THE GORDON WILSON LECTURE

MITOCHONDRIA AND THE PHYSIOLOGY OF CA^{2+}

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In this paper I shall sketch a trail of research that had its beginnings in an area of fundamental biochemistry perhaps somewhat far removed from the everyday concerns of the physician, but which has since led us successively into some molecular aspects of cell physiology and cell pathology, and it may yet lead us even more directly toward some basic problems in clinical medicine. For some years my students and I have been working on the enzymatic mechanism of oxidative phosphorylation, the process by which metabolic energy in the form of adenosine triphosphate (ATP) is generated from ADP during the combustion of nutrients in cells. This process takes place in the mitochondria, in which are located all the enzymes concerned in the tricarboxylic acid cycle, electron transport to oxygen, and the coupled phosphorylation of ADP to ATP. In all types of cells, the mitochondria have the basic function of serving as "power plants".

Today we know that the inner membrane of the mitochondrion plays a very fundamental role in the mechanism of ATP formation, since the enzymes catalyzing electron transport and ATP formation are embedded in it and this membrane must be intact for ATP to be formed. Moreover, recent work indicates that the inner mitochondrial membrane also participates in a number of transport activities vital in the metabolic economy of cells. During respiration such fuel molecules as pyruvic acid and fatty acids must pass from the surrounding cytoplasm through the membranes and enter the inner compartment of mitochondria, the matrix, and the end-products of respiration, bicarbonate and water, must then leave the matrix and pass back to the cytoplasm. Concurrently, phosphate and ADP must enter the matrix and ATP must leave, to complete the process of respiration and phosphorylation. Mitochondria also support certain biosynthetic activities of cells; they can manufacture citrate and malate in the matrix for export to the cytoplasm. Moreover, during the synthesis of urea in the liver, ornithine enters the mitochondria and becomes trans-

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formed to citrulline, which then passes out of the mitochondria again before it is converted in the cytoplasm into arginine, the immediate precursor of urea. Thus there is a very considerable metabolic traffic across the mitochondrial membranes. This traffic is by no means random; it is highly regulated. In fact, it is literally computerized, since these molecules pass through the mitochondrial membrane in precise molar ratios to each other through the action of a set of highly specific permeases or metabolite transport systems located in the inner membrane. Transport systems specific for phosphate, ADP, malate, citrate, α-ketoglutarate, aspartate, and glutamate are now known; recently we have also identified permeases for ornithine, citrulline, and Ca^{2+}. Actually, it is the transport of Ca^{2+} across the mitochondrial membrane that is the basic process to which this lecture is devoted.[1, 2]

The trail of our research on Ca^{2+} began in 1948, when we found that Ca^{2+} completely inhibits the generation of ATP from ADP by respiring mitochondria, without interfering with their consumption of oxygen. Thus Ca^{2+} was believed to be a toxic uncoupling agent, resembling 2,4-dinitrophenol. For many years thereafter all investigators of mitochondrial function scrupulously avoided the presence of Ca^{2+} in their test systems. It was not until 1961 that we learned we had all along missed a very important attribute of mitochondria. In that year my colleague Dr. Frank Vasington discovered what really happens when Ca^{2+} is added to a suspension of mitochondria respiring in a medium containing ADP, phosphate, and a substrate of the tricarboxylic acid cycle. Oxygen is consumed, but no ATP is formed, precisely as we had earlier found; however, the Ca^{2+}, instead of remaining unchanged in the medium, disappeared from it and was found to be present in the mitochondria when they were recovered from the medium by centrifugation. Ca^{2+} uptake did not occur, however, if respiration was inhibited by cyanide. When Ca^{2+} was not added to the medium, oxygen consumption occurred and ATP was formed. Vasington concluded that the energy of electron transport can be used either to phosphorylate ADP or to accumulate Ca^{2+}; they are thus alternative processes. Soon Rossi and I found that the accumulation of Ca^{2+} by mitochondria proceeds in a strict molar relationship to electron transport. Passage of each pair of electrons from substrates of the tricarboxylic acid cycle to oxygen via the electron transport chain can cause either the accumulation of 6 Ca^{2+} ions or the phosphorylation of 3 molecules of ADP. When Ca^{2+} is accumulated by mitochondria, phosphate ions also enter from the medium to yield an internal Ca^{2+}:phosphate ratio of about 1.5–2.0. Such an accumulation of Ca^{2+} in the mitochondrial matrix represents true active transport against a gradient of concentration, since internal:external Ca^{2+} concentration ratios exceeding 500:1 can be

achieved. Moreover, we have found that Ca^{2+} accumulation does not damage mitochondria; they are still capable of normal oxidative phosphorylation of ADP after they have accumulated a load of Ca^{2+}. In addition, we have found that Ca^{2+} accumulation is not an irreversible process, since Ca^{2+} will rapidly leak out of previously loaded mitochondria if we should limit availability of substrates or oxygen.

The energy-linked accumulation of Ca^{2+} into mitochondria under these conditions is relatively specific. Only Sr^{2+} is accumulated at a rate comparable to the rate of transport of Ca^{2+}; in fact, they compete with each other. Mn^{2+} and Ba^{2+} are only slowly accumulated, whereas Mg^{2+} and a variety of other cations fail to enter. Specific inhibitors of mitochondrial Ca^{2+} transport have been found. La^{3+} and other rare earth cations, such as Pr^{3+} and Ho^{3+}, are potent inhibitors of Ca^{2+} uptake by mitochondria.[3] Although La^{3+} blocks accumulation of Ca^{2+}, it does not inhibit respiration or phosphorylation.

Because of the specificity of Ca^{2+} transport and the specificity with which Ca^{2+} transport can be inhibited, as well as other evidence we have acquired, we have postulated that the mitochondrial inner membrane contains a specific permease or transport system for the Ca^{2+} ion, which translocates Ca^{2+} across the membrane into the matrix in response to a transmembrane electrochemical gradient generated by electron transport. It has recently been possible to extract a heat-labile Ca^{2+} binding protein from mitochondrial preparations and to purify it considerably.[4] There is reason to believe that this substance, which has a molecular weight exceeding 150,000 and contains sialic acid, hexosamine, and lipids, represents at least part of the Ca^{2+}-carrier system in the inner membrane.

Mitochondria isolated from every mammalian tissue we have tested to date, including brain, heart, muscle, adrenal cortex, thyroid, and spleen, all show the same capacity for Ca^{2+} accumulation.[5] Indeed, we have also found this capacity in mitochondria from tissues of the other great classes of vertebrates: birds, fishes, reptiles, and amphibia. In mitochondria from invertebrate tissues the distribution of Ca^{2+} transport activity is not universal. We have found it in mitochondria from tissues of some crustacea mollusks, and worms; it is found in some insects but not in others. We have found Ca^{2+} transport activity in mitochondria of some plants, such as the white potato and sweet potato, but not in bean seedlings, nor in some fungi, yeasts, and molds. Ca^{2+} transport is, however, highly developed in some protozoa.

We may now ask What is the significance of the energy-linked accumulation of Ca^{2+} by isolated mitochondria: Does it have a biological function? We have made two sets of observations which strongly indicate that energy-linked accumulation of Ca^{2+} by mitochondria plays a very fundamental

role in the cell physiology of all vertebrates. The first set of observations demonstrated that mitochondria greatly prefer to accumulate Ca^{2+} rather than to phosphorylate ADP. In fact, we have found that Ca^{2+} accumulation always takes primacy over ADP phosphorylation, even when the concentration of Ca^{2+} added to the test medium is as low as 10 μM and the ADP concentration as high as 4000 μM, approximately the maximum level it ever reaches in the intact cell. Under these conditions nearly all the Ca^{2+} will be accumulated before any ADP is phosphorylated. The dominance of Ca^{2+} transport over ATP formation has been observed in mitochondria of all mammalian cell types we have examined. Moreover, the affinity of respiring mitochondria for Ca^{2+} is extremely high; they will keep removing Ca^{2+} from the medium until the remaining concentration is less than 1×10^{-6} M. We are therefore left with the conclusion that the energy-linked accumulation of Ca^{2+} by mitochondria must be an extremely important process because it always takes precedence over oxidative phosphorylation, for long considered to be the primary and characteristic function of mitochondria.

We have postulated a biological role for this property of mitochondria; we propose that mitochondria participate in the mechanisms by which the Ca^{2+} concentration in the cytoplasm of cells is regulated.[2] We believe that mitochondria function as membrane-surrounded Ca^{2+} reservoirs or buffer systems in the cytosol, each capable of segregating Ca^{2+} reversibly, thus supplementing the action of the outward-directed Ca^{2+} pumps of the plasma membrane. Thus the mitochondria, working together with the plasma membrane, keep the concentration of Ca^{2+} in the cytosol very low and in a homeostatic condition at levels of about 1 μM or less, in the face of the exceedingly high concentrations of Ca^{2+} present in the blood and interstitial fluid, which is about 2 mM or 2000 times higher than in the cytoplasm. Moreover, because the total volume of the cytoplasm occupied by the mitochondria is of the order of 20% in liver and 40% in heart muscle cells, and because mitochondria are very numerous and are distributed in a characteristic geometry throughout the cytoplasm, we also suggest that they can regulate the Ca^{2+} concentration in different local domains of cytoplasm at different levels. As we shall see, there are many Ca^{2+}-dependent activities in cells, which are presumably located in different portions of the cell.

Why must cells regulate their cytoplasmic Ca^{2+} concentration so tightly and at such low levels? The answer is clear from much recent work on the regulation of cell activities (reviewed in[6]). As is shown in Table 1, the Ca^{2+} ion is an extremely important chemical "messenger" in the regulation of such activities as mobilization and combustion of fuels, contraction and relaxation of contractile and motile systems, in the release of hormones, in

TABLE 1

Some Ca^{2+}-dependent Activities

Mobilization of fuels
Activation of phosphorylase *b* kinase
" " lipases and phospholipases
" " glycerol phosphate oxidation
Activation of contractile and motile systems
Actomyosin ATPase
Cilia and flagella
Microtubule function
Cytoplasmic streaming
Pseudopod formation
Endocrine regulation
Formation and/or function of cyclic AMP (GH, LH, TSH, MSH, PTH)
Release of insulin, steroids, vasopressin
Membrane-linked functions
Excitation-secretion coupling
Neuromuscular coupling
Synaptic transmission
Action potential
Regulation of Na^+, K^+-ATPase
Regulation of permeability
Regulation of tight junctions

the function of the cyclic AMP system as a cellular mediator of endocrine action, and in the regulation of membrane functions, to say nothing of the role of Ca^{2+} in calcification processes.

We have undertaken an investigation of the role of mitochondria in one of these processes, namely the regulation of contraction and relaxation of certain types of muscle by Ca^{2+}. It is now well known that contractile sliding of actin and myosin filaments along each other is activated by free Ca^{2+} released by the wave of depolarization which sweeps through the T-system after the muscle is excited by the incoming nerve impulse. This Ca^{2+} becomes bound to troponin molecules, which activate myosin ATPase activity; lateral translocation of one filament on the other then ensues. Relaxation occurs when the free Ca^{2+} is absorbed from the sarcoplasm again through the action of an ATP-induced Ca^{2+} pump present in the membrane of the sarcoplasmic reticulum. The picture I have just outlined represents the course of events in white muscles.

For some time we have wondered whether the sarcoplasmic reticulum represents the site of reversible Ca^{2+} segregation in all types of muscles. There can be no doubt that it is the major if not sole organelle for Ca^{2+} segregation in white muscles, which have a very profuse sarcoplasmic reticulum, but very few mitochondria. White muscles produce the ATP required for

contraction largely by glycolysis, not respiration. But red muscles, such as those in the flight muscles of birds, are a different matter, as is cardiac muscle. The latter types have very little sarcoplasmic reticulum, which is sometimes even rather difficult to find in electron micrographs, but they have enormous numbers of mitochondria. Nearly 60 percent of the volume of cardiac muscle consists of mitochondria, and over 50% of the flight muscle of the pigeon. These rapidly respiring muscles thus have an enormous potential capacity for segregating Ca^{2+} in their mitochondria and we have accordingly set out to see if mitochondria play a role in the contraction and relaxation of red muscle and cardiac muscle.

We have found mitochondria of red muscles such as the masseter and heart muscle of the rabbit are extremely active in segregating Ca^{2+} *in vivo* as well as *in vitro*. For example, parenteral administration of $^{45}Ca^{2+}$ to rabbits is followed by extremely rapid incorporation of the Ca^{2+} into the mitochondria of red muscles which attain a much higher specific activity of any of the Ca^{2+} pools of these tissues; in contrast, the microsome fraction is only sluggishly active. On the other hand, microsomes of white muscles show a much more pronounced uptake of Ca^{2+} than those of red muscles.[7] Moreover, Haugaard and his colleagues and Fehmers (see[2]) have shown that Ca^{2+} enters the mitochondria of heart muscle extremely rapidly from the perfusing medium. Administration of epinephrine or isoproterenol further stimulates the entrance of Ca^{2+} into the mitochondria of heart muscle.

While much work remains to be done on the difficult experimental problem of following precisely the movements of Ca^{2+} in intact muscle tissue, much evidence now supports the idea that mitochondria play an important role in regulating the contraction and relaxation of cardiac muscle and some specialized red muscles, either to supplement the role of the sarcoplasmic reticulum or to substitute for it. Table 2 shows that the mitochondria of heart muscle easily have the Ca^{2+}-binding capacity, the necessary affinity

TABLE 2

Parameters of Ca^{2+} Segregation by Mitochondria in Rat Heart

	nmoles/g wet
Free Ca^{2+} in cytoplasm (rest)	0.1
Threshold concentration for contraction	0.3
Maximum free Ca^{2+} release on excitation	300
Ca^{2+}-segregating capacity of mitochondria in 1 g heart (maximum)	>24,000
Rate of Ca^{2+} segregation required for heart rate = 200 min^{-1}	300 nmoles sec^{-1} g^{-1}
Observed rate of Ca^{2+} segregation by isolated mitochondria	~2000 nmoles sec^{-1} g^{-1}

for Ca^{2+}, and can accumulate Ca^{2+} at a high enough rate to account for the Ca^{2+} segregation and release cycle.

The second set of observations which suggested that mitochondrial Ca^{2+} transport is an important physiological process relates to the very large capacity of mitochondria to accumulate Ca^{2+}. When provided with an ample supply of respiratory substrate and both Ca^{2+} and phosphate in the suspending medium, mitochondria of liver and kidney, and indeed all mammalian tissues, can accumulate up to 2500 nmoles of Ca^{2+} per mg of mitochondrial protein, which is equivalent to something over one-quarter their dry weight.[1, 2] This extremely large amount of Ca^{2+} and phosphate, when referred to the water content of the mitochondrial matrix, is equivalent to a 2.5 M solution. Since this concentration is far above the solubility product of calcium phosphate it meant to us that calcium phosphate must precipitate out of solution in the mitochondrial matrix. Electron microscopy of liver mitochondria massively loaded in this fashion revealed many electron-dense granules throughout the matrix, which we proved to be insoluble deposits of calcium phosphate. From electron micrographs taken at different stages during loading of the mitochondria in this fashion, precipitation of small granules was found to begin near the inner surface of the inner mitochondrial membrane; these ultimately grow by accretion to become large, boulder-like deposits, which still retain a granular substructure. Ca^{2+}-loaded mitochondria are easy to isolate since they are heavier than normal mitochondria and may be separated from them by density gradient centrifugation. Such electron-dense granules have been isolated from the matrix of disrupted mitochondria; they contain Ca^{2+} and phosphate in the ratio 1.5–2.0. They also contain about 15% organic matter, which appears to be largely protein.

We became interested in the question as to whether the calcium phosphate deposits in such loaded mitochondria are crystalline or amorphous. We accordingly prepared large amounts of such mitochondria, dehydrated them with acetone, and turned them over to Dr. Aaron Posner of New York Hospital, an expert x-ray analyst of bone mineral. He could find no regular x-ray diffraction pattern, indicating that the intramitochondrial deposits were amorphous and thus contain no significant amount of hydroxyapatite. This finding was confirmed in a parallel study by my colleagues Thomas and Greenawalt who used microincineration procedures. We believe that the calcium phosphate deposited in the matrix of normal mitochondria is probably amorphous tricalcium phosphate. The amorphous nature of these mitochondrial deposits is reminiscent of the work of Dr. John Eager Howard and his successive colleagues, particularly Dr. Thomas, on the presence in normal urine of a biochemical factor capable of inhibiting the precipitation or crystallization of calcium phosphate, a factor whose deficiency in the

urine appears to be the cause of recurrent kidney stone formation in some individuals. We have speculated that the mitochondria must also contain a calcification-inhibitor which prevents formation of crystalline hydroxyapatite from amorphous calcium phosphate; indeed, we are searching for such a substance.

Mitochondria from all mammalian tissues we have examined, including kidney, liver, brain, and heart, are all capable of forming such massive deposits of amorphous tricalcium phosphate *in vitro*. The formation of these deposits is not a spontaneous artifact, since it very specifically requires electron transport and the complete integrity of the energy-coupling inner membrane of the mitochondria. Moreover, such massive accumulation proceeds in precise stoichiometric equivalence with electron transport.

Can such dense granules of calcium phosphate form in mitochondria in intact living tissues? The answer is now quite certainly yes. Electron microscopic investigations in many different laboratories have revealed that such granules readily form *in vivo* in specific tissues under specific kinds of physiological or pathological stress.[2] They were first observed in mitochondria of osteoclasts in the rat femur during the healing of experimental fractures. They have not only been observed when bone mineral is deposited or mobilized at high rates, but also in mitochondria of certain tissues in situations well known to cause changes in Ca^{2+} metabolism, particularly in carbon tetrachloride poisoning, in which the mitochondria lose K^+, which is replaced with Ca^{2+}, in mitochondria of heart muscle cells following administration of certain inotropic agents such as isoproterenol, in the kidney following the administration of excess Vitamin D, parathormone, or after specific bacterial toxins, and particularly following the administration of various heavy metals and their derivatives, such as mercury, uranium, lanthanum, and other rare earths, which are known to cause calcification of some soft tissues.

These and other observations have raised the question whether mitochondria might not play an important role in calcification, not only normal calcification in bony tissues, but also pathological calcification of soft tissues. We must recall that nearly all soft tissues of the mammal are intrinsically capable of undergoing calcification when they are appropriately insulted. In fact, Selye and his colleagues have described empirical procedures which will produce calcification of virtually any tissue at will.[8] It therefore appears that the molecular mechanisms for calcification must be inherently present in all mammalian tissues, but are dormant or "turned off" during their normal activity and come into play only following some special triggering insult.

Starting from these premises we have constructed a hypothesis of the mechanism of normal and pathological calcification in animal tissues in which the ubiquitously-occurring mitochondria play a central role.[2] This

hypothesis also provides a role for the Howard factor and, in particular, takes account of a relatively recent development in the chemistry and thermodynamics of hydroxyapatite formation, which we must very briefly review. Posner and others have found that formation of crystalline hydroxyapatite under conditions relevant to biological calcification occurs in two major stages. In the first, Ca^{2+} and phosphate ions, when present in sufficiently high concentration to exceed the solubility product, precipitate to form amorphous (i.e. non-diffracting) tricalcium phosphate. In the second stage, the amorphous tricalcium phosphate undergoes a transition in which some of the phosphate is replaced by hydroxyl ions to form crystalline hydroxyapatite, which of course shows a characteristic x-ray diffraction pattern. This second stage is quite spontaneous and rapid and may occur in pure chemical systems within minutes. However, amorphous tricalcium phosphate is an absolutely obligatory precursor of hydroxyapatite. Since Ca^{2+} and phosphate ions in the extracellular fluid are not high enough to precipitate amorphous tricalcium phosphate, hydroxyapatite cannot form spontaneously unless the concentration of Ca^{2+} and phosphate can be raised high enough to exceed the solubility product of tricalcium phosphate.

These recently developed facts have led us to propose that the mitochondria are the site at which the first stage of calcification takes place, namely the conversion of soluble Ca^{2+} and phosphate into insoluble amorphous tricalcium phosphate. Mitochondria, so far as we know, are the only structures, intracellular or extracellular, capable of concentrating Ca^{2+} and phosphate to sufficiently high concentrations to precipitate amorphous tricalcium phosphate. Moreover, this capacity of mitochondria is found in all mammalian tissues, consistent with the fact that all tissues can undergo calcification. Our hypothesis also suggests a mechanism and site for the second stage of calcification, the conversion of amorphous tricalcium phosphate to crystalline hydroxyapatite. We have postulated that this conversion normally takes place outside of the cell at the sites of deposition of bone mineral provided by the appropriate connective tissue elements. But how can the amorphous tricalcium phosphate generated within the mitochondria pass to these extracellular sites? Clearly it would not accomplish anything if it simply redissolves to form ionic Ca^{2+} and phosphate again and then passes in this soluble form via the cytoplasm and the plasma membrane to the extracellular space. We have postulated that the amorphous tricalcium phosphate is generated in the mitochondrial matrix in a stabilized colloidal form, possibly in very small "micropackets" having the dimensions of the unit crystal, some 10–20 Å in diameter. Only in massive loading do such micropackets aggregate to form visible "boulders". We suggest that the stabilization of amorphous tricalcium phosphate micropackets is brought about by a factor identical or similar to the Howard factor

in normal urine. We postulate that such colloidal micropackets of calcium phosphate pass through the mitochondrial membranes, into and through the cytoplasm, and ultimately leave the cell as such or in an aggregated form to the extracellular sites where calcium phosphate and hydroxyapatite are deposited.[2]

The weak point in our hypothesis when it was originally stated was lack of evidence regarding the mechanism of passage of colloidal calcium phosphate out of the mitochondria and then out of the cell. However, recently evidence has arisen which supports our hypothesis. Perhaps the most spectacular is the discovery of Ali and his colleagues[9] that the epiphyseal cartilage contains numerous small extracellular vesicles staining for calcium phosphate. These membrane-surrounded vesicles have been isolated in highly-enriched form from homogenates of epiphyseal cartilage. By marker enzyme analysis they have shown that the membrane surrounding these extracellular vesicles derives from the plasma membrane. They concluded that these calcium phosphate-bearing vesicles were secreted from bone-forming cells by reverse phagocytosis, a conclusion which supports our hypothesis.

We are now studying the release of colloidal calcium phosphate from mitochondria, which is experimentally a rather difficult problem. However, we have been encouraged by recent studies of Trump and his colleagues, who have shown that toxic doses of mercuric salts cause calcification of the kidney, which begins with the formation of electron-dense granules of Ca^{2+} phosphate in the mitochondria. Electron microscopic examination of cells in successive stages of this process has revealed that the electron-dense calcium phosphate granules appear to undergo exit from the mitochondrial matrix into the cytoplasm, through an out-pocketing of the inner mitochondrial membrane.[10] My colleague Dr. Carafoli is studying other types of induced calcification of soft tissues, particularly after administration of uranium salts.

Recently we have undertaken study of Ca^{2+} transport in other organisms capable of rapid calcification and decalcification processes. Of special interest is the molting cycle of the Maryland blue crab and the land crab of the Caribbean islands. At molt these crustaceans lose their shells, which are rich in calcium carbonate, and then lay down, very rapidly, a new and larger shell. During this process much Ca^{2+} moves through the hepatopancreas en route to the newly forming shell. The land crab, unlike his delectable cousin in the Cheasapeake Bay, who is exposed to Ca^{2+} in the bay water, must practice extreme economy in the use of Ca^{2+}. Just preceding molt, the very large amount of Ca^{2+} present in the old exoskeleton is resorbed via the blood stream and transported to the hepatopancreas and other soft tissues where it is stored as crystalline deposits of calcium phos-

phate, to be used over again to form the next and larger shell of the animal. In a very dramatic 24-hour period, several grams of calcium pass from the soft tissues of the land crab to the new shell. We are carrying out combined biochemical and electron microscopic study of movements of Ca^{2+} into and out of the mitochondria of the hepatopancreas during this cycle, a biological situation that is easily controlled in the laboratory and which represents the calcification process in one of its most dramatic manifestations.

Our hypothesis for calcification builds on Dr. John E. Howard's concept, first proposed in the 1950's and since supported by most workers, that cells must first *do* something to Ca^{2+} and phosphate before they participate in extracellular calcification events. I would not want to suggest that the intracellular phase of calcification we postulate represents all that matters. Indeed, I expect that the arrangement of micropackets of amorphous calcium phosphate into the epitaxial matrix of collagen fibers and the subsequent events leading to hydroxyapatite formation are exceedingly complex and vital processes in the inorganic and organic biochemistry of calcification.

In conclusion, I trust you can see that the trail of research I have described has led a long way from the purely biochemical problem of the mechanism of energy coupling in the mitochondrial membrane which was its origin. But the trail has been very interesting and challenging, and may give us a glimpse of the molecular events in one of the most ubiquitous pathological changes afflicting the human organism.

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