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Review

The fateful encounter of mitochondria with calcium: How did it happen?

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ABSTRACT

A number of findings in the 1950s had offered indirect indications that mitochondria could accumulate Ca^{2+}. In 1961, the phenomenon was directly demonstrated using isolated mitochondria: the uptake process was driven by respiratory chain activity or by the hydrolysis of added ATP. It could be accompanied by the simultaneous uptake of inorganic phosphate, in which case precipitates of hydroxyapatite were formed in the matrix, buffering its free Ca^{2+} concentration. The properties of the uptake process were established in the 1960s and 1970s: the uptake of Ca^{2+} occurred electrophoretically on a carrier that has not yet been molecularly identified, and was released from mitochondria via a Na^+/Ca^{2+} antiporter. A H^+/Ca^{2+} release exchanger was also found to operate in some mitochondrial types. The permeability transition pore was later also found to mediate the efflux of Ca^{2+} from mitochondria. In the mitochondrial matrix two TCA cycle dehydrogenases and pyruvate dehydrogenase phosphate phosphatase were found to be regulated in the matrix by the cycling of Ca^{2+} across the inner membrane. In conditions of cytoplasmic Ca^{2+} overload mitochondria could store for a time large amounts of precipitated Ca^{2+}-phosphate, thus permitting cells to survive situations of Ca^{2+} emergency. The uptake process was found to have very low affinity for Ca^{2+}: since the bulk concentration of Ca^{2+} in the cytoplasm is in the low to mid-nM range, it became increasingly difficult to postulate a role of mitochondria in the regulation of cytoplsmic Ca^{2+}. A number of findings had nevertheless shown that energy linked Ca^{2+} transport occurred efficiently in mitochondria of various tissues in situ. The paradox was only solved in the 1990s, when it was found that the concentration of Ca^{2+} in the cytoplasm is not uniform: perimitochondrial micropools are created by the agonist-promoted discharge of Ca^{2+} from vicinal stores in which the concentration of Ca^{2+} is high enough to activate the low affinity mitochondrial uniporter. Mitochondria thus regained center stage as important regulators of cytoplasmic Ca^{2+} (not only of their own internal Ca^{2+}). Their Ca^{2+} uptake systems was found to react very rapidly to cytoplasmic Ca^{2+} demands, even in the 150-200 msec time scale of processes like the contraction and relaxation of heart. An important recent development in the area of mitochondrial Ca^{2+} transport is its involvement in the disease process. Ca^{2+} signaling defects are now gaining increasing importance in the pathogenesis of diseases, e.g., neurodegenerative diseases. Since mitochondria have now regained a central role in the regulation of cytoplasmic Ca^{2+}, dysfunctions of their Ca^{2+} controlling systems have expectedly been found to be involved in the pathogenesis of numerous disease processes.

1. From pre-history to early history

Considering the impact it has had on the biology of cells, the "encounter" was fateful indeed: Ca^{2+} signaling is now one of the hottest topics in cell research, and the interplay between mitochondria and Ca^{2+} has a central position in it. Curiously, however, the beginnings of the story and the unusual aspects of its early development are poorly known, as they are covered only incompletely, or not covered at all, in the abundant literature on the topic. In the following pages I will recapitulate them: it will be a first hand account, as I was present when and where it all started. That was in 1961–1962, when Vasington and Murphy [1,2] and De Luca and Engstrom [3] discovered that isolated mitochondria were able to take up large amounts of Ca^{2+} in a process that was driven by respiratory energy. It was the first direct demonstration that the exposure of mitochondria to Ca^{2+} resulted in the penetration of the latter in the organelle. It was a landmark discovery but, in the years that had preceded it, compelling indications had appeared, most of them from experiments on mitochondrial swelling (a very popular topic in the 1950s), that something special was going on when mitochondria were exposed to Ca^{2+}. Surprising as it may now seem, however, no one had understood what was really happening: evidently, times were not ripe, and findings which, in the light of what is known today should have spoken for themselves, were forced back within the mainstream thinking of bioenergetics of those early days: which, one must remember, were the pre-chemiosmotic days. Raaflaub [4], Hunter and Ford [5], Tapley [6] had for instance found that Ca^{2+} caused a swelling

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of large amplitude in isolated mitochondria. Even if it was generally accepted that mitochondria behaved as osmotic systems [7] and would swell and shrink when the osmolarity of the external medium changed, the effect of Ca^{2+} was related to non-osmotic causes, e.g., to the stimulation of the synthesis of an endogenous uncoupling agent, the so called U-factor [8], or to its action on hypothetical contractile elements in mitochondria [4]. However the swelling results, suggestive as they may have been, were indirect. Those published by Edward C. (Bill) Slater and Kenneth W. Cleland in 1953 [9], instead, were direct: Slater and Cleland had found that isolated mitochondria (called sarcosomes in these early days) contained practically all the Ca^{2+} of the heart homogenate. When exposed to Ca^{2+}, they accumulated large amounts of it: the synthesis of ATP became depressed in the process, and was restored to normal by the removal of Ca^{2+} with EDTA (Table 1). Striking results, indeed: in effect, Slater and Cleland had discovered the process that in today's parlance would be defined as the uptake of Ca^{2+} by mitochondria as an alternative to ADP phosphorylation. They had actually considered the possibility that the uptake of Ca^{2+} "could be brought about by coupling with an energy- yielding mechanism such as respiration," but had discounted it because the uptake of Ca^{2+} had occurred at 0°, i.e., under conditions in which the sarcosomes were "not respiring or carrying out any other obvious metabolic activity." They had thus concluded that the uptake of Ca^{2+} was "an artifact not representing the state of affairs of the intact fibre," attributing the inhibition of phosphorylation to the possible sequestration of a high energy intermediate – the mytical ~ P- by Ca^{2+} that had entered the sarcosomes. Or to the displacement by Ca^{2+} of some metal prosthetic group from an enzyme that operated in conjunction with ~ P: conclusions that were not too surprising, if one considers that the 1950s were the golden era of the high energy intermediates of oxidative phosphorylation: at that time the now infamous "squiggle" was the *Holy Graal* of all bioenergeticists of any prominence. Explanations of Ca^{2+} effects on mitochondria in terms other than the conventional schemes of the chemical theory of energy coupling [10] would have probably been taken as oddities. Yet, one of the most authoritative proponents of the high energy intermediate concept, Britton Chance, had interpreted a stunning experiment he had performed in 1955 [11] as we would interpret it today: as an indication, indirect but compelling, that mitochondria were able to take up Ca^{2+} in an energy-driven process. Chance was studying the effects of Ca^{2+}, which was one of the commonly used uncouplers of oxidative phosphorylation, on the consumption of oxygen and on the oxidation of NADH (called DPNH at that time) by isolated mitochondria. The amazing observation he had made was that Ca^{2+}, at variance with all other uncouplers, activated respiration reversibly. As is well known, uncouplers induce a phase of activated respiration (the State 4/State 3 transition) that lasts until the oxygen in the system has been consumed. A pulse of Ca^{2+}, instead, elicited a burst of oxygen consumption that ceased after a while (Fig. 1): only the addition of a second pulse of Ca^{2+} at this point activated the consumption of oxygen (and the oxidation of DPNH) undefinitely, an effect that Chance attributed with remarkable foresight to profound changes in the mitochondrial structure. On comparing the amount of extra oxygen consumed in response to Ca^{2+} to that induced by the addition of ADP (which is used to calculate the ADP/0, the conventional way of expressing the coupling degree of mitochondria), Chance found that the Ca^{2+}/0 stoichiometry was two to three times the ADP/0 stoichiometry: in other words, the amount of Ca^{2+} required to induce the same extra consumption of oxygen was two to three times that of ADP. In keeping with the prevailing views of the times, Chance had suggested that the reversible activation of the consumption of oxygen by Ca^{2+} was due to the release of bound ADP or to the breakdown of ATP, which could have then activated the increase of oxygen consumption (and of DPNH oxidation). But it was clear from the experiment that Ca^{2+} had been somehow "consumed" in the process, i.e., it had become bound to mitochondria. Yet, Chance did not explicitly say so. Perhaps he had thought it was obvious, but the point was important, thus, a few years ago, as I was preparing a review on the topic of Ca^{2+} and mitochondria [12], I thus asked him directly. His e-mail reply was "the very fact that successive additions of calcium up to overload gave the same stoichiometry of respiratory stimulation, made it apparent to us that calcium was gone, or, in modern terminology must have been translocated from the external medium, just where in the mitochondria or on the mitochondria was not shown by our studies."

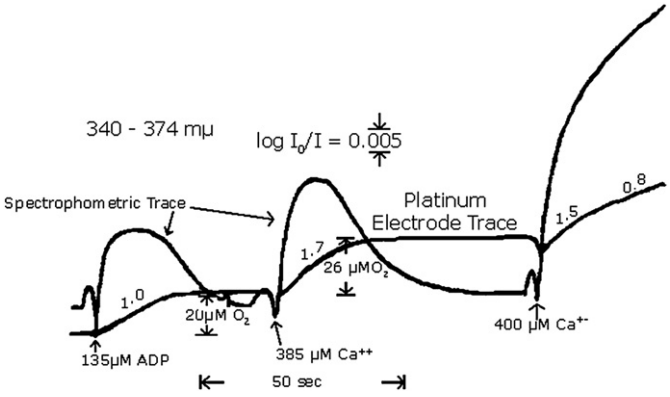

Fig. 1. Simultaneous recordings of respiration (black trace) and NADH (diphosphopyridine nucleotide, called DPNH in the 1950s) oxidation (grey trace) in isolated guinea pig liver mitochondria. Oxygen (O_2) consumption was recorded as an upward of the platinum microelectrode trace: the medium (K^+- free, isotonic, temperature not specified) contained a respiratory substrate (glutamate), but the respiration was maintained at a low level (State 4 rate) owing to the absence of the phosphate acceptor ADP. The legend does not state it explicitly but 4.5 mM phosphate was also present, judging from the legends to other figures in the article. Addition of ADP caused a burst of O_2 consumption (State 3 rate) that ceased when all ADP had been transformed into ATP. The addition of a pulse of Ca^{2+} induced a similar reversible increase of the respiratory rate. A second addition of Ca^{2+} activated respiration indefinitely. NADH (DPNH) oxidation (not discussed in the text) was recorded as an upward deflection of the spectrophotometric trace, which reflects the decrease of optical density of the mitochondrial suspension at 340 mμ. The oxidation of NADH (DPNH), which accepted the reducing hydrogen from the substrate at the proximal end of the respiratory chain, was an alternative way of expressing the changes in respiratory rate induced by ADP and by Ca^{2+}. Reprinted from Ref. [11].

The article of Chance describing the 1955 experiment was published in 1956, i.e., a full 6 years before the articles of Vasington and Murphy and De Luca and Engstrom. After three years, it was followed by a contribution in which Nils E. Saris, a Finnish post-doctoral fellow in the Laboratory of Chance in the summer of 1959, had studied the hydrolysis of ATP by mitochondria using the pH shift method, and had noticed an anomaly in the pH traces when Ca^{2+} was added to the medium. He had discovered that the addition of a pulse of Ca^{2+} to mitochondria in the absence of ATP caused a pH drop in the medium, which was followed by a stationary phase. The cycle occurred with a Ca^{2+}/H^+ stoichiometry of about 0.8 and could be repeated until the total amount of Ca^{2+} added exceeded 200–300 μM (Fig. 2). Saris published his observations after he had returned to Finland in an obscure Finnish journal [13], and he collected them in comprehensive form only in 1963 in his impressive Ph.D. Dissertation

Table 1
Ca^{2+} uptake by heart mitochondria.

Ca^{2+} Content Of Mitochondria (μmol/mg protein)				
Uptake medium	EDTA-washed preparation	% Δ	Saline preparation	% Δ
No Ca^{2+}	0.0015		0.083	
0.1 mM Ca^{2+}	0.068	+~450	0.127	-~50

Heart mitochondria were prepared either in saline, or in saline plus 10 mM EDTA. The reaction medium contained 0.135 M KCl, 20°mM phosphate, 0.6 mM Ca^{2+}. Incubation was at 0°. After 30 min mitochondria were sedimented by centrifugation, and Ca^{2+} was determined in the supernatant. Modified from [9].

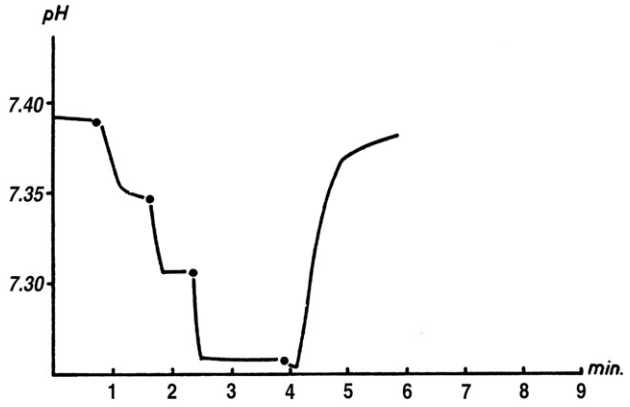

Fig. 2. pH changes induced by the addition of Ca^{2+} to a mitochondrial suspension. Rat liver mitochondria were prepared by a conventional cell fractionation procedure. The medium contained 50 mM sucrose, 40 mM KCl, 4.5 mM succinate, 1.9 mg of mitochondrial protein per ml. The starting pH was 7.4, and the temperature 20–23 °C. The additions of Ca^{2+} were made at the points of deflection of the trace (dots). The final concentration of Ca^{2+} added each time was 91 μM. Adapted from Ref. [14].

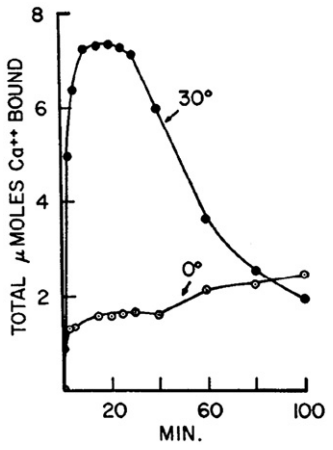

Fig. 3. Uptake of Ca^{2+} by isolated rat kidney mitochondria. Mitochondria were isolated by a conventional cell fractionation procedure. The reaction medium (isotonic NaCl, pH 7.0) contained a respiratory substrate (succinate), ATP, Mg^{2+}, and 2.5 mM radioactive Ca^{2+}. At the end of the incubation mitochondria were sedimented by centrifugation, and radioactivity was measured in a Geiger counter. Reprinted from Ref. [2].

[14], in which he demonstrated that mitochondria had actually taken up Ca^{2+} in exchange for H^+. The observations in the Dissertation thus only appeared after the publication of the seminal papers of Vasington and Murphy and De Luca and Engstrom in 1961, but it is remarkable that they anticipated explanations of the mitochondrial Ca^{2+} transport process which were in line with the chemiosmotic concept, which was about to swipe away from bioenergetics the once domineering dogma of chemical coupling in energy transduction. Even Saris, though, could not do entirely away with the still all-pervasive ways of thinking of the chemical coupling concepts: he attributed the active transport of Ca^{2+} to an ATP-powered Ca^{2+}/H^+ pump based on a mechanism of vectorial hydrolysis of the mythical "squiggle" in the active sites of the membrane, followed by exchange reaction and active transport. To complete the story, it should be mentioned that the pioneering observations of Saris on the movement of H^+ appeared simultaneously with similar observations of Bartley and Amoore on Mn^{2+} (a close relative of Ca^{2+}) [15]. They had found that mitochondria could take up large amounts of Mn^{2+}, displacing H^+ in the process. The pH drop was very significant: in the estimate of the authors 60 to 90% of the uptake of Mn^{2+} was accounted for by the loss of H^+.

2. The golden 1960s

2.1. Establishing the basic phenomenology

Pre-history ended in 1961–1962, when the papers by De Luca and Engstrom and of Vasington and Murphy established directly and conclusively that isolated kidney mitochondria took up Ca^{2+} in an energy-driven process [1-2,3] (Fig. 3). Actually, the energy-dependence was unambiguously demonstrated only in the contribution by Vasington and Murphy. That of De Luca and Engstrom described some puzzling findings, e.g., some respiratory chain inhibitors (antimycin A) abolished the uptake, of Ca^{2+} while CN^- did not. More disturbingly, the classical uncoupler dinitrophenol had no effect on the uptake, whereas another uncoupler, dicumarol, abolished it. De Luca and Engstrom were thus led to conclude, much as Slater and Cleland had done 8 years earlier [9] that the Ca^{2+} uptake process did not require oxidative phosphorylation, but only the functionality of the mid-portion of the respiratory chain. In hindsight, one can now consider these inconsistent observations and conclusions as minor incidents: the important finding was that isolated mitochondria could take up large amounts of Ca^{2+} in a process that was an alternative to ATP synthesis as a means to harvest respiratory energy$^+$. Vasington and Murphy, instead, had unambiguously shown that the uptake of Ca^{2+} required coupled respiration, since it was inhibited by respiratory chain blockers and by uncouplers, but not by the inhibitor of the synthesis of ATP oligomycin. One year later, Rossi and Lehninger [16] closed the circle by showing that the uptake of Ca^{2+} could be energized by ATP in the absence of respiration, in which case it was inhibited by oligomycin, and not by inhibitors of respiratory chain. Those were exciting findings: thus having just joined the Laboratory of Albert Lehninger at Johns Hopkins where Frank Vasington had made his landmark discovery, I had decided to work on the new topic.

All these initial studies had established that the amount of Ca^{2+} that could be accumulated was hundreds of times greater than the initial mitochondrial content, but they had also found that these very large amounts could only be accumulated in the presence of phosphate. In its absence, the accumulated amounts were much more limited (leading to the definition of two uptake conditions: "limited" uptake as opposed to "massive" uptake). One odd finding (made also by De Luca and Engstrom) was the necessity of ATP for the Ca^{2+} uptake process even when the source of energy was the respiratory chain (Fig. 4). The finding was all the more surprising as the inhibitor of the "coupling" ATPase oligomycin had no effect on the

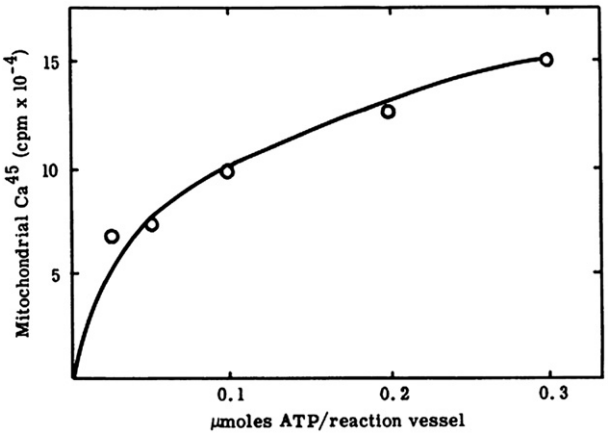

Fig. 4. Uptake of Ca^{2+} by isolated rat kidney mitochondria. Mitochondria were isolated by a conventional cell fractionation procedure from vitamin-D deficient rats. The reaction medium (isotonic sucrose-KCl pH 7.4) was supplemented with respiratory substrates, 0.3 mM radioactive ^{45}Ca, $MgCl_2$, inorganic phosphate, and the amounts of ATP shown in the figure. At the end of the incubation mitochondria were sedimented by centrifugation, and radioactivity was measured in a Geiger counter. Reprinted from Ref. [3].

uptake process: it was only understood about two years later when it was shown that ATP was taken up by mitochondria alongside with Ca^{2+} and inorganic phosphate (see below).

In agreement with predictions Saris had made in his Dissertation [14], Lehninger and coworkers [16] soon found that phosphate was the major anion that accompanied Ca^{2+} on its way to the mitochondrial matrix, to precipitate it there as an insoluble phosphate salt. This observation, regarded at that time essentially as a convenient device to increase the amount of Ca^{2+} that could be stored by mitochondria in Laboratory experiments, soon came to be recognized as physiologically very important when, much later, it was discovered that the matrix contained enzymes that were allosterically regulated by μM Ca^{2+} [17]: two TCA cycle dehydrogenases (α-ketoglutaric dehydrogenase and NAD^+- isocitric dehydrogenase) and the phosphatase that dephosphorytates pyruvic-dehydrogenase (reviewed in [18]). Therefore, the operation of the TCA cycle, i.e., the delivery of reducing equivalents to the respiratory chain and, ultimately, the very synthesis of ATP, depend on the proper regulation of these three enzymes by Ca^{2+}. The simultaneous uptake of inorganic phosphate was thus recognized as a means to buffer matrix Ca^{2+}, modulating its free concentration in keeping with the variable demands of reducing equivalents by the respiratory chain.

2.2. The massive uptake of Ca^{2+} and phosphate

The massive uptake of Ca2+ and phosphate was soon also proposed to be important for an entirely different reason: i.e., as a means to permit the temporary accumulation of very large amounts of Ca^2 whenever Ca^{2+} storms in the cytosol would threaten the life of the cell: mitochondria would thus be essential Ca^{2+} sinks that would buy precious time for the cell. The phosphate precipitates in isolated mitochondria that had accumulated massive amounts of Ca^{2+} and phosphate were visualized in the matrix as osmiophilic granules (Fig. 5) that resembled closely those that had been observed within the mitochondrial profiles of various tissues in numerous disease conditions (reviewed in [19]): the precipitation initiated on the cristae, as was documented using the Ca^{2+} analogue Sr^{2+} (Sr^{2+} has higher atomic number than Ca^{2+} and could be visualized in the electron microscope at lower concentrations) [20], and then migrated to freer spaces in the matrix (Fig. 6). Rossi and Lehninger [21] went on to establish that the molar Ca^{2+}: phosphate accumulation ratio averaged 1.67, which is precisely that of hydroxyapatite, the principal mineral component of bone. The formation of hydroxyapatite in the matrix was confirmed directly slightly afterward by the isolation of the dense electron-opaque granules [22,23] by Weinbach and Von Brand. The granules were indeed found to contain hydroxyapatite [$(Ca_{10}(P04)_6(OH)_2$], but also whitlockite [calcium carbonate $Ca_3(PO4)_2$]. They also contained organic components, among them sugars (ribose) and ATP/ADP: significantly, synthetically prepared hydroxyapatite crystals bind ATP/ADP and can even perform the non-catalytical cleavage of ATP [24]. The presence of adenine nucleotides in the granules explained the requirement for ATP in the medium even when the uptake process was energized by the respiratory chain [2]: no granule formation occurred, and no granules could be isolated, in the absence of ATP. We soon found [25] that ATP(ADP) was indeed accumulated by mitochondria along with Ca^{2+} at a molar ratio to the latter of about 1 to 10. The uptake of adenine nucleotides only occurred when phosphate was also taken up, and when the amounts of accumulated Ca^{2+} and phosphate were massive. It was only partially inhibited by the inhibitor of the phosphate carrier atractyloside, compatible with the idea that the route for their intake could be different from that mediated by the ATP/ADP carrier. To this day, the reasons for the uptake of the nucleotides are still obscure, but it could be reasonably suggested that they would either promote the formation of the dense granules, or somehow stabilize them. Significantly, matrix hydroxyapatite was found to be amorphous, and to remain so indefinitely: evidently, some component in

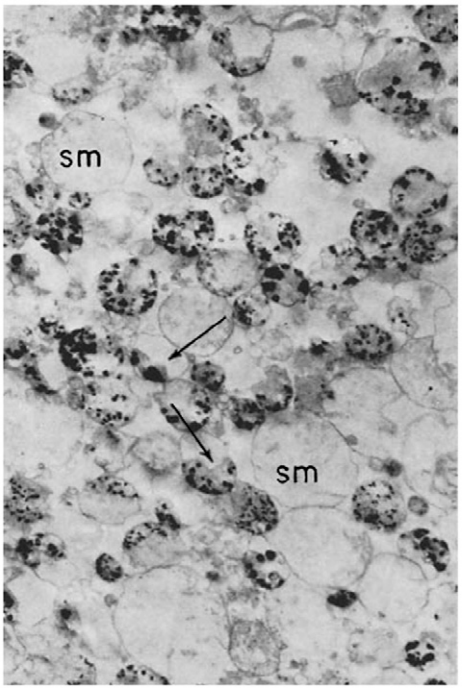

Fig. 5. Electron-dense calcium-phosphate precipitates in isolated rat liver mitochondria after accumulation of Ca^{2+} and phosphate. Mitochondria were isolated by a conventional cell fractionation procedure. The composition of the medium for the uptake reaction is not specified in the legend but, according to the reference quoted, it contained isotonic NaCl at pH 7.0, a respiratory substrate (succinate), 4.0 mM phosphate, 3.0 mM ATP and 4.0 mM Ca^{2+}. The incubation lasted 20 min at 30°. Mitochondria were then sedimented by centrifugation, fixed in OsO_4, and observed in an electron microscope, ×8.000. Reprinted from Ref. [16].

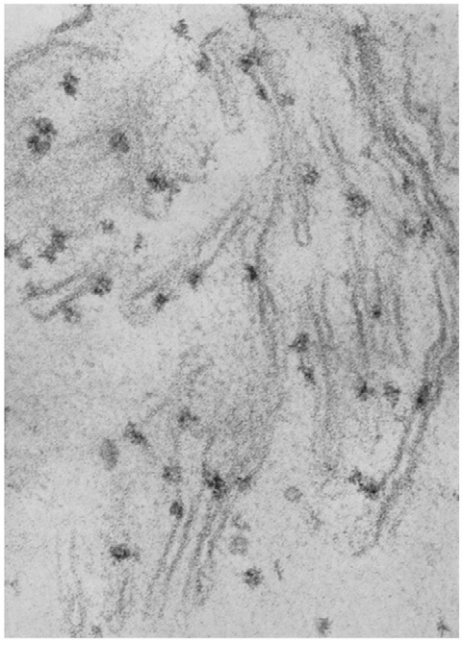

Fig. 6. Accumulation of Sr^{2+} and phosphate by isolated rat liver mitochondria. Mitochondria were isolated by a conventional cell fractionation procedure. The medium (isotonic NaCl, pH 7.0, temperature 30°) contained a respiratory substrate (succinate), 4.0 mM phosphate, and 3 mM ATP. Mitochondria were added last, and pulsed three times with Sr^{2+} to reach a concentration of 0.475 mmol $Sr^{2+}\times$mg of mitochondrial protein. At the end of the incubation (10 min after the last Sr^{2+} addition) mitochondria were sedimented by centrifugation, fixed in OsO_4, and observed in an electron microscope, ×260.000. Reprinted from Ref. [20].

mitochondria acts as an inhibitor of its crystalline transition. This component could be phosphocitrate, a very potent inhibitor of the growth of hydroxyapatite crystals [26]: when administered to animals treated with large daily doses of calcium gluconate or parathyroid hormone, a regimen that causes the massive accumulation of calcium and phosphate in the mitochondria of renal tubule cells, it prevented the appearance of the dense granules within the mitochondrial profiles [26]. The failure of mitochondrial hydroxyapatite to become crystalline could be important, as it would presumably facilitate the dissolution of the granular deposits and the gradual release of Ca^{2+} from mitochondria to permit its export out of the cell once the Ca^{2+} emergency is over. The precipitation of the electron dense Ca^{2+} deposits in the mitochondrial matrix may also be important outside the pathology area. It could have a role in the physiological process of calcification of hard tissues. The biological potential to undergo calcification is ubiquitously present in tissues of higher animals: tissues must thus possess appropriate mechanisms for promoting and regulating the process. Lehninger had proposed that these mechanisms could be linked to the transport of Ca^{2+} in mitochondria, and in particular to the deposition of Ca^{2+}-phosphate deposits [27]. Mammalian bone contains two phases of calcium-phosphate, one amorphous (tricalcium phosphate), one crystalline (hydroxyapatite) [28], amorphous tricalcium phosphate probably being an obligatory step in the formation of crystalline hydroxyapatite. Prior to this, calcium and phosphate must be concentrated to exceed the solubility product of calcium triphosphate. This could happen in mitochondria, which could concentrate Ca^{2+} and phosphate to a level which could not be attained spontaneously in the extracellular fluids. Lehinger had suggested that the initial deposition of Ca^{2+} and phosphate in the mitochondrial matrix could occur in the form of micropackets of amorphous tricalcium-phosphate similar to those present in the amorphous phase of bone mineral and bone cells, which will grow to yield the large electron dense granules, and which would be stabilized by an inhibitor (phosphocitrate ?). Micropackets of colloidal dimensions would dissociate from the large granules and somehow "pass" through the mitochondrial membrane to be exported from the cell, to diffuse to extracellular calcification sites. Clearly, the transfer of the Ca^{2+} deposits from the mitochondria to the sites of mineralization is the main problem [29] that must be solved before the proposal of mitochondrial participation in the calcification process will gain general acceptance. Findings supporting the proposal have become available over the years: for instance, the presence of dense (calcium-phosphate) granules in the mitochondria of cells known to have special roles at the calcification sites (condrocytes, osteoblasts, osteocytes, osteoclasts [30–32]), or the histochemical demonstration of the migration of the Ca2+-phosphate deposits from an intracellular, mainly mitochondrial location to an extracellular location in the epiphyseal growth plate [33]. Since these supporting findings are all indirect, the proposal still needs final experimental validation.

2.3. Understanding the mechanism of the uptake process

In the wake of the exciting findings described above, mitochondrial Ca^{2+} transport rapidly became a very popular topic. In hindsight, one important reason for the wide explosion of interest should have been the obvious and essential support the findings were providing to the then emerging chemiosmotic concept of energy transduction [34,35]. However, it wasn't so: for a long while, findings in numerous Laboratories continued to be interpreted within the framework of the "chemical theory" of mitochondrial energy transduction. Even Laboratories later due to become staunch supporters of Mitchell's theory still interpreted their results using the high energy intermediate concept: one could for instance quote a 1965 paper on Ca^{2+}-induced mitochondrial swelling [36] in which Chappell and Crofts had at long last disposed of the old U-factor interpretation (see above), neatly showing that the swelling was due to the uptake of Ca^{2+}: remarkably, however, they had attributed the swelling to the lowering of the level of the high energy intermediates brought about by the uptake of Ca^{2+}. Chemiosmosis thus crept into the field laboriously and did not take it over until well into the 1960s. In meanwhile, a large number of findings were rapidly made, so that the phenomenology of the Ca^{2+} uptake process became essentially established by the mid-late 1960s. Thus, Rossi and Lehninger [21] had measured the stoichiometry of Ca^{2+} uptake to oxygen consumption, finding that two Ca^{2+} ions were required to elicit the same amount of extra oxygen consumption elicited by one molecule of ADP: the finding was neatly reminiscent of the earlier conclusion of Chance [11] that the Ca^{2+}/0 stoichiometry in the State4/State3/State4 respiratory transition was 2–3 times the ADP/0 stoichiometry. It also became soon clear that in the absence of phosphate the limited amounts of Ca^{2+} that could be taken up were maintained in mitochondria in a dynamic steady state in which the "leak" of Ca^{2+} was balanced by its re-uptake during the phase of resting oxygen consumption (State 4 rate) that followed the burst of activated respiration [37]. The finding was important, as it fostered the concept that Ca^{2+} cycled continuously across the inner membrane, its long-term storage in the matrix only occurring under conditions that favored its massive uptake together with phosphate. At that time, the role of Ca^{2+} as a universal regulator of cell activities was beginning to be recognized, and it was becoming clear that the precise regulation of cell Ca^{2+} was an absolute necessity of cell life. Thus, the finding that mitochondria could dynamically move Ca^{2+} to and from the cytoplasm in response to the demands of the Ca^{2+} modulated processes placed mitochondria in a key position as potential regulators of cell functions. However, in the 1960s one important element of the concept was still missing, and that was the route that released Ca^{2+} from mitochondria: when the route was eventually discovered in 1974 [38] the concept of the "mitochondrial Ca^{2+} cycle" [39] became firmly established. The "limited" uptake of Ca^{2+} in the absence of phosphate was used in a number of Laboratories to extend and refine the old observations of the exchange of Ca^{2+} for H^+, as it would have allowed the movements of Ca^{2+} to be interpreted within chemiosmotic principles. Numerous measurements [40-46] showed that the number of H^+ ejected per Ca^{2+} ions taken up was about 1, a figure that had to be integrated within the concept of electrophoretic uptake of Ca^{2+} that was being proposed. An interesting observation was made during the study of H^+ ejection during the phase of Ca^{2+} cycling that followed the respiratory burst: the movements of Ca^{2+} and H^+ had oscillatory behaviour [47], a finding that predated by a number of years the observations, which are now routine, of oscillations in the cellular movements of Ca^{2+}.

By the end of the 1960s the chemiosmotic theory of energy conservation had become widely accepted, and the process of Ca^{2+} uptake by mitochondria had been instrumental in fostering its success. It had for instance been important to show that Ca^{2+} influx could be promoted in the absence of respiration or ATP hydrolysis by imposing a diffusion potential across the inner membrane by a gradient of the permeant anion SCN-, or of H^+ in the presence of uncouplers that permitted electrogenic H^+ permeation [48]. Similarly, Ca^{2+} influx could be promoted by a diffusion potential created by a gradient of K^+ in the presence of valinomycin [49]: clearly, Ca^{2+} moved electrophoretically across the inner membrane in response to the electrochemical gradient. Later on, Rottenberg and Scarpa [50] compared the distribution of Ca^{2+} between energized mitochondria and medium to that of Rb^+, which, in the presence of valinomycin, is assumed to attain electrochemical equilibrium according to the Nernst equation. The data were in agreement with a purely electrophoretic mechanism, with a net charge transfer of two per Ca^{2+} ion transported. Based on these and other results the presence of an electrophoretic component in the uptake process thus became

generally accepted, even if uncertainties of various type complicated experiments designed to establish whether the mechanism of the process was purely electrophoretic or partially charge-compensated. At this point, then, the process of Ca^{2+} transport, which had initially been described as consisting of an active uptake and a passive release leg, became an energetically downhill uptake process (occurring on a hypothetical uniporter) coupled to an uphill efflux against an electrochemical gradient. The studies of the $Ca2^{+}$ uptake process were greatly facilitated by the discovery of a specific inhibitor of the hypothetical uniporter. This was a commonly used histochemical stain, the polycation ruthenium red. It inhibited other Ca^{2+} linked processes at concentrations higher than 5–10 μM, whereas it inhibited the mitochondrial uptake process at sub-μM concentrations [51] (about 20 years later a derivative of ruthenium red, RU360 [52] was found to be even more effective, inhibiting the uptake of Ca^{2+} with an IC_{50} between 0.2 and 2 nM).

2.4. How does Ca^{2+} exit from mitochondria?

The efflux leg of the Ca^{2+} cycling process was only identified in 1974, when our Laboratory discovered that Na^{+} released Ca^{2+} from heart mitochondria [38]. The original experiment I performed after I had returned to Italy from my stay at Johns Hopkins, is shown in Fig. 7: the effect of Na^{+} was specific, i.e., it was not duplicated by K^{+}, was made more evident if the reuptake of the lost Ca^{2+} was prevented by ruthenium red, and was already evident at 5 mM Na^{+}. This Na^{+} concentration was in the range of that of the cytoplasm, and it was thus plausible to suggest that the route could be operative in the cell ambient (see below). Since the experiment had been performed on heart mitochondria, the releasing effect of Na^{+} could have perhaps offered an alternative explanation for the positive ionotropic effect of cardiac glycosides: the results of the work were thus published in a molecular cardiology journal, rather than in a classical biochemistry journal. In the following few years I explored in detail the Na^{+}-induced Ca^{2+} release with my colleague Martin Crompton at the ETH in Zurich [53-55]. We characterized the process as a carrier mediated antiport that exchanged Na^{+} for Ca^{2+}; it may be interesting to point out that we suggested the main role of the carrier to be the regulation of mitochondrial Ca^{2+}: at the time, the consensus was becoming general that mitochondria had little to say on the regulation of cytoplasmic Ca^{2+}. The curve relating the rate of Na^{+}-induced Ca^{2+} release to the concentration of external Na^{+} had a pronounced sigmoidal character, with an EC_{50} of about 8mM, and a Vmax of Ca^{2+} release of about 18 nmol per mg of mitochondrial protein per minute. That is, the rate of Ca^{2+} efflux was changed significantly by relatively small changes of the Na^{+} concentration in the 4–10 mM range. This finding thus reinforced the suggestion that the efflux could be regulated by physiological changes of the cellular Na^{+} concentration. We found that the exchanger was particularly active in the mitochondria of excitable tissues [53-55]. A few years later, a Ca^{2+}/H^{+} exchanger was instead found to mediate preferentially the release of Ca^{2+} from mitochondria which were less responsive to Na^{+}, e.g., those of liver [56]. We had originally found that the Na^{+}/Ca^{2+} exchanger was sensitive to lanthanides [57]. Later work by others showed that it was also inhibited by a number of L-type Ca^{2+} channel blockers [58], this dual specificity limiting their use in studies on the exchanger in intact cells. It was then found that benzodiazepines also blocked the Na^{+} induced Ca^{2+} efflux, the most effective being clonazepam [59]. Benzodiazepines, however, also inhibit GABA receptors, and are further limited as tools of work by the presence of a benzodiazepine receptor in the mitochondrial outer membrane [60]. A very selective (and effective) inhibitor of the Na^{+}/Ca^{2+} exchanger was identified in 1988 by Chiesi and coworkers as a benzothiazepine (CGP-37157) [61], which became the inhibitor of choice for the studies on the antiporter. One problem which is still debated is the stoichiometry of the exchange: the antiporter must operate in the presence of the large negative membrane potential that is maintained inside mitochondria by the vectorial operation of the respiratory chain, and must therefore either be electroneutral, or operate electrogenically exchanging more than 2 Na^{+} per Ca^{2+}. Our early report [48] had shown that the efflux of Ca^{2+} was dependent on the energy state of mitochondria, and had indicated the presence of three Na^{+} binding sites on the antiporter [62]. A number of studies (for instance [63]) later concluded that the antiporter operated electroneutrally, but more recent results indicate instead a 3 Na^{+} per 1 Ca^{2+} stoichiometry [64, 65].

2.5. Clouds on the horizon: the problem of the affinity of the uniporter for Ca^{2+}

Another important question was that of the affinity of mitochondria, i.e., of the hypothetical uptake uniporter, for Ca^{2+}. The question

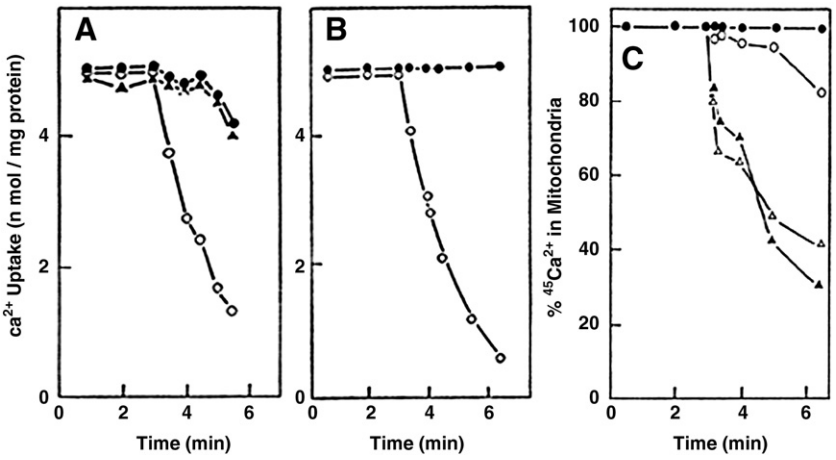

Fig. 7. Na^{+}-induced Ca^{2+} release from rat heart mitochondria. Mitochondria were isolated by a conventional cell fractionation procedure. The release of Ca^{2+} was followed isotopically by rapid Millipore filtration after mitochondria had been loaded with Ca^{2+}, labeled with $^{45}Ca^{2+}$. The medium (pH 7.4, 25°) contained 210 mM mannitol, 70 mM sucrose, and a respiratory substrate (succinate). No substrate was present in panel (a), in which mitochondria had also been pre-incubated with rotenone to block the reuptake of the lost Ca^{2+} driven by endogenous respiratory chain activity. In panel (b), the reuptake of the lost Ca^{2+} had instead been blocked by 10 nm ruthenium red, added 30 s before the addition of Na^{+}. The amounts of Ca^{2+} added to the medium were extremely small (between 5 and 10 nmol per mg of mitochondrial protein). In panel (c), no Ca^{2+} was added, as the rats had been injected intraperitoneally with 180 μci of $^{45}Ca^{2+}$ 5 min before being killed. Panel (a): open circles, 100 mM Na^{+}, triangles, 100 mM K^{+}, closed circles, equal concentration of reaction medium. Panel (b): open circles, 50 mM Na^{+}, closed circles, equal concentration of reaction medium. Panel (c): open circles, 5 mM Na^{+}, closed triangles, 20 mM Na^{+}, open triangles, 50 mM Na^{+}, closed circles, 100 mM reaction medium. Adapted from Ref. [38].

was of obvious importance: if mitochondria were to play any role in the regulation of cytosolic Ca^{2+} they had to be able to interact efficiently with it in the concentrations known to prevail in the cytosol, which oscillate around mid nM values. The matter of the affinity was a turning point in the research on mitochondria and Ca^{2+}, as problems soon surfaced that begun to dampen significantly the excitement of the early days. Isolated mitochondria could lower the concentration of Ca^{2+} in the medium well below 1 μM, but the concentration of Ca^{2+} necessary for half-maximal uptake velocity (the K_m) turned out to be significantly higher than that value. Measurements were performed on mitochondria from different tissues and with different methods (it had soon become clear that Ca^{2+} transport was a general property of mitochondria), but the K_m values measured in a number of Laboratories (including ours) were invariably higher than 1–2 μM. They were frequently much higher, up to 50–70 μM (see [66] for a review): a problem was certainly Mg^{2+}, since it decreased the affinity very significantly when added to mitochondrial suspensions in the concentrations found in the cytoplasm [67]. Natural factors were later discovered that increased the affinity of the uniporter, e.g., spermine, which was claimed to make mitochondria responsive to Ca^{2+} concentrations in the physiological range [68]. But the importance of mitochondria as physiological regulators of cytosolic Ca^{2+} started to be seriously questioned, and the feeling became gradually widespread that they would be essentially inactive in the Ca^{2+} ambient of the cytoplasm. The feeling had ground, since in the 1960s and 1970s it was generally assumed that the concentration of Ca^{2+} in the cytoplasm was uniformly in the 100–200 nM range. Another element in the mounting skepticism on the importance of mitochondria as regulators of cytosolic Ca^{2+} was provided by findings made much later that showed that the amount of Ca^{2+} within mitochondria in situ, which could be conceivably mobilized in response to physiological demands, was lower than the figures derived from early measurements in isolated mitochondria: reliable in vivo tests, ranging from electron microprobe analysis [69] to the measurement of the amount of Ca^{2+} released with metabolic inhibitors [70] produced figures as low as 1–2 nmol per mg of mitochondrial protein. These measurements appeared in the 1980s, i.e., at a time when mitochondrial Ca^{2+} transport had already lost a significant portion of its appeal, but were instrumental in decreasing it even further. Thus, in the 1980s the vast majority of those working in the field of cellular Ca^{2+} regulation became convinced that mitochondria had nothing important to say in it. The regulation of the three internal Ca^{2+} dependent dehydrogenases came to be considered as the only rationale for the existence of the mitochondrial Ca^{2+} transporting system. The role of mitochondrial Ca^{2+} transport would in other words essentially be the translation of Ca^{2+} signals inside mitochondria to modulate energy production. Which was of course logical; while other systems could have had the primary role in the regulation of cytosolic Ca^{2+}, only mitochondria could obviously regulate their own internal Ca^{2+}. Nevertheless, the conclusion that mitochondria had nothing serious to do with the regulation of cell Ca^{2+} was still somewhat surprising, as it had overlooked important evidence that had shown that the process of energy-linked mitochondrial Ca^{2+} transport, irrespective of the poor affinity of the uptake uniporter for Ca^{2+}, did indeed operate with great efficiency in situ. Ample evidence had been available for a while that mitochondria were very active in transporting Ca^{2+} in situ, but, somehow, it was not taken into account. The 1953 paper by Slater and Cleland [9] had for instance shown very clearly that mitochondria sequestered essentially all the Ca^{2+} of the heart homogenate, i.e., even if all other organelles were present, Ca^{2+} ended up in mitochondria. And at about the same time a similar study of Maynard and Cotzias [71] had shown that the same was true of Mn^{2+}, a cation that is taken up by mitochondria using the same mechanistic principles of Ca^{2+}: liver fractionation tests had shown that injected radioactive Mn^{2+} was concentrated primarily in the mitochondria. Our Laboratory had also performed detailed studies along these lines, and had found that more than 50% of the $^{45}Ca^{2+}$ injected into rats was recovered in the mitochondrial fraction of the liver homogenate, and less than 20% in the microsomal fraction that contained the endoplasmic reticulum [72] (Table 2). Importantly, the percentage of $^{45}Ca^{2+}$ recovered in mitochondria dropped to about 20% if the rat had been previously injected with the uncoupler pentachlorophenol: the finding showed that the association of Ca^{2+} with mitochondria *in vivo* had indeed occurred via the energy linked uptake process. We had made similar findings on heart [73], and found that 60 to 70% of the injected $^{45}Ca^{2+}$ was recovered in the mitochondrial fraction of the homogenate, but only 20 to 25% if the rats had been previously treated with the uncoupler. And we had extended the work to skeletal muscle, finding that the uptake of Ca^{2+} by the intact rat diaphragm was favored if the muscle was incubated under conditions that would have promoted the uptake of Ca^{2+} by mitochondria, and was inhibited by respiratory inhibitors and uncouplers. Instead it was not inhibited by agents known to block the uptake of Ca^{2+} by sarcoplasmic reticulum [74].

Table 2
Distribution of injected $^{45}Ca^{2+}$ in the subcellular fractions of rat liver.

Fraction	% distribution	
	Control	Rat pre-injected with DNP
Residue	22.6 ± 1.12 (9)	21.5 ± 4.33 (3)
Mitochondria	55.5 ± 1.84 (9)	22.2 ± 2.40 (3)
Heavy microsomes	4.3 ± 028 (9)	12.8 ± 1.65 (3)
Microsomes	15.3 ± 290 (9)	30.3 ± 0.46 (3)
Supernatant	2.5 ± 038 (9)	13.2 ± 052 (3)

$^{45}Ca^{2+}$ (10 μc) was injected intraperitoneally 6 min before the death of the animal, and 3 min before the injection of pentachlorophenol (20 mg/kg). Livers were homogenized in a Potter–Elvehjem homogenizer and subcellular fractions separated with a conventional fractionation scheme. Data are given ± standard errors. The number of experiments is in brackets. Modified from Ref. [72].

3. The next 20 years: the field slides into lethargy

The abundant and sophisticated phenomenology that had been amassed, and the evidence that, somehow, the process of mitochondrial Ca^{2+} transport was alive and well in situ, could not outweigh the general skepticism on the physiological importance of the process. The topic gradually lost appeal, leaving center stage to the vigorously advancing tide of findings and conclusions sparked by the seminal findings on Ca^{2+} regulation by endoplasmic reticulum [75]. Research on mitochondrial Ca^{2+} continued, but center stage was gone, and did not come back until well into the 1990s (see below). Significant findings were nevertheless made during the times of obscurity, e.g., correlating changes in mitochondrial Ca^{2+} with those of NADH and of NADH-linked activities [76-78], and showing that the uniporter had two Ca^{2+} conductivity states: when liver or heart mitochondria were exposed to Ca^{2+} pulses lasting from 1 to 10 s, Ca^{2+} uptake was very fast for pulses of less than 1 s, and slowed down when the pulse lasted more than one second [79]: the dual state of the uniporter was suggested to enable it to perform the rapid uptake of short Ca^{2+} pulses, and to sequester in a slower mode larger pulses of Ca^{2+} offered to it over longer times. Another important development was the discovery that Ca^{2+} could also be released from mitochondria by an entirely different route, the permeability transition pore (PTP). The PTP had been first proposed by Hunter and Haworth in 1979 [80], and found to mediate the quick release of Ca^{2+} from mitochondria. It became a popular object of study in the 1980s, particularly after it was found that it mediated the release of apoptotic factors, among them cytochrome C, to activate the caspase cascade: it thus became recognized as an important actor in the mitochondrial cell death pathway (reviewed in [81]). The PTP was characterized as a non-specific channel consisting of a number of components that span both mitochondrial membranes and permit the passage of solutes with

molecular mass <1500 Da. A number of factors were found to promote its opening, chiefly among them increased matrix Ca^{2+}, while other factors, chiefly among them cyclosporin [82], promoted instead its closure. Even if not specific, and large enough to let through solutes of much larger mass than Ca^{2+}, the channel nevertheless appears to have some Ca^{2+} specificity: Ca^{2+} could quickly leave mitochondria through it, possibly in a low-conductance state of the channel [83]. The PTP has now become an intensively studied topic in mitochondrial research thanks to the finding of its involvement in a large number of disease conditions, particularly those of the nervous system.

4. From oblivion to resurrection

Throughout the long period of obscurity, reports kept occasionally appearing that showed that mitochondria could buffer physiological Ca^{2+} loads in situ: evidently, and in agreement with the findings described above on the sequestration of Ca^{2+} by mitochondria in situ, conditions prevailed in the cell cytoplasm that could somehow overcome the poor affinity of the uniporter. This was clearly at odds with what was generally accepted on the concentration of Ca^{2+} in the cytoplasm. A conceptual breakthrough was evidently necessary to solve the paradox, and it came in 1992, when Rosario (Sarino) Rizzuto and his coworkers in the Laboratory of Tullio Pozzan showed that the sub-μM concentration of Ca^{2+} in the bulk cytoplasm needed not reflect its concentration in the vicinity of mitochondria [84]. The conclusion was based on experiments in which the Ca^{2+} sensitive recombinant protein aequorin had been specifically targeted to the mitochondria of bovine endothelial cells, and could thus monitor directly the uptake of Ca^{2+} *in situ*. It was found that the exposure of cells to a plasma membrane agonist known to promote the elevation of cytoplasmic Ca^{2+} unexpectedly elicited the rapid and transient increase of mitochondrial Ca^{2+}, which was prevented by the addition of uncouplers. It was the first direct indication that the energy-linked Ca^{2+} mitochondrial Ca^{2+} transport responded rapidly to physiological stimuli that increased cytoplasmic Ca^{+} in living cells (Fig. 8). The authors then extended the finding by showing that stimuli that generated InsP3 [85] rapidly increased mitochondrial Ca^{2+}, an effect that could be duplicated by adding InsP3 to permeabilized cells, but not by adding to them Ca^{2+} in the concentrations known to exist in the bulk cytoplasm. [85]. The obvious conclusion of the last experiments was that the opening of the InsP3-gated channel in the endoplasmic reticulum had generated spots of high Ca^{2+} concentration that would be sensed by nearby mitochondria and activate the low affinity uniporter. It was the " Ca^{2+} micropools " concept, which was rapidly corroborated by the demonstration of the intimate physical interplay between mitochondria and the endoplasmic reticulum (Fig. 8) ([86], reviewed in [87]).

The findings on the Ca^{2+} micropools attracted wide attention, triggering work on a number of cell types and with different targeted indicators. They rapidly resuscitated mitochondrial Ca^{2+} transport from the comatose state in which it had languished for so long, bringing it back to the center stage it had occupied in the old days. They were thus very important: but not, as one sometimes reads in the literature, for having demonstrated that mitochondrial Ca^{2+} transport indeed occurred in vivo: this had already been established by the experiments carried out in the old days [9,71,72-75]. Their value was the clarification of the mechanism that permitted the uniporter to be fully operational in situ in spite of its poor affinity. A second important merit was the possibility they afforded to test how dynamically, i.e., how rapidly, the mitochondrial system reacted to changes in cytosolic Ca^{2+}. The matter was of obvious importance: a significant role of the mitochondrial Ca^{2+} pool in the regulation of cellular (and of course mitochondrial) Ca^{2+} demanded a reactivity compatible with that of Ca^{2+} modulated cytoplasmic processes. The finding that upon stimulation the Ca^{2+} concentration in the perimitochondrial space could rise to values approaching 20 μm [88] was thus important: these concentrations permitted the uptake of Ca^{2+} to occur at higher rates that had been established from the early studies on isolated mitochondria. The behavior of the mitochondrial Ca^{2+} pool during the contraction/relaxation cycle of heart cells, i.e., in the 150–200 ms time range, thus received much attention. The earliest study on the problem was published 1 year after the appearance of the Ca^{2+} micropools reports: however, it had used electron probe microanalysis (a technique that monitored total Ca^{2+}, not only free Ca^{2+}), not targeted Ca^{2+} probes [89], and had shown beat-to-beat changes of mitochondrial Ca^{2+} in guinea-pig ventricular myocytes. However,

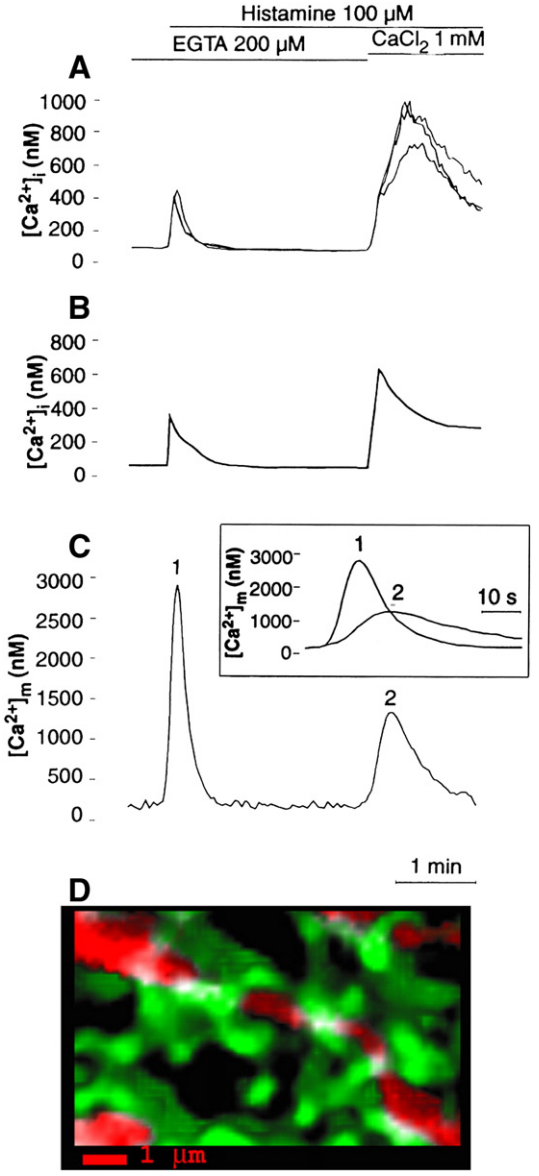

Fig. 8. Changes of cytoplasmic Ca^{2+} (Ca_i^{2+}) and mitochondrial Ca^{2+} (Ca_m^{2+}) in a HeLa cell clone that stably expressed mitochondrial aequorin or cytoplasmic aequorin. The cells were trypsinized, plated on glass cover slips, left in culture for two days, and loaded with fura-2 and coelenterazine (to reconstitute aequorin). Full experimental conditions, including observation of cells, are described in Refs. [84,85]. (A) Measurement of ($Ca^{2+}{}_i$) in three representative single cells. (B) Measurement of Ca^{2+} in a monolayer of cells. (C) Measurement of (Ca_i^{2+}) in a monolayer of cells. In the inset, the (Ca_m^{2+}) peaks (1, mobilization, 2, influx), are compared on an expanded time scale. (D) Combined 3D imaging of mitochondria and endoplasmic reticulum in a HeLa cell transiently expressing mitochondrial GFP and endoplasmic reticulum GFP. The two 3D images were superimposed. The mitochondrial and endoplasmic reticulum images are represented in red and green, respectively. The overlaps of the two images are white (8°-nm pixel). Full experimental conditions are described in Ref. [86].

other results with targeted indicator methodologies were conflicting. The issue was eventually solved by a study that used simultaneously two novel recombinant Ca^{2+} indicators (ratiometric pericams, [90]) targeted to the mitochondria and to the nucleus (which was taken as an equivalent of the cytoplasm) of neonatal rat cadiomyocytes [91]: the results showed unequivocally that mitochondrial Ca^{2+} oscillated synchronously with cytoplasmic Ca^{2+} during the contraction-relaxation cycle.

It was logical to expect that the newly demonstrated rapid changes of mitochondrial Ca^{2+} would affect the Ca^{2+}-dependent matrix dehydrogenases, and thus the NAD(P)H and ATP production. The matter was promptly investigated in a number of Laboratories, using once again heart as a preferred object of study. Various preparations were used, from whole hearts to isolated myocytes. The increases of NAD(P)H production in various preparations, from whole hearts to isolated myocytes, were taken as an indirect indication of ATP production (although ATP was also in some cases measured directly with targeted luciferase). The correspondence with the changes in mitochondrial Ca^{2+} upon rapid stimulation of cells, i.e., following a sudden increase in energy demand, was not always clear-cut [92]: in general, however, an initial drop of NAD(P)H and ATP was observed, which was followed by an increase, that occurred as soon as mitochondrial Ca^{2+} rose to levels that activated the Ca^{2+} sensitive matrix dehydrogenases [92,93].

The discussion above has concentrated on the activation of the uptake uniporter as a means to modulate cytoplasmic (and mitochondrial) Ca^{2+}. The in situ role of the Ca^{2+} efflux pathways has now also attracted much interest. It has for instance been shown that the inhibition of the Na^+/Ca^{2+} efflux antiporter in isolated guinea pig cardiomyocytes by CGP-37157 potentiated diastolic mitochondrial Ca^{2+}accumulation [94]. Interestingly, artificially elevating cytoplasmic Na^+ from 5 to 15 mM accelerated the decrease of mitochondrial Ca^{2+}. The effects on NADH production were also studied: at 5 mM Na^+ the levels of reduced NADH were maintained, but at 15 mM the NADH pool became partly oxidized [94]: evidently, the decrease of mitochondrial Ca^{2+} had limited the ability of the Ca^{2+} sensitive matrix dehydrogenases to generate NADH. These findings on the elevated intracellular Na^+ are interesting, as cytoplasmic Na^+, which in large animals, including man, varies between 5 and 10 mM [95], increases for instance significantly in response to the increase of heart rate [96]. Clearly, the changes in cytoplasmic Na^+ could play an important role in the in situ regulation of the activity of the mitochondrial Na^+/Ca^{2+} exchanger, and thus of cytoplasmic Ca^{2+}: one could thus mention again the possibility of a role of the mitochondrial Na^+/Ca^{2+} exchanger in mediating the positive inotropy effects of cardiac glycosides (see above). The finding that cytoplasmic Na^+ increases under cardiac pathological conditions like hypertrophy, ischemia/reperfusion injury, and failure [94,95,97–100] has led to the suggestion that the Na^+ dysregulation may be a primary cause of the mitochondrial decreased ability to retain Ca^{2+}. In a guinea pig model of heart failure (aortic constriction) intracellular Na^+ was found to increase from 5 to 16 mM (with a concomitant decrease of NADH which was prevented by the exchanger inhibitor CGP 37137) [101]. Earlier work had shown that the exposure of isolated mitochondria to Na^+ shifted the activation curve of the dehydrogenases to the right [102]: as a result of these findings, the mitochondrial Na^+/Ca^{2+} exchanger has thus become a plausible target for cardioprotection. It may be involved in other disease conditions as well: in a genetic form of Parkinson's disease caused by a PINK1 mutation the exchanger has been found to be dysfunctional, i.e., less Ca^{2+} was released by mitochondria in permeabilized neurons exposed to 50 mM Na^+. The ensuing condition of mitochondrial Ca^{2+} overload caused the premature opening of the permeability transition pore, profound mitochondrial depolarization, and eventual neuronal death [103].

5. The newest frontier: mitochondrial Ca^{2+} and the disease process

The involvement of the mitochondrial Ca^{2+} controlling systems in the onset and development of diseases briefly introduced in the paragraphs above underlines the ambivalent nature of the Ca^{2+} signal: the choice of Ca^{2+} as a determinant for function forces cells to control its level very carefully and precisely: if control somehow fails, Ca^{2+} becomes a conveyor of death [104]. It is thus hardly surprising that dysfunctions of the ability of mitochondria to control Ca^{2+} should be harmful to cells. The topic of mitochondrial Ca^{2+} transport in the disease process is now becoming very popular and the examples above show that the Na^+/Ca^{2+} exchanger is attracting particular attention. An interesting contribution on a mitochondrial disease caused by a mutation in a mitochondrial tRNA (lys) (myoclonic epilepsy with ragged red fibers, MERRF) has nicely underlined its importance in the homeostasis of Ca^{2+} in the cytoplasm and within the mitochondria [105]. The disease caused a respiratory chain defect that prevented muscle mitochondria from efficiently taking up Ca^{2+}, thus depressing the synthesis of ATP. Both the Ca^{2+} response of mitochondria, and the production of ATP were restored by CGP 37 157. The other mitochondrial system that is now attracting increasing attention as an actor in the disease process is the permeability transition pore. Even if it is not in a strict sense a specific Ca^{2+} permeable structure, it is sensitized by matrix Ca^{2+}, and lets Ca^{2+} through. The opening of the pore causes the release of cytochrome C and other pro-apoptotic proteins from the intermembrane space, and is now assumed to have a critical role in cell necrosis and apoptosis from numerous toxicants and from stressing conditions of various origin. Of particular interest are recent reports on the role of the permeability transition pore in the pathogenesis of neurodegenerative diseases which are now being increasingly considered as mitochondrial stress diseases. Factors of various types, e.g., reactive oxygen species, or even Ca^{2+} itself, would somehow create a condition of mitochondrial stress that hampers their ability to correctly handling Ca^{2+}. That is they would promote conditions that would be conducive to the premature opening of the permeability transition pore and to the exit of proapoptotic factors, and of Ca^{2+} itself, to the cytoplasm. Such a chain of events has now indeed been described, e.g., in Alzheimer's [106] and Huntington's [107] diseases. In addition to the PTP, other mitochondrial Ca2+ controlling systems could also be dysfunctional in neurodegenerative diseases and contribute to their pathogenesis: interesting work on cells that lack endogenous mitochondria and that are repopulated with mitochondria from Alzheimer's disease patients (called AD *cybrids*) has shown that they did not display the mitochondrial membrane potential flickering that is normally induced by cyclosporine. The Na^+/Ca^{2+} exchange inhibitor CGP 37157 eliminated the flickering in control cells, reproducing the behaviour of Alzheimer's disease cells [108]: it was an indirect indication that a dysfunction of the mitochondrial Na^+/Ca^{2+} exchanger may have a role in the pathogenesis of Alzheimer's disease. Further indications for a role of the exchanger were provided by the finding that CGP 37157 blocked the potentiation by β-amyloid of the Ca^{2+} response induced by nicotinic stimulation in forebrain neurons [109]. The possibility of a role of the mitochondrial Na^+/Ca^{2+} exchanger in the pathogenesis of Parkinson's disease has been mentioned above. Results on fibroblasts of patients with inherited defects of complex I of the respiratory chain (a defect commonly associated with Parkinson patients) offer some support for such a role: CGP 37157 restored to normal the deficient increase of ATP synthesis induced by the stimulation with bradykinin [110]

6. A still unsolved problem: The molecular nature of the mitochondria Ca^{2+} transporters

One aspect of the mitochondrial Ca^{2+} story that has not been handled in the pages above is the molecular nature of the systems that

transport Ca^{2+} across the mitochondrial membranes. The reason was quite simple: nearly 50 years after the opening of the field still very little is known on them. One could perhaps forget the permeability transition pore, which is not specific for Ca^{2+}. But the uniporter, and the two exchangers, particularly the more popular Na^+/Ca^{2+} exchanger, have been intensively investigated: so far, however, with the possible exception of the exchangers (see below) they have disappointingly defeated all attempts of molecular characterization. The uniporter has recently been shown to be a novel, highly selective Ca^{2+} channel that binds Ca^{2+} with extremely high affinity, thus accounting for the high Ca^{2+} sensitivity despite the very low cytoplasmic Ca^+ concentrations [111]. Patch-clamping of COS-7 cell mitochondria freed of the outer membrane (mitoplasts) has shown that the channel was sensitive to ruthenium red and to RU360, and had the same relative divalent conductance selectivity of the uptake reaction in isolated mitochondria [111]. These were interesting findings, but the molecular nature of the uniporter/channel is still unknown. Earlier attempts to establish it had only produced enriched fractions labeled with RU360 that exhibited Ca^{2+} transport activity [112], or preparations that reconstituted a ruthenium red-sensitive channel in planar lipid bilayers [113]. Much excitement had also been generated by a more recent report [114] that had suggested that the uncoupling proteins UCP2 and UCP3 were the uniporter. Mitochondria isolated from the livers of $UCP^{-/-}$ mice had no uptake of Ca^{2+}, but overexpression of UCP2 and UCP3 in an endothelial cell line increased the mitochondrial Ca^{2+} response to an InsP3 linked agonist. The finding was naturally exciting, but was soon challenged by a study in which no differences with respect to wild type were found in Ca^{2+} uptake by mitochondria of liver, kidney, and muscle of $UCP2^{-/-}$ and $UCP3^{-/-}$ mice [115]. Therefore, the identification of the mitochondrial uniporter with the UCP2 and UCP3 proteins must be considered as doubtful at best.

Excitement was also generated in 1992 by a report that described the purification and reconstitution in liposomes of the Na^+/Ca^{2+} exchanger of beef heart mitochondria [108]: the fraction reconstituted in liposomes supported Na^+/Ca^{2+} exchange activity, which was monitored with the help of Na^+ and Ca^{2+} sensitive probes. The reconstituted exchange activity was inhibited by TPP^+ and by diltiazem, as expected of the mitochondrial transporter. Polyclonal antibodies against the protein fraction that supported the reconstituted exchange activity reacted with a protein of molecular weight 110 kDa [116]. The Na^+/Ca^{2+} exchange activity of the 110 kDa protein was studied in some more detail in a later report by one of the authors of the 1992 paper [117]. However no other studies on it have appeared. Very recently, however, a study has provided convincing evidence that a mammalian member of a phylogenetically ancestral branch of the Na^+/Ca^{2+} exchange superfamily, the NCLX [118] could be the mitochondrial Na^+/Ca^{2+} exchanger [119]. The study has shown that the NCLX is enriched in mitochondria, where it localizes to the cristae. It has also shown that the Na^+/Ca^{2+} exchange activity of mitochondria is enhanced by the overexpression of the NCLX, and reduced by silencing its expression. Finally, the NCLX-mediated mitochondrial Na^+/Ca^{2+} exchange is inhibited by CGP 37 157, and inhibited in cells overexpressing an inactive mutant of the NCLX.

To complete the discussion on the molecular nature of mitochondrial Ca^{2+} transporters one recent report must be mentioned [120] which described the search for genes that regulate mitochondrial Ca^{2+} and H^+ transport using a genome-wide *Drosophila* RNAi screening. The search led to the identification of a protein (Letm1) with no appreciable homology to bacterial and plant Ca^{2+}/H^+ and Ca^{2+}/Na^+ exchangers that mediated Ca^{2+} uptake coupled to H^+ extrusion in mitochondria of permeabilized S2 cells. Knocking down Letm1 markedly reduced the uptake of Ca^{2+}. The Letm1 protein was expressed in bacteria, purified, and incorporated into liposomes: it supported the rapid accumulation of Ca^{2+} coupled to the efflux of H^+. Surprisingly, however, the exchange was blocked by RU360, and partially inhibited by CGP 37157. The finding was puzzling, as the insensitivity of mitochondrial Ca^{2+} exchangers to ruthenium red has been established beyond any reasonable doubt. The finding that the Ca^{2+} influx into the reconstituted liposomes demanded a negative inside membrane potential and was inhibited by RU 360 was also puzzling, and more in line with the operation of the uniporter than of a Ca^{2+}/H^+ exchanger. Thus, it seems fair to conclude that the real functional nature of the Letm1 protein is not yet conclusively established.

7. A few final comments

As I had stated in the first paragraphs of this contribution, the story of the encounter of mitochondria with Ca^{2+} is poorly known. With very few exceptions, the accounts in the literature offer an incomplete, and often distorted, picture of its beginnings and early development. In looking for the reasons, it seems clear that the long period of dormancy that followed the early period of excitement during which practically the entire phenomenology of the field was established had something to do with it. The reasons for the long period of essential neglect have been discussed in the pages above, but it is perhaps necessary to add that a factor that contributed to it was the mounting excitement created by the findings on the Ca^{2+} regulation by endoplasmic reticulum: here we had a beautiful system, molecularly and functionally defined, that had the proper affinity for Ca^{2+} and the appropriate rate of transport: it thus had all credentials to demand center stage in the area of cell signaling: endoplasmic reticulum and his Ca^{2+} controlling systems therefore became the real topic to work on in Ca^{2+} signaling. Mitochondrial Ca^{2+} transport had definitely lost momentum, and when it eventually bounced back in the 1990s the old literature had become forgotten or at least hard to consult, as a result of the explosion of the internet literature.

Hopefully, the account offered in the pages above, told by someone who has been there from the very beginning, will be found useful. If I were to put down the essence of the story in a nutshell, I would tell it as follows: practically all we now know of the properties of this exciting and important topic had been established in the pre-oblivion days. That included the demonstration that the process of mitochondrial Ca^{2+} transport did occur efficiently *in vivo*: which was a physiological nonsense, considering the inadequate Ca^{2+} affinity of the system. The way out of the nonsense was only found in the 1990s, when the Ca^{2+} micropools concept reconciled the poor affinity of the mitochondrial uptake system with its demonstrated ability to control cytoplasmic Ca^{2+}. From that moment on mitochondrial Ca^{2+} rapidly regained the attention of the old days: it is now alive and well again.

One last comment is in order at the end of this presentation: reports keep appearing claiming that mitochondria contain a number of extraneous proteins that are known to be linked to the control of Ca^{2+} elsewhere in the cell, from the ryanodine receptor to the SERCA pump. These and other reports have not been discussed in the pages above since no general consensus appears to have been reached on their reality and role.

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