorylation in most tissues. Then I shall outline some experimental work and ideas on the role of mitochondrial Ca^{2+} transport in the excitation–relaxation cycle of certain muscles and in the molecular biology of calcification.

Background
(*for review see Lehninger, Carafoli & Rossi*, 1967)

That mitochondria can accumulate certain ions from the suspending medium was first observed in the early 1950s, but little progress was made in analysing this process quantitatively until my colleagues Vasington & Murphy (1961, 1962), and independently DeLuca & Engstrom (1961), discovered that isolated mitochondria can accumulate large net amounts of Ca^{2+} from the suspending medium during electron transport, up to several hundred times the initial Ca^{2+} content. This was a rather unexpected observation; Ca^{2+} had long been regarded as toxic to mitochondria because it inhibits oxidative phosphorylation and has other deleterious actions (Potter, 1947; Lehninger, 1949; Slater & Cleland, 1953). However, it was found that accumulation of Ca^{2+} replaces and thus is alternative to oxidative phosphorylation; since both processes are inhibited by 2,4-dinitrophenol they apparently depend upon a common high-energy intermediate or state generated by electron transport.

It was then found that phosphate may accompany the respiration-linked accumulation of Ca^{2+} (Lehninger, Rossi & Greenawalt, 1963) and that the uptake of both Ca^{2+} and phosphate is stoicheiometric with electron transport, in such a manner that about 1.7–2.0 Ca^{2+} ions and about 1.0 phosphate ions are accumulated per pair of electrons traversing each of the three energy-conserving sites of the respiratory chain (Rossi & Lehninger, 1963). Moreover, it was found that Ca^{2+} stimulates respiration of mitochondria in a stoicheiometric and cyclic fashion, in such a manner that 2 Ca^{2+} ions yield the same amount of extra oxygen uptake as 1 molecule of ADP (Chance, 1963, 1965; Rossi & Lehninger, 1964). Still another important stoicheiometric relationship was uncovered with the finding that accumulation of Ca^{2+} during electron transport is accompanied by H^+ ejection (Saris, 1963; Engstrom & DeLuca, 1963); in the absence of phosphate or other 'permeant' anions, at least 1 proton is ejected per Ca^{2+} ion accumulated (reviewed by Lehninger *et al.* 1967). Thus during Ca^{2+}-activated electron transport at least 2 protons are ejected as a pair of electrons traverses each of the three energy-conserving sites. In fact, some observations suggest that the true maximum is 4 protons per site (Massari & Azzone, 1970). Accompanying the ejection of H^+ is alkalinization of the mitochondria (Rossi, Bielawski & Lehninger, 1966; Chance & Mela, 1966; Lynn & Brown, 1966*a*,*b*; Addanki, Cahill & Sotos, 1967); direct titrations show that this alkalinization is stoicheiometric with the H^+ ejected and thus with electron transport (Rossi *et al.* 1966; Gear, Rossi, Reynafarje & Lehninger, 1967). These observations have been of central importance in evaluating the role of proton movements in the mechanism of oxidative phosphorylation.

Table 1 summarizes a number of important relationships between Ca^{2+} and anion accumulation, the amounts of Ca^{2+} that may be accumulated under different conditions and the intramitochondrial compartmentation of the accumulated ions. Of special significance is the finding that,

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Table 1. *Characteristics of energy-linked* Ca^{2+} *uptake under different conditions*

Permeant anion(s) in medium	Means of charge compensation	End result	Capacity (nmol/mg of protein)
None	H^+ ejection	Ca^{2+} bound to membrane; 'alkalinization'; State 6	~80 (limited loading)
Acetate or phosphate	Entry of anion	Soluble calcium salts in matrix; swelling	200–300
Phosphate+ATP (ADP)	Entry of anion	Formation of $Ca_3(PO_4)_2$ granules	3000 (massive loading)

when Ca^{2+} is accumulated in the absence of matching permeant anions, it becomes bound to the inner mitochondrial membrane. Only when matching permeant anions are present does the accumulated Ca^{2+} appear in the matrix, together with the anion (Chappell & Crofts, 1965; Rasmussen, Chance & Ogata, 1965; Gear *et al.* 1967; Hackenbrock & Caplan, 1969). Secondly, ATP (or ADP) is required to promote formation of the electron-dense deposits of precipitated calcium phosphate and in thus allowing 'massive loading' of mitochondria to take place (Rossi & Lehninger, 1964; Carafoli, Rossi & Lehninger, 1965). The role of ATP in this process, which we shall return to, is still unknown.

With these principal relationships as background, let us examine the specificity, affinity, and the mechanism of the transport of Ca^{2+} across the mitochondrial membrane.

Specificity and affinity

Only three divalent cations (Ca^{2+}, Sr^{2+} and Mn^{2+}) are accumulated by intact liver mitochondria in an energy-linked process stoicheiometric with electron transport, although Ba^{2+}, Zn^{2+} and Fe^{2+} may be bound in a respiration-independent process. Ca^{2+} and Sr^{2+} are taken up at about equal rates and with nearly equal affinity; Mn^{2+} runs a very poor third (see review by Lehninger *et al.* 1967; Mela & Chance, 1968). Mg^{2+} is not accumulated by intact mitochondria from most tissues, although heart mitochondria may be a special case (Brierley, Bachmann & Green, 1962). Moreover, neither K^+ nor Na^+ is accumulated by intact mitochondria in a net stoicheiometric fashion unless ionophorous antibiotics, such as valinomycin or gramicidin, are present. Only Ca^{2+} of all the common uni- and bi-valent cations present in cells is rapidly and stoicheiometrically accumulated by mitochondria.

That the respiration-dependent accumulation of Ca^{2+} may be a normal biological process is also strongly suggested by the exceedingly high affinity of mitochondria for Ca^{2+}, which greatly exceeds that for ADP. In fact, when an equimolar mixture of Ca^{2+} and ADP is presented to intact rat liver mitochondria, even at very low physiological concentrations, the Ca^{2+} is accumulated in preference to phosphorylation of ADP; no ATP is formed until essentially all the added Ca^{2+} has been accumulated (Rossi & Lehninger, 1964). In the steady state that occurs after accumulation of a pulse of Ca^{2+} in the absence of a permeant anion, only about 1.0 μM-Ca^{2+} remains in the suspending medium (Drahota, Carafoli, Rossi, Gamble & Lehninger, 1965); when ATP and Mg^{2+} are also present, liver mitochondria can decrease the concentration of Ca^{2+} in the medium to less than 0.2 μM.

This exceedingly high affinity for Ca^{2+} not only supports a biological role for mitochondria in Ca^{2+} transport, but it also indicates that this role must be an important one in the energy economy of the cell, since it may take primacy over oxidative phosphorylation.

Tissue and species distribution

Accumulation of Ca^{2+} stoicheiometrically with electron transport was early found to occur in mitochondria from rat liver, kidney, brain and heart (Rossi & Lehninger, 1963). In a more recent survey (Carafoli & Lehninger, 1970) mitochondria from all mammalian tissues examined (Table 2), including the adrenal cortex, testis, spleen and skeletal muscle of such animals as the mouse, rat, guinea pig and rabbit, show the capacity for energy-linked accumulation of Ca^{2+}. Ca^{2+} accumulation also occurs in mitochondria of at least some higher-plant cells (Bonner & Pressman, 1965; Kenefick & Hanson, 1966). However, the mitochondria of two yeasts (*Saccharomyces cerevisiae* and *Torula utilis*) have been found to lack completely the capacity for Ca^{2+} accumulation (Carafoli, Balcavage, Lehninger & Mattoon, 1970), although *Neurospora crassa* mitochondria do show activity (Carafoli & Lehninger, 1970). Since respiring yeast mitochondria eject H^+ and accumulate K^+ in the presence of valinomycin, it appears possible that they possess the electronmotive (or protonmotive) power to accumulate Ca^{2+}, but fail to do so because Ca^{2+} cannot pass through the mitochondrial membrane. These observations were among those that led us to consider that Ca^{2+} can readily cross the mitochondrial membrane only on a specific, genetically determined, carrier or porter that is not

Table 2. *Phenotypic distribution of capacity for respiration-linked* Ca^{2+} *accumulation and high-affinity* Ca^{2+} *binding*

Source of mitochondria	Ca^{2+} accumulation	High-affinity Ca^{2+} binding
Livers of rat, guinea pig, mouse, rabbit, cow	+	+
Rat kidney	+	+
Rat heart	+	+
Rat brain	+	+
Rabbit heart	+	+
Rabbit kidney	+	+
Rabbit brain	+	+
Rabbit spleen	+	+
Ox adrenal cortex	+	+
Blowfly flight muscle	Slow non-stoicheiometric uptake	0
N. crassa	+	+
S. cerevisiae	0	0
T. utilis	0	0

itself a component of the respiratory chain. Let us now review the evidence for such a Ca^{2+} carrier.

Models of Ca^{2+}*-transport mechanisms*

Two types of models have been proposed for the mediated transport of Ca^{2+} across the mitochondrial membrane. One assumes that Ca^{2+} combines directly and stoicheiometrically with a hypothetical high-energy intermediate generated by electron transport in such a way that the unidirectional transfer of Ca^{2+} across the membrane into the mitochondrion is coupled to discharge of the high-energy bond. Such a model was proposed by Chance (1965), who also suggested that the high-energy intermediate is protonated, in order to account for the simultaneous ejection of protons to the external medium as Ca^{2+} is transported inward, according to the vectorial equations:

$$\underset{\text{(outside)}}{2\,Ca^{2+}} + \underset{\text{(membrane)}}{H_2(X\sim I)} \rightarrow \underset{\text{(membrane)}}{Ca_2(X\sim I)} + \underset{\text{(outside)}}{2\,H^+}$$

$$\underset{\text{(membrane)}}{Ca_2(X\sim I)} \rightarrow \underset{\text{(membrane)}}{X+I} + \underset{\text{(inside)}}{2\,Ca^{2+}}$$

The hallmarks of this type of model are obligatory stoicheiometry between Ca^{2+} uptake and electron transport and a specific directionality.

The other type of model is a passive reversible carrier or porter, independent of the respiratory chain, that binds Ca^{2+} in a specific saturable manner and can transport it in either direction across the membrane in response to a transmembrane electrochemical gradient. Such a Ca^{2+} carrier would be analogous to other carriers known to be present in the mitochondrial membrane, for example the atractyloside-sensitive carrier for ADP and ATP (for review see Chappell, 1968). A Ca^{2+} carrier could be either neutral or electrogenic. If neutral, it would carry a charge-compensating counteranion with the Ca^{2+}, or exchange a charge-compensating cation such as H^+. In an electrogenic carrier, which is the more likely model, the electrical charge of Ca^{2+} would not be compensated and the carrier would thus respond to either a chemical or an electrical gradient, i.e. an electrochemical gradient. Because such a hypothetical Ca^{2+} carrier is not a member of the respiratory chain and is not chemically coupled to the latter, it can be expected to function independently of electron transport, in either direction, depending on the electrochemical gradients of all relevant ionic species. Both models provide for respiration-dependent transport of Ca^{2+} against a gradient, the former by direct chemical coupling, the latter in response to an electrochemical gradient generated by electron transport. The evidence strongly supports the second model.

Independence of Ca^{2+} *movements and electron transport*

Although the $Ca^{2+}:\sim$ accumulation ratio is normally 2.0 during electron transport, this rigid stoicheiometry is observed only when phosphate or some other 'permeant' anion is present in the medium and is accumulated with the Ca^{2+}. When no permeant anions are present, the $Ca^{2+}:\sim$ accumulation ratio may vary considerably, depending on conditions. At elevated pH (approx. 8.0) or when the potassium chloride concentration exceeds 100 mM, the $Ca^{2+}:\sim$ ratio may very greatly exceed 2.0, a condition known as super-stoicheiometry (Carafoli, Gamble, Rossi & Lehninger, 1967). At low pH and low potassium chloride concentrations the $Ca^{2+}:\sim$ ratio is less than 2.0, and damped oscillatory movements of Ca^{2+} into and out of the mitochondria may occur without change in the rate of oxygen uptake (Carafoli, Gamble & Lehninger, 1966). Such observations are clearly inconsistent with a stoicheiometric chemical interaction of Ca^{2+} with a high-energy intermediate followed by unidirectional inward transport. They are, however, wholly consistent with the independent carrier model. The variations in the $Ca^{2+}:\sim$ accumulation ratio that are induced by changes in pH or potassium chloride concentration are apparently caused by alterations in the electrochemical gradient across the mitochondrial membrane.

High-affinity binding sites for Ca^{2+}

Further evidence for the occurrence of a specific carrier for Ca^{2+} has come from the study of the number and affinity of specific Ca^{2+}-binding sites

in mitochondria. Scatchard plots of the binding of Ca^{2+} to non-respiring rat liver mitochondria have revealed that at least two sets of respiration-independent Ca^{2+}-binding sites exist (Reynafarje & Lehninger, 1969). One set has rather low affinity (K_m 100 μM) but is very numerous (40–60 nmol/mg of mitochondrial protein); this type of Ca^{2+} binding is believed to involve non-specific anionic binding groups of membrane proteins and lipids. However, the other set is much less numerous (<1.0 nmol/mg of protein) and has a very high affinity for Ca^{2+} (K_m 0.1–1.0 μM), comparable with the affinity of respiring mitochondria for Ca^{2+}. The high-affinity Ca^{2+}-binding sites have a number of other properties consistent with the view that they represent the binding sites of Ca^{2+}-carrier molecules in the membrane. They can bind Ca^{2+} in the absence of electron transport or ATP hydrolysis. They agree in specificity since they bind Ca^{2+}, Sr^{2+} and Mn^{2+}, but not Mg^{2+}, K^+ or Na^+ (Reynafarje & Lehninger, 1969). The high-affinity Ca^{2+}-binding sites are present only in those species of mitochondria that are capable of accumulating Ca^{2+} stoicheiometrically during respiration (Table 2). Mitochondria from blowfly muscles, or from the yeasts *S. cerevisiae* and *T. utilis*, which are unable to accumulate Ca^{2+} during respiration (Table 2), possess no high-affinity Ca^{2+}-binding sites, although they are of course capable of oxidative phosphorylation. Moreover, La^{3+} ions, which inhibit respiration-linked Ca^{2+} accumulation (Mela & Chance, 1968; Lehninger, 1969), also prevent high-affinity Ca^{2+} binding. Much evidence therefore supports the view that high-affinity Ca^{2+} binding reflects the action of a specific Ca^{2+} carrier in the membrane. Independent evidence for a Ca^{2+} carrier has been brought forward by Chance & Azzi (1969).

Extraction of a Ca^{2+}-binding protein from mitochondria

Although none of the specific anion carriers of the mitochondrial membrane, such as those for phosphate, malate, citrate or ATP, have yet been extracted in soluble form and purified, we have recently obtained extracts of rat liver mitochondria, after osmotic shock with distilled water, which show high-affinity Ca^{2+}-binding activity (Lehninger & Carafoli, 1969). The binding of Ca^{2+} is measured by means of equilibrium dialysis carried out in micro-cells or by an ultrafiltration procedure. Scatchard plots showed that the soluble Ca^{2+}-binding factor has capacity for both high- and low-affinity binding of Ca^{2+}, the former with an affinity for Ca^{2+} about equal to that of intact mitochondria. The soluble preparations also bind Sr^{2+}, Mn^{2+} and La^{3+}, but are much less active towards Mg^{2+}. The Ca^{2+}-binding activity is heat-labile, non-diffusible on dialysis and, from its behaviour on molecular-exclusion columns, has a Stokes radius corresponding to a molecular weight of 150000, assuming that the molecule is spherical (M. Hollenberg & A. L. Lehninger, unpublished work). The mitochondrial Ca^{2+}-binding protein is apparently not identical with the vitamin D-dependent Ca^{2+}-binding protein of intestinal mucosa (Wasserman, Corradino & Taylor, 1968). With a more purified preparation of the mitochondrial Ca^{2+}-binding protein we shall attempt to reconstitute transmembrane transport of Ca^{2+} in mitochondrial membranes or in synthetic phospholipid bilayers.

Now let us examine two important Ca^{2+}-dependent processes in cells in which the specific transport of Ca^{2+} by mitochondria may be a central element.

Mitochondrial Ca^{2+} transport and the excitation–relaxation cycle of muscle

Recent experiments strongly suggest that mitochondria may play a significant role in the excitation and relaxation of muscles, either supplementing the established role of the sarcoplasmic reticulum in segregation of Ca^{2+} or possibly even supplanting it in some types of muscles. In particular, the high affinity and specificity of the Ca^{2+}-transport mechanisms in mitochondria may enable them to function in the release and segregation of Ca^{2+} in red muscles, which are profuse in mitochondria but have a rather sparse sarcoplasmic reticulum, whereas the sarcoplasmic reticulum may be dominant in this function in white muscles, which have few mitochondria but are rich in reticulum. Patriarca & Carafoli (1969) have provided some evidence for this view. They injected rabbits with $^{45}Ca^{2+}$ and from samples of white muscle (adductor magnus) and red muscle (masseter) isolated the mitochondrial and microsomal fractions. They found that both the specific radioactivity of the Ca^{2+} and the radioactivity per unit protein were very much higher in mitochondria than in microsomes of the red muscle; indeed, the microsomes contained only a few per cent of the $^{45}Ca^{2+}$. In white muscle the microsomes were far more active in binding Ca^{2+}, but even in this case the mitochondria contained a surprisingly large amount of $^{45}Ca^{2+}$. Appropriate control experiments showed that these differences were not artifacts taking place during isolation of mitochondria. Other investigators (Haugaard, Haugaard, Lee & Horn, 1969; Fehmers, 1967) have also considered the possibility that mitochondria may play a significant role in Ca^{2+} movements in the excitation and relaxation of the actomyosin system in heart muscle.

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 physio-

logical role in the excitation process. It is already known that a large fraction of previously loaded Ca^{2+} can be released from mitochondria very rapidly by uncoupling agents, indicating that intramitochondrial Ca^{2+} is largely very mobile. One possible mechanism for the release of Ca^{2+} from 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. This aspect of Ca^{2+} transport in mitochondria clearly requires much further study.

Formation of calcium phosphate granules in mitochondria

Another possible role for the Ca^{2+}-transport system of mitochondria is in biological calcification processes. Such a role was first suggested by the enormous amounts of calcium phosphate that may be sequestered by mitochondria, up to 3.0 μmol/mg of protein, which represents an increase of over 25% in their dry weight. Since this would amount to an 0.8M solution, which vastly exceeds the solubility product of calcium phosphate, if dissolved in the matrix water, it was a foregone conclusion that calcium phosphate is deposited in a solid form in massively loaded mitochondria. Electron microscopy indeed showed that many large electron-dense osmiophilic granules appear in the matrix of rat liver mitochondria when they are massively loaded with calcium phosphate (Lehninger *et al.* 1963; Greenawalt, Rossi & Lehninger, 1964; Brierley & Slautterback, 1964; Weinbach & von Brand, 1965). Deposition of such granules requires electron transport; they do not form in the absence of an energy source no matter how high the concentration of Ca^{2+} in the suspending medium. Moreover, such granules do not form if phosphate is replaced by other permeant anions such as acetate or arsenate. It is particularly noteworthy that ADP or ATP, in addition to a respiratory substrate, is required for the deposition of the granules. If ADP is omitted, the mitochondria simply swell and fail to accumulate more than a few hundred nmol of Ca^{2+}/mg of protein. ADP or ATP cannot be replaced in this function by any other nucleoside di- or tri-phosphates. Since atractyloside prevents the accumulation of calcium phosphate it is probable that the ATP or ADP must actually enter the mitochondria in order to support calcium phosphate accumulation; indeed, the adenine nucleotide content of mitochondria was found to rise some tenfold during accumulation of calcium phosphate (Carafoli *et al.* 1965). Despite much research (Rossi & Lehninger, 1964; Carafoli *et al.* 1965; Caplan & Greenawalt, 1967; Bass & Lehninger, 1970) the role of ADP or ATP in the deposition of calcium phosphate in the matrix is still not understood; perhaps it is required to prepare the cristae or matrix in some manner to initiate the precipitation of calcium phosphate in well-defined granules. The energy-linked deposition of calcium and phosphate in mitochondria occurs with a Ca/P ratio between 1.51 and 2.00, with an average of about 1.71, or close to the Ca/P ratio 1.67 for hydroxyapatite (Rossi & Lehninger, 1963).

Are such large electron-dense deposits of calcium phosphate in the mitochondrial matrix merely artifacts of the test tube, or can they also be observed in mitochondria in intact tissues? The answer is now quite clear. Such granules have been repeatedly observed in mitochondria *in situ* in thin sections of various tissues under both normal and pathological conditions (Table 3). They are normally present in mitochondria of osteoclasts in healing bone fractures, in chondrocytes of cartilage, in some protozoa and in the shell gland of the fowl. They also form under some pathological conditions leading to calcification: poisoning by carbon tetrachloride, administration of excess of vitamin D, Ca^{2+}, parathyroid hormone, or isoproterenol, and in some malignant tissues.

A series of investigations on the dynamics and sequence of events in massive loading of mitochondria with calcium phosphate and strontium phosphate has been carried out in our Department with the aid of chemical and isotopic analysis, density-gradient centrifugation to follow the increase in apparent density of the mitochondria, electron microscopy after fixation with osmium or

Table 3. *Some observations of electron-opaque deposits of insoluble calcium salts in mitochondria* in situ

Observation	Reference
Osteoclasts in healing bone fractures	Gonzalez & Karnovsky (1961)
Shell-gland epithelium of fowl	Hohman & Schraer (1966)
Chondrocytes in calcifying cartilage	Martin & Matthews (1969)
Kidney after calcification induced by parathyroid hormone and excess of Ca^{2+}	Caulfield & Schrag (1964)
Kidney after administration of excess of vitamin D	Scarpelli (1965)
Liver after carbon tetrachloride poisoning	Reynolds (1965)
Calciferous gland of earthworm	Crang, Holsen & Hitt (1968)
Protozoan *Spirostomum*	Jones (1967)
Heart after isoproterenol administration	Bloom & Cancilla (1969)
Skeletal muscle after tetanus toxin	Zacks & Sheff (1964)

glutaraldehyde and a micro-incineration procedure. In the last-named technique the location of minerals in mitochondria in various stages during Ca^{2+} loading is ascertained by plating the mitochondria on a grid and subjecting them to low-temperature incineration with electrically excited oxygen, a process that leaves the mineral components on the grid in positions corresponding to their location in the intact mitochondria (Thomas & Greenawalt, 1964).

From such approaches it has been found (Greenawalt *et al.* 1964; Greenawalt & Carafoli, 1966; Thomas & Greenawalt, 1968) that there are at least three stages in the energy-linked deposition of Ca^{2+} in rat liver mitochondria. In the first, a few very small electron-dense granules begin to appear on the inner surface of the inner membrane, particularly on the cristae. In the second, small isolated granules begin to appear free in the matrix. In the third stage, the granules grow by accretion to form larger masses, which may range up to $0.3\,\mu m$ in diameter. At all times in the growth of these masses they show fine granularity, as though they are formed by accretion of many small units. Contrary to many speculations, it is unlikely that such calcium phosphate deposits arise from the 'dense granules' normally found in the matrix of some mitochondria. Not only are the 'dense granules' too few in number to account for the very large numbers of calcium phosphate granules that may form, but they leave no mineral deposit after micro-incineration of normal mitochondria (Ronchetti-Pasquali, Greenawalt & Carafoli, 1969).

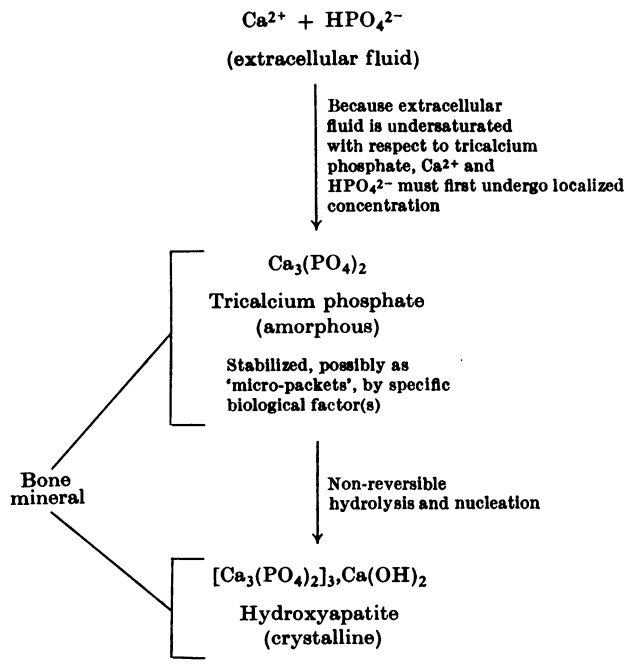
Are the electron-dense calcium phosphate granules amorphous or crystalline? Through the co-operation of Professor Aaron Posner of New York Hospital, X-ray diffraction analysis of freeze-dried or acetone-dried rat liver mitochondria massively loaded with calcium phosphate was carried out, but not even a faint hydroxyapatite pattern could be seen. Either the calcium phosphate deposits were totally amorphous or the crystals were too small to yield visible bands (Greenawalt *et al.* 1964). Very similar results were later obtained by Thomas & Greenawalt (1968), who observed no diffraction bands from the granular mineral deposit left after low-temperature micro-incineration of Ca^{2+}-loaded mitochondria. However, if such deposits were heated to 900°C and cooled, a process known to convert amorphous calcium phosphate into crystalline diffracting hydroxyapatite, a well-defined hydroxyapatite diffraction pattern appeared, demonstrating that the precursors of hydroxyapatite are present in the amorphous calcium phosphate granules. Curiously, loading of rat liver mitochondria with strontium phosphate, even in the absence of ATP, results in crystals that are easily seen with the electron microscope (Greenawalt & Carafoli, 1966).

Mitochondria and calcification

Although we had long considered the possibility that mitochondria might participate in some way in the process of calcification of hard tissues (Greenawalt *et al.* 1964), as have others since (for example Halstead, 1969; Shapiro & Greenspan, 1969), these speculations have been largely intuitive and have lacked a chemically sound rationale for the role of the mitochondria. But it is now possible to develop a much more satisfactory hypothesis in the light of more recent developments in the study of the calcification process. First we must recall that the capacity to form solid deposits of calcium salts is by no means a highly specialized process limited only to bone; it is in fact a very broadly distributed property, as is already suggested in Table 3. Deposition of insoluble calcium salts occurs in the formation of eggshells, in the formation of exoskeletons of many invertebrates, as in crustaceans and molluscs, and in the hardening of the stalks and stems of higher plants. Indeed, the potential to undergo calcification is inherently present in most tissues of higher animals. It is well known in human pathology that the brain, blood vessels, the kidney and the liver are quite prone to undergo calcification as a response to injury. Because the biological potential for calcification is ubiquitous in the tissues of higher animals, they must possess, not only some ubiquitous biostructure or process capable of promoting deposition of insoluble calcium salts, but also appropriate mechanisms for regulation of this process.

One of the most important new developments in the study of calcification is the discovery, with more sophisticated physical methods, that living mammalian bone contains two phases of calcium phosphate, one amorphous and non-diffracting, which has the statistical composition of tricalcium phosphate, and the other crystalline, with a composition approaching that of hydroxyapatite (reviewed by Posner, 1969). The amorphous phase, which is more labile, may be as high as 70% of the total bone mineral in young animals; it declines with age to as little as 36%.

A second major development is a better understanding of the physical chemistry and mechanism of hydroxyapatite formation. Much evidence now indicates that amorphous tricalcium phosphate is probably an obligatory first stage in the formation of crystalline hydroxyapatite (Scheme 1). Although the conversion of tricalcium phosphate into hydroxyapatite is a relatively fast and spontaneous process in the test tube in the absence of biological matter, under biological conditions there are two bottlenecks in the overall process. One is the fact that amorphous calcium phosphate is much more



Scheme 1. Major stages in formation of bone mineral.

soluble at pH 7.0 than is hydroxyapatite. Although the concentrations of Ca^{2+} and phosphate in blood plasma and interstitial fluid are such as to render them supersaturated with respect to crystalline hydroxyapatite, they are substantially undersaturated with respect to amorphous tricalcium phosphate. If amorphous calcium phosphate is an obligatory precursor for the formation of hydroxyapatite, it is at once obvious that hydroxyapatite cannot form from the free Ca^{2+} and phosphate of interstitial fluid unless the latter are first concentrated to such an extent that the solubility product of amorphous tricalcium phosphate is exceeded.

The second bottleneck in formation of bone crystal is the existence of specific biological inhibitors for this process (Scheme 1). My Johns Hopkins colleague, Professor J. E. Howard of the Department of Medicine, has made some very interesting and important biochemical discoveries concerning the inhibition of calcification. These began from the clinical observation that patients who repeatedly form stones of calcium salts in their kidneys lack a specific chemical component in their urine that normally inhibits calcification. This factor, which is present not only in the urine, but also in the blood, milk and some tissues of normal individuals, is assayed by its capacity to prevent the calcification of pieces of rib cartilage, incubated in a buffered solution containing Ca^{2+} and phosphate, from young ricketic rats. Normal urine added to such a system prevents calcification of the cartilage, whereas the 'evil' urine from stone-forming patients has little or no inhibitory action. This inhibitor has been considerably purified by Howard and his colleagues. Although it was first thought to be a small peptide (Howard, Thomas, Barker, Smith & Wadkins, 1967), it is now clear that its intrinsic activity is independent of peptide linkages, although the inhibitor may conceivably be attached to a peptide or protein. The inhibitor is organic in nature, highly acidic, and contains a functional group whose methylation causes loss of activity. Its molecular weight, deduced from its behaviour on molecular-exclusion columns, is about 600. Not only does this substance prevent living cartilage from calcifying *in vitro*, but it can also prevent the spontaneous non-biological formation of hydroxyapatite from free ionic Ca^{2+} and phosphate at pH 7.4, in the complete absence of enzymes or living cells (Barker *et al.* 1970). Indeed, this factor appears to be responsible for the fact, long known in the construction industry, that freshly voided urine can prevent concrete from setting. This inhibitory substance is not merely a chelating agent for Ca^{2+}, since one molecule of the inhibitor can prevent hundreds of Ca^{2+} and phosphate ions from undergoing transition into hydroxyapatite; presumably it acts to produce a crystal defect, since the large deposits of calcium phosphate formed in mitochondria *in vitro* are amorphous; it may be

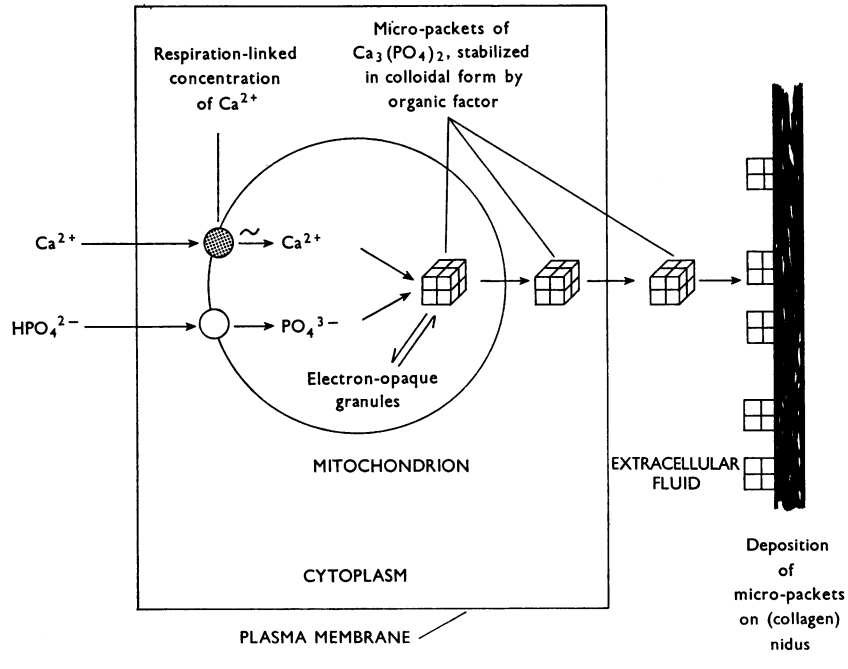
inferred that the mitochondria also contain such an inhibitory substance that prevents formation of diffracting hydroxyapatite.

There is a third development in our knowledge of the mechanism of calcification relevant to the role of mitochondria. For some time there have been two opposed views regarding the role of specific cells in the formation of bone mineral. One hypothesis, favoured by many in the past, is that bone crystal is formed extracellularly on specific acceptor regions of collagen fibrils from free Ca^{2+} and phosphate in the extracellular fluid, without intervention of any intracellular steps. The other hypothesis is that a living cell must first 'do' something to Ca^{2+} and phosphate, presumably inside the cell, before they will deposit on the extracellular matrix (Howard, 1956). The latter hypothesis is increasingly coming to the fore (Posner, 1969).

Role of mitochondria in calcification

We have adopted the working hypothesis (Scheme 2) 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, a process that cannot occur spontaneously in extracellular fluid, nor for that matter in the extramitochondrial cytoplasm. 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. Let us trace the stages in the overall process we postulate.

In the first stage Ca^{2+} and phosphate originating from the blood plasma pass into the cytoplasm, in which their concentrations are much lower than in the plasma, perhaps about 1 μm. Both Ca^{2+} and phosphate are then 'pumped' into the inner compartment of the mitochondrion from the cytoplasm, on specific carriers such as we have described, in response to an electrochemical gradient generated by energy-yielding electron transport. When the solubility product of tricalcium phosphate is exceeded, its precipitation in the form of micropackets begins on or near the inner surface of the inner membrane, but continues, in an ATP-dependent process, to yield electron-opaque granules, which we suggest are composed of many micropackets of tricalcium phosphate, in the matrix. We propose that these micro-packets in the mitochondrial matrix are similar to or identical with those present in the amorphous phase of bone mineral and in bone cells (see Hirschmann & Nichols, 1970) and that they consist of small aggregates of tricalcium phosphate molecules stabilized by an inhibitor, possibly the Howard factor or its mitochondrial equivalent. We also propose that such micro-packets are the smallest transport-



Scheme 2. Working hypothesis for the role of mitochondria in biological calcification.

able form of solid calcium phosphate and that they are about the size of the smallest observable subunits of amorphous calcium phosphate or of crystalline hydroxyapatite seen with the electron microscope, perhaps 20–30 Å in diameter (cf. Posner, 1969). The formation of such micro-packets in mitochondria we suggest to be easily reversible, in consonance with earlier findings that much of the Ca^{2+} can leave massively loaded mitochondria rather rapidly (Greenawalt *et al.* 1964). ATP (or ADP) is apparently required in the synthesis or activation of some biostructure or macromolecule in the mitochondrion that serves as a nidus or growing point to organize a number of molecules of tricalcium phosphate into a micro-packet, or to organize many micro-packets into larger granules. Another possibility is that ATP promotes the entry or formation of a specific mitochondrial inhibitor similar to or identical with the Howard factor in urine and blood.

But we come to an important question. How do the calcium and phosphate deposited in the mitochondrial matrix pass to those extracellular sites where calcium phosphate is ultimately deposited? There seems to be little point in postulating the dissolution of the micro-packets within the mitochondria and the passage of the free Ca^{2+} and phosphate ions through the mitochondrial membrane back into the cytoplasm and thence into the extracellular compartment, as has been proposed (Shapiro & Greenspan, 1969). Rather 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. Two possible mechanisms are open. It is possible that the micro-packets may pass directly through the mitochondrial membrane. Such a process is not so unlikely as it may first seem, since they are presumed to be much smaller than the thickness of the membranes and are also probably electrically neutral. Or they may leave by reverse phagocytosis, i.e. by an out-pocketing of a membrane vesicle containing one or more micro-packets. We have already obtained evidence that some of the Ca^{2+} deposited in massively loaded liver mitochondria *in vitro* may leave the mitochondria in a form other than ionic Ca^{2+}, i.e. a form that is sedimentable from the suspending medium at high centrifugal speeds (J. Tsai & A. L. Lehninger, unpublished work; Hirschmann & Nichols, 1970). Once such micro-packets pass through the plasma membrane they may diffuse to specific extracellular calcification sites where they attach to epitaxial structures in collagen fibrils and ultimately become part of the amorphous bone mineral fraction. At some later stage the amorphous bone mineral is converted into crystalline hydroxyapatite, a process that might be accompanied by removal of the stabilizing Howard factor, which may be enzymically inactivated or destroyed.

Although this working hypothesis has obvious points of weakness, particularly with regard to the mechanism by which the micro-packets leave the mitochondria, it does account in a chemically satisfactory way for the fact that the potential for calcification is a broadly distributed property of cells and that calcium phosphate granules often appear in the mitochondria during active calcification or decalcification. It also accounts for the formation of insoluble tricalcium phosphate, the required precursor of hydroxyapatite, from concentrations of Ca^{2+} and phosphate that are otherwise too low for precipitation, and for the observed biological inhibition of the transition of tricalcium phosphate into hydroxyapatite. It also provides a possible explanation for the essential role of ATP in the formation of calcium phosphate granules.

The experimental work and the working hypotheses outlined in this Lecture with regard to a specific Ca^{2+} carrier and the possible role of mitochondria in calcification obviously require much further development. Nevertheless the information already at hand is very promising. Certainly there must be some biological rationale for a mitochondrial process that is so rapid and dramatic as energy-linked Ca^{2+} accumulation, particularly since it may take primacy over oxidative phosphorylation. We now see mitochondria as something more than phosphorylating power plants, as we first viewed them 20 years ago. What is remarkable is that the same molecular apparatus of the electron-transport chain that generates the driving force for oxidative phosphorylation may also be used to transport ions against gradients, to change the conformation of mitochondria and to carry out reductive biosynthesis. If the ideas we have developed here are correct, mitochondria may also function as packaging plants, in which are manufactured the bricks and mortar of hard tissues; moreover, mitochondria may also harbour the secret as to why we do not all turn into stone.

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