Amarcord: I Remember

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Ja, Kalzium, das ist alles!–Otto Loewy (1959)

I have tried to offer a historical account of a success story, as I saw it develop from the early times when it interested only a few aficionados to the present times when it has pervaded most of cell biochemistry and physiology. It is of course the story of calcium signaling. It became my topic of work when I was a young postdoctoral fellow at The Johns Hopkins University. I entered it through a side door, that of mitochondria, which had been my area of work during my earlier days in Italy. The 1960s and 1970s were glorious times for mitochondrial calcium signaling, but the golden period was not going to last. As I have discussed below, mitochondrial calcium gradually lost appeal, entering a long period of oblivion. Its fading happened as the general area of calcium signaling was instead experiencing a phase of explosive growth, with landmark discoveries at the molecular and cellular levels. These discoveries established that calcium signaling was one of the most important areas of cell biology. However, mitochondria as calcium partners were not dead; they were only dormant. In the 1990s, they were rescued from their state of neglect to the central position of the regulation of cellular calcium signaling, which they had once rightly occupied. Meanwhile, it had also become clear that calcium is an ambivalent messenger. Hardly anything important occurs in cells without the participation of the calcium message, but calcium must be controlled with absolute precision. This is an imperative necessity, which becomes unfortunately impaired in a number of disease conditions that transform calcium into a messenger of death.

The village where I was born is close to the Slovenian border in the Italian province of Friuli. This is an enclave of about 700,000 people who speak an ancient and beautifully musical language that bears little resemblance to Italian. My father, Umberto, was the doctor responsible for the people of the village and of some smaller communities. My mother was a school teacher. To marry my father, she had come to the village from the cosmopolitan city of Trieste, one of the pearls of the Austrian empire. At the age of 23, she had already given birth to three children and had given up teaching to stay at home to tend and raise them. My mother’s given name had an interesting history. Her father had emigrated to Trieste at the end of the nineteenth century from the Friulian village where my father was also born. He was penniless but had a strong drive and uncommon abilities, and in a short time, he had managed to create a small commercial empire. He had evidently maintained strong pro-Italian feelings, so, when in 1912 my mother was born, he had decided to name her “Cirene” to celebrate the conquest of the Libyan city of Cirene by the Italian army, which at that time was trying to wrestle Libya from the Ottomans. However, in 1912, Trieste was still Austrian, and the civil officer had refused to accept such a blatantly pro-Italian name. My grandfather had thus decided on “Margherita,” which was the name of the queen mother of Italy, so my mother’s official name had become Margherita. To everybody, however, she remained Cirene. At the time of my birth in 1932, Friuli was a sleepy agricultural region that suffered from poverty and emigration. Unlike most villagers, we lived in a large house with a stable and a kitchen garden, where we grew all sorts of vegetables, yet our house
lacked heating or running water, and we had to walk for water to the fountain in the square. The village had a grade school but no high school; for that, one had to go to boarding schools, generally run by priests, in the capital city of Udine. In 1932, with the Fascist regime enjoying its peak of popularity, the teaching programs in grade schools were heavily imbued with its propaganda. To us children, of course, the militaristic rhetoric, with uniforms, fake arms, and physical exercises on Saturdays, was great fun.

Italy entered the war when I was about to begin second grade, and by the time I was ready for high school, the situation had deteriorated dramatically. The German army had occupied Italy after the proclamation of armistice by the Italian government in 1943, and Friuli had actually been incorporated into the Third Reich. Life had become very difficult, also because our village was next to a military airport, which was bombed nearly every day (or night). We had built a bomb shelter in our kitchen garden and spent night after night in it. The idea of sending me to a boarding school had of course to be abandoned, so my private teacher for the first three years of high school was the chaplain of our village church. He had studied mathematics at a university but had been crippled by a bone disease and had decided to become a priest. He was a man of culture: apart from mathematics, he had profound knowledge of Latin and scientific matters. His lessons must have been effective because, at the end of the war, I had no problems in passing the admission exam for senior high school and was sent to a boarding school in Udine run, as I wrote, by priests. I was there (with some interruptions) for five years. Because my interests were mainly in the humanities, I chose the classic high school line. Unlike many of the colleagues who have written Reflections articles, I was not interested in collecting crystals, nor did I ever attempt to set up a chemistry laboratory at home during the vacation periods. As years went by, however, I decided to become a medical doctor. This decision was motivated in large part by tradition because doctors had been common in my family for generations. After the “Matura,” I thus enrolled in the School of Medicine of the University of Modena, which was the city where my father’s family had originally come from. Modena was not one of Italy’s premier universities, the city being far more famous for gastronomy (balsamic vinegar was invented there) and, above all, as the place where the Ferrari and Maserati sport cars were built. When I enrolled, I was determined to become a “real” medical doctor, and the idea of a research career had never crossed my mind. I was conscientiously attending the preclinical courses, and I passed the first ten or so mandatory exams. Then, at the beginning of the third year, something happened. While attending the general pathology course given by a new professor, Massimo Aloisi, my determination to become a practicing doctor suddenly started to waver. Day after day, my fascination with the lectures of this charismatic teacher was increasing. He was taking us into the cell, discussing its functional mechanisms and the ways in which diseases would change them, something I had not heard in any of the other courses. One day at the end of his lecture, I asked him whether he would accept me as an internal student in his Institute of General Pathology. He said yes, and the answer changed my life. Aloisi was a man of enormous charm (Fig. 1) and a first-rate intellectual whose culture spanned from science to philosophy and all the way to the humanities, and it showed in his lectures. He was a convinced Marxist who had spent time in fascist jails, and that, for young students in post-war Italy, was an additional point of attraction. In the 1930s, he had done research in the laboratory of Otto Warburg in Berlin and in that of Earl J. King in London, and he had become a top leader in the Italian cell biology/biochemistry scene. His research topic was the biochemistry of muscular dystrophy, an area in which he had made important contributions. However, when I joined his institute, he had probably passed his scientific prime and was becoming more and more interested in philosophical and political issues. I had never been part of a research institute, but I immediately found the atmosphere very much to my liking. Of course, I had to learn the ABCs of laboratory life, from the preparation of solutions, to pipetting, to the now forgotten routine methods of analytical chemistry such as the Kjeldahl determination of nitrogen or the glorious Fiske-Subbarow assay for phosphate. Especially, I had to learn practical histology, as Aloisi had asked me to assist him in his work on the negative birefringence of microscope slices of rabbit dystrophic muscles. The first histology slides I had prepared from paraffin-embedded samples of rabbit dystrophic muscles made us very excited, as they showed numerous deposits of negatively birefringent crystals. Aloisi immediately interpreted these crystals as the deposits of “sick” proteins, but the excitement was unfortunately short-lived: we soon found that the crystals were not sick proteins, but paraffin deposits. The finding brought my first scientific project to an inglorious end and was of course a big letdown: mitigated only partially by the observation that the “artifact” occurred only with dystrophic muscles. Eventually, Aloisi still managed to have the useful artifact published in a rather prestigious British journal (1).
A few months after I had joined the institute, a young assistant by the name of Giovanni Felice (Licio) Azzone arrived from Rome, and Aloisi had put me to work with him. We made a small research team in which I had as colleagues Alfredo Margreth and Umberto Muscatello, who have remained my friends ever since. The approach of Azzone was decidedly biochemical, and I discovered that hunting down molecules using biochemical means was more exciting than looking through a microscope. At that time, I knew next to nothing about biochemistry and thus had to steal time from the experiments and the lectures to digest every single page of the newly published biochemistry textbook by Joseph Fruton and Sofia Simmonds, which was the biochemical bible of the time. With Azzone as my new supervisor, I kept working on the biochemistry of muscle proteins, including those from dystrophic muscles. We published some of our results in *Biochimica Biophysica Acta* (BBA) (2), which made us very proud. However, after a while, Azzone felt that muscle biochemistry, at least in the ways it was pursued in the 1950s, was sort of boring. He had cast his eye on the much more fashionable topic of bioenergetics, and thus, we timidly started working on mitochondria, with the idea of studying them in dystrophic muscles. It took some time before we mastered their isolation in a functionally competent state from the livers and hearts of rats, but by the time I was approaching graduation, we had become reasonably competent mitochondrial professionals. We had none of the modern sophisticated equipment that big laboratories used in studying these organelles. All we had was a venerable Warburg respirometer, but we did our best with it. We focused on muscle mitochondria, which at that time were not routine study objects, and we managed to publish papers that had some merit (3, 4). In the meantime, my interest in practical medicine had all but evaporated, and I decided that the only branch of practical medicine worthy of consideration was psychiatry. When I obtained my M.D. degree in 1957, I thus decided to give psychiatry a try, and I accepted a junior assistant position at the Neuropsychiatric Clinic. Unfortunately, that was before the arrival of psychoactive drugs, which meant that the only weapons against serious psychiatric disorders were electroshocks and insulin comas. In a short time, therefore, I became convinced that there was no real science in the psychiatry of those days, and after about two years, I decided to return to the safe haven of the Institute of General Pathology. Aloisi took me back without a grudge, supporting me with a fellowship of the Muscular Dystrophy Association of America, and I went back to my beloved mitochondria. Shortly after my return, however, Aloisi moved to the University of Padova, and I stayed in Modena with his successor, Paolo Buffa. Azzone had gone to the Wenner-Gren Institute at the University of Stockholm, where he rapidly became an international authority on mitochondria. While attending the Fifth International Congress of Biochemistry in Moscow in 1961, he thus recommended me to Albert Lehninger as a potential postdoctoral fellow. Lehninger’s lab would have been a top choice for anyone wanting to...
become a professional “mitochondriac,” but at that time, I had also become very interested in the work of Phil Siekevitz at The Rockefeller Institute and had applied to him for a postdoctoral position. The situation was obviously embarrassing, but a few weeks later, the embarrassment was happily resolved by Siekevitz’s reply that, regrettably, he had no space for me. I thus wrote to Lehninger, who confirmed that he would be happy to have me join his laboratory beginning in the summer of 1963. He sent me a nice letter of support, which I used in my search for a fellowship. A few months later, I was awarded a Fogarty International fellowship and thus was set to go. As I was waiting for the time to set sail for the United States, I kept working on muscle mitochondria, focusing on the changes induced by denervation. I was now more or less on my own but had one or two students as helpers, and I collaborated with other members of the institute. The results I obtained were rather interesting (5), but I was still a real greenhorn when it came to understanding the ways science functions in the real world. For instance, when I decided to publish some results on the changes of pigeon breast mitochondria following nerve section, I picked a journal more or less at random. It happened to be Nature, but to me it could equally well have been any other journal. Incredibly, the paper was accepted in only a couple of weeks without a single change (6). I was happy of course, but only moderately so, as it took years before it dawned on me that publishing in Nature was the unfulfilled dream of many of my colleagues. A few weeks before my scheduled departure, I married Annamaria Benucci, my sweetheart since my student days, and then at the end of August, I left for Baltimore. She was to join me later that year.

I knew very little about America, and my spoken English was primitive at best. I remember stopping in awe in front of the domed Johns Hopkins Hospital on my way to the Department of Physiological Chemistry, which occupied two floors of a building at 725 North Wolfe Street. Lehninger was not there the day I arrived, so Peggy Ford, his secretary, gave me a thick folder describing the projects in progress in the laboratory. She introduced me to some of the colleagues who would later become my good friends, and I started talking to them. I met Lehninger the next day, and it was quite an emotional moment: here I was, talking to a stellar figure in biochemistry, a myth to everybody working in bioenergetics (Fig. 2)! He told me about the various research programs of the department, saying that I could take all the time I needed to choose one. However, he added that the transport of calcium by mitochondria might be a wise choice because, he said, this topic had a great future. He went on talking about the work of Frank Vasington, who had just discovered the process. I told him that I already knew about the mitochondrial calcium work (I had read Vasington’s Journal of Biological Chemistry (JBC) paper in Italy) and that I was indeed thinking of working on it. “Good decision,” he said, and he then added that a gifted Italian postdoctoral fellow by the name of Carlo Stefano Rossi had been working on the project for over a year. Rossi was about to return to Padova, but he would help me getting started. And then, as I was about to leave the office, Lehninger nonchalantly said, “By the way, in about two months, I will go to Italy for a six-month sabbatical.” He must have noticed the disappointment in my face because he quickly added that I needn’t worry because he would be in constant touch with me by mail, and he would leave his technician, a graduate student, and two Ph.D./M.D. students in my hands. And with that, my scientific fate was sealed: calcium would remain my intimate companion for the rest of my professional life.

I started working the next day, getting used to equipment I did not know, including γ-scintillation counters. I intended to check whether mitochondria could also han-
dle strontium, for which \( \gamma \)-emitting isotopes were readily available. Rossi was very helpful, and so were all of the other members of the lab: Jack Greenawalt, Paulette and Pierre Vignais, Gerry Gotterer, Bob Glaze, Jim Mattoon, and Simon Van den Bergh. I put all of the energy I could muster into the work. I had daily discussions with Lehninger, which proved to be an invaluable bonus: it was then that I became aware of his all-encompassing knowledge and his amazing knack for interpreting results and suggesting ways to proceed. My wife, Annamaria, was due to arrive in November, so for two months, I worked with ferocious intensity on at least three simultaneous projects. By the time Lehninger left for Europe, we were ready to submit a paper on the anion and cation balance in mitochondria during the uptake of calcium. I had put together a draft of the paper just before he left, and what followed after three or four days was what all of us postdoctoral fellows came to call the process of “lehningerization”: going to him with a disjointed draft of a paper, only to receive it back, impeccably rewritten, after a couple of days. He was not only a superb writer but also incredibly well organized and efficient. Sure enough, my draft came back from Italy in no time, ready to be sent off to JBC. It was accepted with only minor amendments. It was not my best paper from Johns Hopkins, but it was my first in JBC: something never to be forgotten.

In those “pre-Mitchellian” days, all bioenergeticists of any status were engaged in the search for their Holy Grail: the high-energy intermediate of oxidative phosphorylation, the now infamous “squiggle.” I still remember the long hours I had spent in Italy on a forty-three-page review that Edward C. (Bill) Slater had published in 1958 (7), in which he had discussed in excruciating detail whether, for instance, the intermediate of oxidative phosphorylation was DPN~H or DPNH~H (in those days, NAD was still called DPN for diphosphopyridine nucleotide). Even though this review was a cult piece among bioenergeticists, it was very difficult to get hold of, as it had been published in an obscure Australian journal. I had been so proud to get hold of a copy! Of course, we now know that the squiggle as a chemical entity was a myth and that the countless discussions around it were nonsense. However, this truth only became established later, when a relatively unknown British bacteriologist by the name of Peter Mitchell (Fig. 3), armed mainly with a secondhand pH meter, performed a few embarrassingly simple experiments that swept all that nonsense away. Lehninger’s very large lab was of course also going after the mythical intermediate. However, the department also housed other research groups that did not work on mitochondria. Several colleagues destined to rise to prominence, such as Bill Lennarz, Ed Heath, Tom Thompson, and Gary Ackers (and others later on, including Dan Lane and Paul Englund), performed first-class work on general topics of enzymology, on biological membranes, on carbohydrates, and, especially, on lipids. They occupied the fifth floor of the department, whereas we “phosphorylators” occupied the fourth floor. In essence, the department was split into two echelons: we phosphorylators were the “nobles,” working on the most exciting topic of the time, whereas the fifth floor colleagues were the “grease people.” There was nothing personal in these derisory definitions: friendships were still there, but we phosphorylators certainly considered ourselves the elite. Now, decades later, it still surprises me that we did not see the cloud looming on the horizon. Signs of the impending chemiosmotic catastrophe were there if one only cared to see them, but even the most obvious indications, such as the findings we and others were making on the transport of calcium by mitochondria, were somehow forced to fit into the framework of the chemical theory of oxidative phosphorylation.

While Lehninger was away, I managed to complete a number of experiments on the transport of strontium by
liver mitochondria. True to his promise, Lehninger was in constant mail contact with me, and the strontium project eventually ended up with four articles in BBA (8–11). Drafts were exchanged between Baltimore and Italy, with the lehningerization process being particularly laborious with the draft of the fourth paper. It described the effects of strontium on the swelling of mitochondria, which, very interestingly, were opposite to those of calcium. I had asked Arnold Caplan, the graduate student who had performed the work, to prepare the draft. Caplan, who was to become a prominent bone biochemist at Case Western Reserve University, had produced a sixty-page affair, which Lehninger, who by then had returned to Baltimore, mercilessly reduced to fewer than ten pages! Meanwhile, a number of other postdoctoral fellows (Zdenek Drahota from Prague, Czech Republic, and Józef Bielawski from Poznań, Poland) had joined the lab, and our work on mitochondrial calcium transport gathered speed. It was a very exciting period, as this process had become a hot topic, with numerous laboratories working on it. Lehninger’s lab had of course retained interest in the main bioenergetics topic of the day, which was the mechanism of oxidative phosphorylation. André Goffeau (Fig. 4) and Pete Pedersen, with whom I developed a long-lasting friendship, were working on it, but Lehninger’s interest in calcium was rapidly growing. We turned out paper after paper and gradually started to see that our observations were in line with the basic concepts of the chemiosmotic theory, which was laboriously gaining ground among bioenergetacists. We established the most important properties of the calcium uptake process, among them the distinction between the limited and the massive uptake. The former showed clearly that calcium was taken up as an alternative to the synthesis of ATP and that it was maintained within mitochondria in a dynamic steady state that did not damage the organelles but made calcium available to exchange with the cytosol (12). We also found that mitochondria took up adenine nucleotides alongside calcium, the process being essential for long-term maintenance of calcium in the matrix (13). Naturally, we had all become convinced that mitochondria had an essential role in the regulation of cytosolic calcium, and I remember how emphatic I was in defending the proposal at a number of symposia I had started to attend. However, there was something disturbing: we and others had found that the affinity of the uptake process for calcium was pitifully low, an observation that was hard to reconcile with the accepted submicromolar concentration of cytosolic calcium. Then, at about that time, Denton, McCormack, and their associates in Bristol found that three dehydrogenases participating in the tricarboxylic acid cycle in the mitochondrial matrix were regulated by calcium (reviewed in Ref. 14). Much to our disappointment, the idea thus gradually gained momentum that the process of calcium uptake by mitochondria was irrelevant to the regulation of cytosolic calcium, its only role being the regulation of calcium within the mitochondrial matrix. As a result, when the two years of my fellowship were about to expire, the excitement for the topic of mitochondrial calcium had started to decline. For me, it was time to decide on whether to go back to Italy or to stay in the United States. Britton Chance had sounded me out on a very attractive position in Philadelphia, and I had trouble making up my mind. The work at Johns Hopkins had gone very well, and Annamaria and I had been very happy in Baltimore and had established lasting friendships there. However, I felt I owed a debt to my home country, and we eventually decided to return to Italy. The position I had had in Modena was still available, and I resumed it. Of course, it was a bit of a shock to compare the conditions I had enjoyed at Johns Hopkins with those I had to cope with now. However, I managed to buy an Oxygraph, and the essential equipment for working on mitochondria was available in the institute. I had some enthusiastic students who looked with great admiration at the young man who had published several JBC papers and had made contributions to a hot topic. I was still convinced

FIGURE 4. With André Goffeau in a laboratory at Johns Hopkins in 1964. I was still using a slide ruler to calculate something. Decades were to pass before computers invaded research laboratories. The woman in the background working on the Oxygraph was Paulette Riley, the technician Lehninger had assigned to me at that time.
that, despite the low calcium affinity problem, the process of mitochondrial calcium uptake had a significant \textit{in vivo} function and was set to prove my point. In those days, it was possible to use radioactivity in animals without too much red tape, so I injected a radioactive calcium isotope into the peritonea of rats and proceeded to fractionate their livers. To my great delight, a large portion of the injected radioactive calcium was recovered in the mitochondrial fraction (Table 1). Most importantly, the amount of calcium associated with mitochondria dropped dramatically in the livers of rats that had been pretreated with uncouplers of mitochondrial oxidative phosphorylation (15), a convincing proof that the process of energy-linked calcium transport by mitochondria did indeed take place \textit{in vivo}! How this finding could be reconciled with the low affinity of the mitochondrial calcium "uniporter" was an enigma, which was to remain unsolved for twenty-five years, when it was demonstrated that, during cell activation, calcium released from the endoplasmic reticulum formed micropools of high calcium concentration around neighboring mitochondria that satisfied the poor affinity of the organelles’ calcium uniporter (16). I then extended these findings to heart and skeletal muscle mitochondria, but I also kept collaborating with Lehninger on various aspects of mitochondrial calcium transport. I even returned to Baltimore for six months in 1968. By then, it had become clear that the transport of calcium by mitochondria provided strong support for Mitchell’s chemiosmotic theory, and Lehninger was investing increasing time and resources on it. Calcium transport and calcium signaling in general were becoming more and more popular among biochemists, even though the emphasis shifted from mitochondria to more general aspects of the process, in particular cellular signal transduction. During my second stay at Johns Hopkins, we discovered that yeast mitochondria were unable to transport calcium (17). It was a neat finding, but at that time we did not consider it so important. However, we were wrong: forty years later, the finding was resurrected as a key element in the work that led to the molecular identification of the calcium uptake uniporter (18, 19).

Over the next few years in Modena, I kept working on mitochondria and calcium. My laboratory had become larger and better known, and colleagues even joined me from abroad as sabbatical visitors. One of them was Frank Vasington, with whom we did interesting work on the inhibition of mitochondrial calcium uptake by ruthenium red. Then, in 1973, we hit on an important finding. The missing link in mitochondrial calcium transport was the mechanism for calcium release, and we discovered that this release was triggered by sodium. I had thought of sodium simply because its coordination chemistry had similarities to that of calcium and also because I was impressed by the recent discovery of a sodium/calcium exchange system in the plasma membrane of heart cells and neurons. The idea of similarities between the coordination chemistry of sodium and calcium may have been naïve, but it worked for us. External sodium indeed released mitochondrial calcium, and it did so at concentrations known to exist in the cytoplasm (Fig. 5). Potassium, cesium, and rubidium did not release calcium. However, lithium did, which was a clear demonstration that the mitochondrial system was different from the sodium/calcium exchanger of the heart membrane, which Reuter and Seitz (20) had found not to accept lithium. We had thus discovered the missing step in the mitochondrial calcium transport cycle, and I was very excited. Because the experiment had been performed on heart mitochondria, I speculated that our findings could also explain the mechanism of digitalis, which is known to increase cytoplasmic sodium in the heart and was suggested to reverse the calcium extrusion operation of the sodium/calcium exchanger of the plasma membrane. Thus, I decided to send the paper to a molecular cardiology journal (21), rather than to JBC, which would have been the obvious choice. As a result, the paper got less recognition than it would have deserved from a biochemical readership, and cardiologists ignored mitochondrial calcium release as a mechanism for digitalis.

At about the same time, at a symposium at Lake Balaton in Hungary, Giorgio Semenza, a professor of biochemistry at the Eidgenössische Technische Hochschule (ETH) in Zürich, told me that his institute was looking for another professor of biochemistry. Might I be interested? I felt flattered. How could anyone feel otherwise, given that he was talking about the famed ETH, and I replied that I was indeed interested. The idea of leaving Italy had actually

**TABLE 1**

**Distribution of injected radiocalcium in the subcellular fractions of rat liver**

$^{45}$Ca$^{2+}$ (10 μCi) was injected intraperitoneally 6 min before the death of the animal and 3 min before the injection of pentachlorophenol (PCP; 20 mg/kg). Liver subcellular fractions were separated with a conventional fractionation scheme. Data are given as means ± S.E. The number of experiments is in parentheses. For full details, see Ref. 15, from which this table has been adapted.

| Fraction       | Control       | Rat pre-injected with PCP |
|----------------|---------------|----------------------------|
|                | %             | %                          |
| 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 ± 0.28 (9)| 12.8 ± 1.65 (3)            |
| Microsomes     | 15.3 ± 2.90 (9)| 30.3 ± 0.46 (3)            |
| Supernatant    | 2.5 ± 0.38 (9)| 13.2 ± 0.52 (3)            |
been with me for a while. I had become increasingly impatient with the limitations of the Italian research system and kept telling myself that I had repaid my debt to Italy. I first wanted to talk it over with my wife, who also was at the symposium. That evening, we talked for a long time. Moving to a German-speaking environment would not be easy, especially because we now had two small boys, but we eventually felt the idea had many positive aspects. Thus, I went to Zürich for the routine seminar, interviews, and negotiations. It all went well, and I was offered the position. Albert Claude had been one of my sponsors; I had met him during a visit to the Johnson Foundation in Philadelphia and on several other occasions after my return to Italy (Fig. 6), developing a friendly relationship. In September 1973, I moved to Zürich, with my family joining me a few months later. My new institute occupied the top four floors of the newly built twelve-story chemistry building, within easy walking distance of the town center. Zürich is a hilly city, and the building was up on a hill, offering a breathtaking panorama of the city and the distant Alps. The working conditions were nothing less than superb: I had one and a half floors of the building at my disposal, plenty of money to buy equipment, and three permanent positions to fill, with the promise that I would inherit a fourth one from Carl Martius, who would retire about two years later. I started low key, with one technician and my Italian colleague Paolo Gazzotti as my only co-worker. However, I could rapidly secure funds from the National Research Council for some graduate students. We continued working on mitochondrial calcium transport, trying to become more molecular. I was in the right place to do it.

Our chemistry tower housed glamorous organic chemists such as Albert Eschenmoser, Duilio Arigoni, Vladimir Prelog, Jack Dunitz, Willy Simon, and others. Actually, at the time of my arrival, the relationship between the famed
Organic Chemistry Institute (it was still called “Laboratorium”) and the newer Laboratorium of Biochemistry was less than optimal due to historical misunderstandings that went back to the time when Leopold Ružička had selected Martius as the head of the newly established Laboratorium of Biochemistry. For myself, I felt that the proximity of all those top-level organic chemistry colleagues was a big asset because I intended to give a more molecular turn to the analysis of mitochondrial calcium transport. Thus, I rapidly developed excellent relationships with all my organic chemistry colleagues, particularly with Arigoni: our common language and a number of common cultural interests helped a lot. I daresay that I even managed to trigger their interest in the “bio” world as something that would nicely complement the straight chemical world in which they were the undisputed rulers. Thus, for instance, two years after my arrival, I organized with Simon and Jean-Marie Lehn (who was a frequent visitor to ETH) the Table Ronde Roussel on Transport Processes in Chemistry and Biology in Paris (Fig. 7).

In 1973, the essential phenomenology of mitochondrial calcium transport had been established, and the obvious next step was to take the system apart and isolate its components. A number of laboratories were busy doing just that; thus, we tried to identify the uptake uniporter. However, we did not get very far: a promising glycoprotein that we had isolated eventually turned out to be just one of several molecules of unknown function that other laboratories erroneously identified as the elusive calcium uniporter. In hindsight, it is now clear that our efforts were doomed. It was to take four decades and great conceptual and technological advances before the uniporter was eventually tracked down (18, 19). However, we also kept working on the properties of sodium-promoted release of calcium from mitochondria, and we characterized it as a process mediated by a novel exchanger, thanks to the work of a series of students led by Martin Crompton, a British co-worker that had joined the laboratory as an assistant (22). We described the properties of the exchange process in great detail, and our papers on this topic still contain all of the essential information on this process. We made no attempt to isolate the exchanger protein, and in hindsight, this was a sound decision, as it would probably also have been a doomed effort. Conceptual and technical advances had to occur that were not available in the 1970s and that only permitted the identification of the exchanger nearly forty years later (23). However, our discovery that the exchange process was responsive to lithium turned out to be an essential element in the work on the identification of the exchanger.

As already mentioned, by the end of the 1970s, interest in mitochondrial calcium transport had diminished significantly, the chief reason being the low affinity of the uniporter. Even if our work had shown that the process did
Occur in vivo (15), it was still widely considered to be irrelevant to the regulation of cytosolic calcium. This was of course a frustrating and disappointing experience. On the other hand, interest in the general role of calcium in cellular signaling was increasing exponentially, thanks to landmark contributions on the role of calcium in the regulation of cell activities. Both the discovery and characterization of calmodulin (24), as well as that of calcium transport by the endo/sarcoplasmic reticulum, contributed to this new interest. The ATPase (the calcium pump) mediating calcium uptake by the endoplasmic reticulum (the SERCA pump) was studied with great impetus in numerous laboratories, and David MacLennan had succeeded in purifying it (25). I had been increasingly thinking of moving to a calcium transport system less controversial than that of mitochondria, and the calcium ATPase of the endoplasmic reticulum seemed a very attractive topic. Unfortunately, the field was quickly getting crowded and very competitive, hardly an ideal choice. I then read an article by Hans Jürg Schatzmann (26) on an ATP-dependent calcium transport system, i.e., a calcium ATPase, in mammalian erythrocytes. It was one of the ATPases that were later defined as “P-type pumps” (27) because they form a phosphorylated intermediate during the reaction cycle. I thus decided to work on the erythrocyte ATPase, trying to purify it by conventional procedures; a number of laboratories were trying to do just this, with disappointing results. Ours were not much better: we only managed to obtain a partially purified preparation of the pump. However, we made a finding that proved to be very important for our future work on the purified ATPase: the partially purified preparations showed activity only in the presence of phosphatidylserine or long chain polyunsaturated fatty acids. Zwitterionic phospholipids did not support activity (28, 29). Then, at about that time, two short papers appeared in which the erythrocyte pump was shown to be activated by calmodulin (30, 31). John Penniston, whom I knew from the days he was still working on mitochondria, was the author of one of them. He came to Zürich for a visit, and we decided to team up and to exploit the activation by calmodulin to purify the pump by means of calmodulin affinity chromatography. One of my postdoctoral fellows, Verena Niggli, went to Penniston’s lab at the Mayo Clinic, prepared the calmodulin column, and in no time purified the erythrocyte pump in an active state (Fig. 8) (32). It was a significant achievement also because the calmodulin column, which is now a routine procedure for calmodulin-dependent proteins, was at that time a novel procedure. However, there was a property of the purified pump that we should have expected in view of our previous finding on the activating role of acidic phospholipids (28, 29): the purified ATPase was no longer activated by calmodulin. The solubilized erythrocyte ghosts had been applied to the calmodulin column in the presence of phosphatidylserine, which evidently had already activated the pump to maximal levels. The activation by calmodulin, as was to be expected, returned when the purification procedure was repeated in the presence of phosphatidylcholine (33); acidic phospholipids evidently activated the enzyme alternatively to calmodulin. This was not a trivial finding, as we calculated that the concentration of phosphatidylserine in the pump’s membrane environment was, in principle, adequate for about half-maximal activation (33). Despite the large amount of work performed in a number of laboratories over the next thirty years, the quantitative significance of the role of phospholipids in the function of

FIGURE 8. Purification of the calcium ATPase from human erythrocyte ghosts. Left panel, a Triton X-100 solubilizate of human erythrocyte ghosts was applied to a calmodulin-Sepharose 4B column in the presence of calcium and phosphatidylserine. The column was washed with a buffer containing both calcium and phosphatidylserine until no protein and no calcium ATPase activity were eluted. The buffer (arrow) was then replaced with one containing EDTA instead of calcium. The eluted protein was assayed for calcium ATPase activity (●) with a coupled enzyme assay spectrophotometric method. Right panel, an aliquot of the EDTA eluate, treated as described (32), was applied to an 8% polyacrylamide gel. TD, tracking dye. Full details are provided in Ref. 32), from which this figure has been adapted.
the pump in the cellular environment is still an open question.

There is an anecdotal aspect of our work on the purification of the pump that is perhaps worth mentioning. As we were preparing the paper that was eventually published in JBC, a colleague whom I shall not name and who had tried in vain to purify the pump visited us in Zürich from abroad. Verena Niggli and I told him about our successful method and showed him all of our experimental protocols in full detail. Three or four weeks later, I was asked by FEBS Letters to referee a manuscript in which this very same colleague described exactly our purification procedure. I was shocked of course and immediately phoned Prakash Datta, who at that time was the managing editor of the journal, explaining to him what was happening. Datta was equally shocked and sent the mischievous colleague a not-so-kind letter explaining why his paper was rejected.

The purification of the pump was the beginning of a long collaboration with John Penniston and his group. The availability of the purified pump led to the detailed definition of biochemical and molecular biology properties of this interesting protein. Here, I only mention the most important findings, as a number of comprehensive reviews offer a complete and detailed panorama of what is now known on the pump (34, 35). The pump of animal cells (acronym PMCA for plasma membrane Ca\(^{2+}\)-ATPase) is the product of four genes. Two of the gene products are ubiquitous (PMCA1 and PMCA4), and two are restricted to some tissues, particularly the nervous system (PMCA2 and PMCA3). PMCA1 is the housekeeping isoform, which ejects calcium from all animal cells. PMCA4 was also considered a ubiquitous isoform, but more recent work has shown that it has specific functions in some cell types, e.g., the spermatozoa (36, 37). Each of the four primary gene transcripts undergoes alternative splicing, increasing the number of pump variants to about thirty. Some of the splice variants acquire properties that are uniquely suited to satisfy the specific calcium homeostasis demands of specialized tissues/organs. A good example of this is the splicing pattern of the PMCA2 transcript in the organ of Corti in the inner ear (38–40). The pump was predicted to be inserted into the membrane with ten transmembrane domains, and this prediction was supported by cloning and sequencing of the corresponding gene in 1988 (41, 42). The sequence showed that the pump has a long cytoplasmic C-terminal tail, unique among all other pumps of the P-type family that contains the calmodulin-binding sequence. Some splice variants of the pump also contain a second domain farther downstream from the first that binds calmodulin with much lower affinity (43). In addition to calmodulin, the PMCA pump is a target of other regulatory mechanisms. Acidic phospholipids have already been mentioned, but phosphorylation by different kinases, a dimerization process, and the proteolytic cleavage by calpain that removes the cytoplasmic C-terminal tail all decrease the apparent \(K_m\) of the enzyme for calcium. The pump is regulated by autoinhibition: the calmodulin-binding domain and the C-terminal tail fold over and bind to two sites next to the pump’s catalytic site (44, 45), keeping it inhibited until calmodulin removes them, relieving the autoinhibition.

The PMCA pump ejects calcium from all animal cells, but in excitable cells such as cardiomyocytes and neurons, its calcium extrusion function is overshadowed by the much more active sodium/calcium exchange systems. The demonstration that a PMCA pump indeed exists in heart cells was thus completely unexpected (46). However, in excitable cells such as those of the heart, the calcium extrusion function of the PMCA pump is not quantitatively significant. It is instead highly significant for the regulation of calcium signaling in selected microdomains within the cell (47). In other words, the PMCA pumps may be more important to the regulation of intracellular calcium signaling than to the export of calcium from excitable cells such as those of the heart. A very recent development, for which the contribution of a very gifted co-worker, Danilo Guerini, has been essential, is the finding that the genes of the pump are differentially regulated, at least in neurons, by calcium itself (48). This emphasizes the concept of autoregulation of the calcium signal, which distinguishes calcium from all other signaling molecules.

The rapid autoregulation was of course implicit in the modulation of the activity of the PMCA pump by calcium/calmodulin, but the genetic regulation adds another dimension to it.

The calcium pump of the plasma membrane has been the main interest of my laboratory for over thirty years, and this was the reason why I have dwelt on it at some length. As the years went by, my laboratory became progressively larger: at its peak, it numbered thirty co-workers or more. It was thus possible to work on something else as well, even on something that had nothing to do with calcium. Thus, perhaps in memory of the times when I was a mitochondriac, I invested time in examining the pumping of protons by cytochrome oxidase. In the 1970s and 1980s, the existence of this process was a hot topic, as it contradicted the orthodox scheme of Mitchell’s chemiosmotic concept. Our work provided convincing support for the proposal of others for a proton-pumping mechanism,
which in the end forced a revision of Mitchell’s original and so elegantly simple chemiosmotic mechanism.

These were nice excursions, but the main theme of the lab of course remained calcium. As the work on the PMCA pump increased exponentially, so did worldwide interest in calcium signaling. Landmark discoveries were responsible for this upsurge: the increase in the number of fundamental cell processes that are controlled by calcium, the mechanisms for the transmission of the calcium signal to targets, the structural definition of proteins (first among them calmodulin) that decode the calcium signal, and the spectacular advances in the area of calcium-dependent protein phosphorylation and dephosphorylation. A place of honor belongs to the discovery in 1983 of inositol trisphosphate (InsP₃), as a messenger that links the action of plasma membrane agonists to the release of calcium from intracellular endo(sarco)plasmic reticulum deposits (49). Equally seminal was the solution of the three-dimensional structure of the calcium ATPase of the sarcoplasmic reticulum about twenty years later (50). Even though the main interest of our laboratory was still the PMCA pump, I had kept a keen interest in developments in other areas of calcium signaling and had for instance invested time on topics like the molecular aspects of calmodulin action and the structural/functional properties of the calcium-dependent protease calpain. I had also become increasingly active in the organization of international events in the general area of calcium signaling. A symposium I organized in Davos, Switzerland, in 1992 on calcium-binding proteins and calcium function was particularly successful (it was the eighth in a series I had initiated with Witold Drabikowski in Poland in 1973) (Fig. 9). Sometimes, I was even tempted to work again on mitochondrial calcium handling, but the situation there was discouraging. After the arrival of InsP₃ in the 1980s, the endoplasmic reticulum had relegated mitochondria to a back corner of cellular calcium regulation. Because I knew that energy-linked handling of calcium by mitochondria did occur in vivo (15), I kept hoping that one day these organelles would somehow bounce back. To my great satisfaction, this happened at the beginning of the 1990s, when Tullio Pozzan, Rosario (Sarino) Rizzuto, and their co-workers (16, 51) discovered that the low calcium affinity of mitochondria was a red herring. Somewhat ironically, it was InsP₃ itself that brought mitochondria back to center stage as regulators of cytosolic calcium. The InsP₃-promoted release of calcium from the endoplasmic reticulum creates micropools of high calcium concentration around neighboring mitochondria that satisfy the low affinity of the uniporter. Thus, mitochondria resumed a central role in the regulation of cell calcium, which I had always felt was their legitimate position.

Ever since the discovery of the release of calcium from heart mitochondria by sodium, I had maintained an interest in the role of calcium in the regulation of heart contractility, even though in those early days mitochondria
were thought not to have a major role in it. Here, the small hydrophobic protein phospholamban had an acknowledged important role, as it regulated the activity of the SERCA pump. This role interested me because the regulation of the SERCA pump had molecular similarities to the reversible autoinhibition of the PMCA pump by its C-terminal tail: we had already found cross-inhibition of the SERCA pump by the C-terminal tail of the PMCA pump and of the PMCA pump, from which we had removed the C-terminal tail, by phospholamban (52). I thus decided to invest time on phospholamban, involving several members of the laboratory in the project. In collaboration with Michihiko Tada, Peter James, and Micky Chiesi, I identified the binding site in the pump for the cytosolic portion of phospholamban (53). Thomas Vorherr achieved the total chemical synthesis of active phospholamban (54). It was the first active membrane protein synthesized by organic synthesis, but the achievement was important also because it provided large amounts of phospholamban for future research. Prior to our synthesis, only micrograms of the protein could be produced with great difficulty. Finally, in collaboration with Christian Griesinger in Göttingen in 2005, Joachim Krebs solved the NMR structure of phospholamban (55).

As the interest in the signaling function of calcium kept increasing, it became gradually recognized that the calcium signal is ambivalent (56). Cells could not possibly function without calcium signaling, but this process demands that the concentration and movements of calcium be temporally and spatially regulated with utmost precision. The concerted operation of membrane channels and transporters ensures the dynamic equilibrium of calcium between the extracellular environment, the lumen of intracellular organelles, and the cytosol. When this equilibrium becomes defective, calcium becomes a negative messenger, triggering a host of detrimental functions. These involve proteases, phospholipases, and nucleases, which are normally kept at bay by precise regulatory circuits. As a result of the disequilibrium, calcium becomes a messenger of doom. In other words, once cells have chosen calcium as a determinant for function, they live in a situation of permanent risk. When the control of cell calcium fails severely and suddenly, e.g. when massive amounts of calcium are admitted into the cytosol following damage to the plasma membrane, calcium rapidly triggers cell death. However, it has now become clear that the control of cell calcium may become distorted in more subtle ways. If this happens, cell life continues, albeit with various degrees of discomfort. The induction of cell death by calcium, the so-called apoptosis, is not necessarily a negative phenomenon. On the contrary, it is one of the positive ways in which the signaling function of calcium is used to safeguard cell renewal and organ modeling.

The area of abnormal calcium signaling in disease has now become impressively large (57). The disease conditions in which the calcium defect is the primary cause, rather than the consequence, are frequently genetic and may involve any of the different regulators of the calcium message. They may affect proteins involved in the buffering of calcium and/or the decoding of its message, such as annexins (58), gelsolin (59), the S100 proteins (60), and the protease calpain (61, 62). The defects may also affect membrane channels for calcium in both intracellular organelles and the plasma membrane: the genetic defects of voltage-gated plasma membrane calcium channels now define a large group of pathologies (the calcium channelopathies) (63). Finally, they may affect membrane transporters of calcium, essentially calcium pumps. Genetic defects of the SERCA pump cause Darier disease (64, 65), and those of the calcium pump in the Golgi membranes (secretory pathway Ca\(^{2+}\)-ATPase (SPCA) pump) cause Hailey-Hailey disease (66, 67). Genetic defects also affect the activity of the PMCA pump. I have expanded the work on the PMCA pump in the direction of pathology after having reached Emeritus Professor status at ETH Zürich. I returned to Italy in 2001, not to Modena but to Padova, as professor of biochemistry at the university (Italian professors retire at an unusually advanced age) and as the co-founder of a semiprivate institute, the Venetian Institute of Molecular Medicine (VIMM). Between 150 and 200 researchers now work there on the molecular etiology of various diseases. My laboratory at VIMM has recently identified and analyzed molecularly a genetic defect in isoform 2 of the PMCA pump in human hereditary deafness (68) and another genetic defect, this time in isoform 3 of the PMCA pump, in X-linked cerebellar ataxia (69). These new exciting forays have kept and still keep my interest in calcium as alive as in the times when I was a young post-doctoral fellow. Ages ago.

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