REFLECTIONS

How Enzymes Handle the Energy Derived from the Cleavage of High-energy Phosphate Compounds

Leopoldo de Meis

From the Instituto de Bioquímica Médica, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Rio de Janeiro 21941-902, Brazil

I was accepted at the Federal University of Rio de Janeiro Medical School when I was seventeen years old. At that time, I was absolutely sure I was going to be a surgeon. One year later, I received an offer for a fellowship to work in a research laboratory. This originally had not been part of my future professional plan, but I was desperately in need of a source of income, so I joined the research laboratory at the age of eighteen. During the medical school course in Rio de Janeiro, I lived the hormonal tempest typical of youth, and my certainties were shaken badly. I soon discovered that I did not have much of a future in either surgery or clinical practice, but my interest in science increased. I received my M.D. degree in 1961, when I was twenty-three years old, and it was time to decide what to do with my life. With a significant degree of uncertainty, I opted for research. Fortunately, I had excellent supervisors who smoothed out my rite of passage into science.

As a medical student in Rio de Janeiro, I worked with Walter Oswaldo Cruz (Fig. 1) in his research laboratory, and shortly after receiving my M.D. degree, I went to the National Institutes of Health (NIH) in Bethesda, where I had the good fortune to work with Herbert and Celia Tabor (Fig. 2) for eighteen months. When I returned to Rio de Janeiro, I was sure that my vocation was in science. I was not yet what one could call a scientist, but I had the basic skills to begin a career.

About Science and Scientists

My major interest throughout the years has been the mechanism by which enzymes interconvert different forms of energy. This involves the concept of high- and low-energy phosphate compounds and the way some proteins regulate how much of the energy available can be used to perform work and how much is converted into heat. When I first started to work in research, I thought of myself as a physiologist with some training in biochemistry. If someone had told me at that time that he worked in the subjects I have just described, I initially would have had difficulty understanding what he was saying. I would have believed that his work required solid physico-chemical training, which I did not have. I would also have thought that the application of this sort of research would be related only remotely to possible medical applications, to which I always hoped to contribute. This juvenile reasoning proved to be completely wrong.

Like any dedicated researcher, I have received some awards for my work, but the sources of these awards often have been unexpected. For example, once I was made an honorary member of the Brazilian Academy of Medicine, and I thought, "I am not ignored by the medical doctors." At home, when I announced that I was still an M.D. after all, my second wife (Fig. 3), my two sons, and my two daughters, who all practice in various medical fields, burst into laughter. I felt their reaction was cruel, but deep down, I had to agree with them because I was a researcher. I then

FIGURE 1. **Walter Oswaldo Cruz, my tutor during my initial period as a medical student.**

FIGURE 2. **Herbert and Celia Tabor, my tutors during my postdoctoral training at the NIH in Bethesda.** They taught me what biochemistry is.

received a nice international award for my work in chemistry, and I was later quoted in several articles published in journals of physics. No one at home laughed at me when I told them of these accomplishments; on the contrary, they said, "Bravo, Papa!"

FIGURE 3. **My second wife, Vivian Rumjanek, and me.** She is an immunologist. My first wife, Regina, was a geologist. We had two daughters and two sons, but unfortunately, she died at an early age.

As time passed and maturity quieted my emotions, I realized that the work itself is what is important, not whether it is categorized as medicine, chemistry, or whatever. When working, there are magic moments in which one suddenly unravels a new finding and has the sensation of understanding a small facet of nature. The magical moment has a short duration, and the new finding is cleared up and substantiated with controls. Then comes the tedious part: writing the paper, submitting it for publication, waiting to know if it has been accepted, and, at times, enduring the rudeness of a bad-tempered referee until finally it is published. At this stage, the magic has faded away, but one goes on struggling to discover something new in the hopes of once more feeling the sensation of a new discovery, no matter the field or how significant it may be. In this search, the main tool frequently is intuition (1). I think scientists are addicts of those glorious moments that follow discovery, and it is with the hope of feeling again the thrill of these moments that they endure long hours of work investigating whatever subject intuition suggests.

I have three major interests related to my profession: research, education, and social studies in science (1–3). I worked most of my life as a university professor and used to select my undergraduate students during my courses on medicine and biology. As my interest in education

increased, I started to organize short experimental courses in different Brazilian universities for young students at what would be the equivalent of the high school level in the United States. These young people live in the lower income neighborhoods of Rio de Janeiro periphery and cannot afford the proper education necessary to attend university. The experimental courses are totally free, and the best students are invited to work in the laboratory under the supervision of M.Sc. or Ph.D. students and eventually enter public universities, which, in Brazil, are free of charge. The students receive a small stipend and all the educational materials they need. Many of these students have graduated and gone on to earn doctorate degrees and have obtained good positions. The system has spread throughout the whole country, and the government is now allocating funds for this activity in the form of science grants. The writer Malcolm Bradbury, in a romance about a professor of history, wrote that although he grew older over the years, his students were always eighteen years old. I have found this to be true, but in my case, I would expand this from fifteen to twenty-five years old. This experience with young people was of great help in educating my own four children and led me to do active research in education and science sociology. In this article, I will refer only to my work in bioenergetics and leave the education affair for another opportunity.

From Italy to Brazil

I was born to an Italian musician's family. My grandfather was a member of the Scala in Milan and the San Martino Theater in Naples. My father played the cello (Fig. 4), and he did well until fascism rose to power in Italy. Because of his antifascist attitudes, he first had problems getting a job and later had to hide from the regime. After the war, the economic situation in Italy was chaotic, and my father decided to emigrate to Brazil and become a member of the Brazilian Symphonic Orchestra (Fig. 4). When we left Italy, I was nine years old, and my father assured us that the move would be for only a short period until he became rich, and we could come back to a free Italy. He never got rich, nor did we ever return to Italy.

As I Became Addicted to Science

I entered into a research laboratory for the first time in 1957, when I was an eighteen-year-old medical student. I worked under the supervision of Dr. Walter Oswaldo Cruz (Fig. 1) at the Institute that carried his family name (from his father). It was not easy to get a position in Dr. Oswaldo Cruz's laboratory. In addition to being an excellent scientist, he paid a hefty fellowship, the equivalent at present of

FIGURE 4. **Ezio de Meis, my father, who taught me to pursue my dreams no matter what.**

300 dollars per month. For someone from a modest family, this was a fortune. Therefore, I applied for a position as soon as one became available, even though, at the time, I envisioned a future as a surgeon, not a researcher.

Dr. Oswaldo Cruz had a peculiar way of selecting his students. The first step of the selection took place at his home. When I arrived, the living room was crowded with candidates, most of whom were far more advanced in the medical course than I was. I feared that I did not stand a chance in comparison, but fortunately, I decided to stay and face the oncoming massacre. When I was finally called for the interview, to my surprise, I found out that Dr. Oswaldo Cruz did not care about my performance at the university or if I knew anything about hematology (his field), and he cared even less if I had any laboratory experience. After a very brief, casual conversation, he showed me several cartoons selected from the American magazine *The New Yorker* and asked me to identify the humorous

point of the cartoon. After the interview, Dr. Oswaldo Cruz informed me that those selected would be called for a second interview. Many did not make it past the first step, but to my surprise, I was called for the second test in his laboratory. Here, he showed us complex pieces of equipment and asked us what they were used for. When I answered that I had no idea, he said, "Good. Now make a guess." A week later I was informed that I had been selected and that I should go to his laboratory the following week. I was an exhilarated new apprentice. At first, I felt like a millionaire as I imagined the 300 dollars I would receive each month. Then, as I cooled down, I had the feeling that, in Dr. Oswaldo Cruz's view, the main ingredients for science were a good sense of humor and a lot of intuition. As the years passed, I found out that Dr. Oswaldo Cruz's recipe was a good one.

I worked in Dr. Oswaldo Cruz's laboratory for six years. He used to design the experiments, and we, the students, performed them. Of course he accepted suggestions and encouraged us to test them, and while there, I learned what it meant to have a good sense of humor in science. When one of my suggestions was accepted, a rare occurrence, I worked crazily, skipping weekends, classes at the university, and encounters with my girlfriend. After a certain amount of time, I usually had to give up because my brilliant idea turned out to be not so brilliant. This was always excruciating and a terrible blow to my ego; I was not as brilliant as I wished to be. So I would go to Dr. Oswaldo Cruz and ask if he had any experiments that needed to be done in the main flux of the laboratory. He would smile (a cruel smile, to my understanding) and ask, "Why, your good suggestion did not work?" With a grotesque attempt at making a humorous face, I would answer, "Nature does not seem to appreciate my ideas." This was stated causally, but inside, I wanted to kill, kick, and do all sorts of malignant acts. Fortunately, this frustration did not persist for a long period: I was soon distracted by the need to catch up on the lessons missed at the university and search for a new girlfriend because the previous one thought I had intentionally ignored her for weeks. I repeated this masochist ritual several times over the years, and a few of my suggestions actually worked out. Years later, during a friendly conversation, Dr. Oswaldo Cruz told me that he used this strategy to see if a trainee was able to face frustration without coming up with false interpretations or, even worse, "adapting" results. To my astonishment, Dr. Oswaldo Cruz also informed me that he also frequently suffered frustrations with ideas that he initially thought to be excellent.

At the end of my medical course, I was a coauthor of several of Dr. Oswaldo Cruz papers that had been published in well known international journals and had authored a couple of small papers of my own. Dr. Oswaldo Cruz felt that I had a reasonable knowledge of physiology and insisted that I should work in the United States for one or two years to be initiated into the field of biochemistry. He helped me in all possible ways to get a fellowship. Thus, I had the good fortune to end up in the laboratory of Drs. Herbert and Celia Tabor (Fig. 2) at the NIH. The Tabors taught me many things, starting with discipline. For a young man raised in a tropical country, waking up at 6:45 a.m. during the winter when it was dark outside and snow was falling was extremely difficult. The working hours at the NIH were 8:30 a.m. to 5 p.m., but I used to come in between 9 and 10 a.m. and work until later in the evening both to compensate for the late arrival and because I enjoyed the work at the laboratory. One day, in a kind but firm voice, Herbert informed me that I had to start at 8:30 a.m. no matter how cold it was outside and then should feel free to work after 5 p.m. if this suited me. In the same kind tone of voice, Herbert told me that I had to attend the journal club meetings they conducted in Building 10. The journal club had been organized by Arthur Kornberg and was attended by distinguished scientists, including Bernard Horecker, Leon Heppel, and Maxine Singer. At the beginning, I could grasp very little of what was being presented, but in a few months, I could understand all of it.

Usually, after the journal club meetings, I would have a generous lunch in the cafeteria. It was usually full, so I had to share the table with other NIH investigators, who were always engrossed in a scientific discussion. Thanks to what I learned at the journal club meetings, I was frequently able to understand most of what was being discussed. At that time, I was quite shy, but one day, I dared to ask a question about the conversation. To my astonishment, they not only answered but involved me in their discussion. Even though the conversations lasted no more than twenty min (after which time, the scientists would leave in a hurry, as they all seemed to have bosses who were watching the clocks), I learned enormously from them. Risking a reprimand from the Tabors, I frequently used to have lunch at one table and then, when the other people seated there left in a hurry, I would get my dessert and choose a different table to engage in more conversation. The Tabors never complained about my lunch delays, and on one particular day when I stayed in the cafeteria a bit longer than necessary, Herbert asked me in a humorous tone (meaning no reproach) if I enjoyed my lunch. I babbled something and quickly went back to the bench. The impression remained,

however, that the Tabors knew all too well what was going on in the cafeteria and the powerful seduction of the conversation going on there. These are only two examples of the rich atmosphere of the Tabors' laboratory.

Celia and Herbert were always very solicitous and ready to discuss any kind of results I managed to get, but I was never able to get many. At the end of my fellowship, all I was able to show for my time in their laboratory was a small chapter in *Methods in Enzymology*, which was written by the Tabors (4). This poor productivity was due to my lack of scientific maturity. There were plenty of opportunities in the Tabors' department, but I was not yet ready to benefit from them. However, despite this apparently meager result, it was in the Tabors' laboratory that I really learned what biochemistry was about and became aware of the vast horizon of the biomedical sciences. Back home in Brazil, it was difficult to explain in reports that I had learned a lot at the NIH journal club meetings and cafeteria conversations. As a result, there was a certain disappointment regarding my seeming lack of performance at the NIH. When I left Brazil, I was considered a bright young fellow who had published several papers with Dr. Oswaldo Cruz, and in the United States, land of opportunity, I was expected to blossom and publish much more. It was difficult to explain how important it was for my scientific education to be one of the Tabors' students.

Shortly after my return from the United States, I obtained a position at the Biophysics Institute of the Federal University of Rio de Janeiro, my alma mater. It was at this time that I married a very attractive and bright geologist. The quality of my life greatly improved after my marriage and migration to the university. In addition to the friendly attitude of the staff, I was allowed to work independently on my own projects. It was at the Biophysics Institute that I became productive, and after two years, I had published four papers in respected international journals. After a long, depressing period without any publications, I had started to doubt my talent; however, with these papers, I proved to myself that I could do it. It was during this period that I stumbled on membrane-bound enzymes and, in particular, on skeletal muscle Ca^{2+}-transport ATPase, a single peptide of 110 kDa that is found in abundance in vesicles derived from the muscle reticulum. This became the main subject of my following working years.

Two of the four papers I published at that time were related to Ca^{2+}-transport ATPases, also named Ca^{2+} pumps. The first one described the finding that the brain microsomal fraction contains a Ca^{2+} pump with the same properties as the muscle Ca^{2+} pump (5). At that time, it was thought that the Ca^{2+} pumps were confined to skele-

tal muscle, and from then on, different isoforms of Ca^{2+} pumps were found in different tissues (5–8) and renamed sarco/endoplasmic reticulum Ca^{2+}-ATPases (SERCA). In the second work, I showed that acetyl phosphate could be used as a substrate by SERCA. Until then, it had been thought that only ATP could be used as a substrate by SERCA. Later, it was shown that like acetyl phosphate, ITP and GTP could be used as substrates, but their affinity was orders of magnitude smaller than that of ATP. The intriguing finding was that the kinetics of the pump varied depending on whether acetyl phosphate or ATP was used as the substrate (9–11).

Background of My Scientific Work

Phosphate Compounds of High and Low Energy—This concept was established in a memorable review by Lipmann in 1941 (12). Based on the data available at that time, it was believed that energy would be concentrated in the chemical bond between the phosphate and the rest of the molecule. Low-energy phosphates were denoted as –P. These phosphates were usually linked to an alcoholic hydroxyl, *e.g.* the phosphoester bonds. The energy released during the cleavage of –P was estimated to vary between 2 and 3 kcal/mol. High-energy phosphate compounds were denoted as ~P. This category included a variety of phosphate compounds with energies of hydrolysis higher than 7 kcal/mol. These included phosphocreatine, phosphoenolpyruvate, and the γ- and β-phosphate phosphoanhydride bonds of ATP. At that time, it was not known how enzymes handled the energy released from the cleavage of a high-energy compound. The general feeling was that energy would be released at the exact moment of the ~P compound cleavage and that the energy released would be concentrated in the region of the protein that would use it, thus optimizing its use with little energy dissipation in the surrounding vicinity.

The Chemiosmotic Theory—Life is sustained by the degradation of high- into low-energy compounds, and among these, ATP was identified as the main carrier of energy through the different cellular compartments. ATP was discovered by Fiske and SubbaRow in 1925 (13), but its central role in biological systems only became apparent several years after its discovery. There are different reaction sequences that can promote the synthesis of ATP from ADP and P_i, but in aerobic cells and in the presence of oxygen, mitochondria are the main cell organelle that continuously produces large amounts of ATP, using the energy derived from the reduction of $\frac{1}{2}O_2$ into HOH. Of key importance in this reaction se-
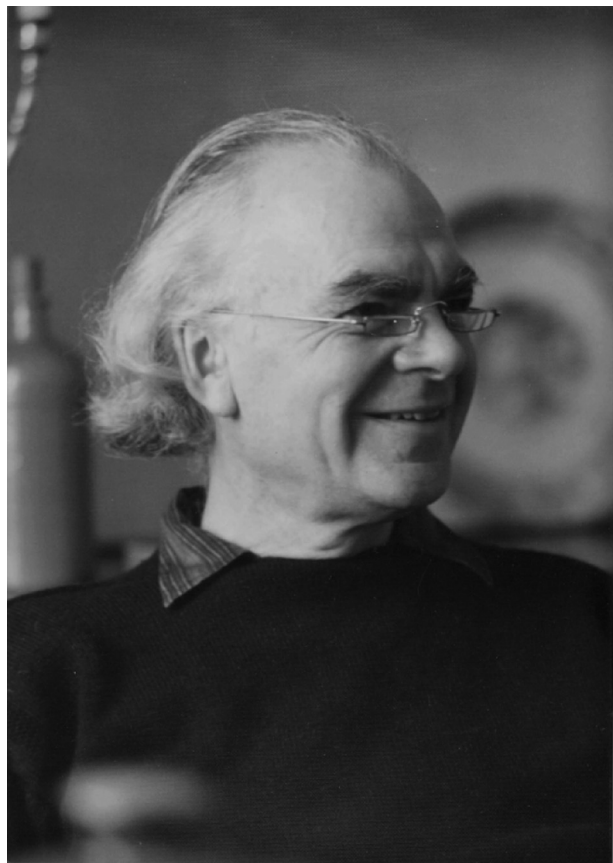
FIGURE 5. **Peter Mitchell, the man who discovered the chemiosmotic energy transduction mechanism.**

quence was the discovery by Keilin of the various cytochromes organized in the inner mitochondria membrane and how the electrons derived from the oxidation of NADH and $FADH_2$ flow through them following an energy gradient until reaching oxygen at the end of the cytochrome chain, reducing it to HOH (14).

After Keilin's discovery, Peter Mitchell (Fig. 5) proposed the chemiosmotic theory to explain the mechanism used by mitochondria to synthesize ATP (15). According to this theory, the energy derived from the flux of electrons through the cytochrome chain is used to exclude protons from the inner membrane into the space available between the two mitochondrial membranes, thus generating a proton gradient across the inner mitochondrial membrane and the matrix (electric and osmotic energies). The H^+ gradient is then dissipated through the mitochondrial F_1F_0-ATP synthase, a complex enzyme that has several subunits, with some embedded into the inner membrane and others protruding from the inner mitochondrial membrane and facing the matrix. During the H^+ efflux through the F_1F_0 complex, the energy derived from the gradient is converted into chemical energy to synthesize ATP from ADP and P_i.

These were the first studies demonstrating that a cell could convert osmotic energy into chemical energy. The entire process of energy interconversion is reversible, *i.e.* the ATP synthase can catalyze both the hydrolysis and synthesis of ATP. In the absence of respiratory substrate, mitochondria hydrolyze ATP and use the chemical energy released to form a proton gradient, and the energy derived from the osmotic energy can be used to revert the electron flux through the cytochrome chain, leading to the reduction of NADH and FAD back into NAD^+ and $FADH_2$. In other words, all of the mitochondrial crucial steps that interconvert energy can operate forward and backward depending on the experimental conditions used. These findings contradicted the concept that during ATP cleavage, the energy released was concentrated in the specific region of the protein that would use it to avoid energy dissipation.

Little was known at that time about the mechanism used by enzymes to convert energy. The ion transport enzymes, also referred to as pumps, were already known prior to Mitchell's proposal (16, 17). Unlike the F_1F_0-ATPase, pumps are usually simple proteins that, at times, are constituted by a single peptide, as in the case of the various SERCA isoforms. Pumps are ATPases that convert the chemical energy derived from ATP cleavage into osmotic energy, but before Mitchell's proposal, it was thought that the process was irreversible. The ionic gradient formed would continuously be lost, mainly through the leakage of ions across specific channels and, at a slower rate, through nonspecific irregularities of the membrane structures. Thus, the pumps were considered to be proteins that continuously cleaved ATP to form and maintain a gradient. The possibility that the mode of a pump could be reversed to synthesize ATP from ADP and P_i using the energy derived from the osmotic gradient formed by the ion transported was not taken into consideration. The Mitchell chemiosmotic proposal quickly spread through the fields dealing with pumps and had a primordial role in the understanding of the mechanisms of energy interconversion in ion transport ATPases.

I had the good fortune to be invited by Peter Mitchell (Fig. 5) for a three-day sojourn to his laboratory at the Glynn Research Centre near Bodmin, Cornwall, United Kingdom. This was an exciting adventure. When I took the train from London, I was expecting to find a tall building crowded with all sorts of laboratories. Instead, the train stopped in a small station in the countryside. The crowded building I had imagined was in fact a lovely mansion located on a farm with a small lake, cows,

FIGURE 6. **Hasselbach (*left*) and Makinose (*right*) at Hasselbach's sixty-fifth birthday celebration.**

ducks, and everything else required for a peaceful and inspiring environment. The research team was restricted to a staff of three: his colleague, Jennifer Moyle; a secretary; and another woman who took care of a rich library. At that time, there was no internet, and we had to go to printed journals to collect information. Peter was waiting for me at the entrance of the mansion and took me directly to his laboratory. The first thing he showed me was a world map full of pins. Red pins located research groups that criticized his theory, white were neutral, and green supported the chemiosmotic theory. I had three lovely days of friendly discussion. On the train back to London, I could not remember clearly what specific topics we had discussed, but I knew that my way of thinking about energy interconversion had changed for the better.

Transport ATPases—Parallel to mitochondrial studies, there was a growing interest in two membrane-bound ATPases that were able to translocate ions across the membrane: the $(Na^+ + K^+)$-transport ATPase discovered by Skou (16) and the sarcoplasmic reticulum Ca^{2+}-transport ATPase from skeletal muscle discovered by Hasselbach and Makinose (Fig. 6) (17). These two enzymes are able to use the chemical energy derived from ATP hydrolysis to transport ions across the membrane, thus forming an osmotic gradient. A common feature of these two transport enzymes is the formation of an acyl phosphate residue during the catalytic cycle.

After binding to the enzyme, ATP transfers its γ-phosphate to an aspartyl residue located in the catalytic site, forming the acyl~P residue. This step was shown to be fully reversible because, in water, the energy of ATP γ-phosphate hydrolysis is the same as that of the acyl~P residue (18). For the Ca^{2+}-transport ATPase, it was thought that chemical energy became available for Ca^{2+} transport only after the cleavage of the aspartyl residue. Isoforms of this enzyme are found in the reticulum of different tissues. Those found in white and red muscle are named SERCA1 and SERCA2, respectively. These enzymes play a key role in muscle relaxation by draining Ca^{2+} from the cytosol into the sarcoplasmic reticulum.

The Brazilian Military Dictatorship, Acetyl Phosphate, and Heidelberg

Shortly after my return from the United States in 1964, the military took over the Brazilian government. These were dark years in Brazil. The new political regime selected a new director for the Oswaldo Cruz Institute, a man who did not have the foggiest idea what science was about but claimed to be very loyal to the new regime. One of the loyal director's deeds was to make the working conditions of the few active laboratories simply unbearable. After a year, I resigned and went to the university to teach and do research at the Biophysics Institute in the department headed by Antonio Paes de Carvalho. The institute was directed by Carlos Chagas Filho. In addition to being a good scientist, Chagas Filho had great diplomatic ability. His family was one of the most traditional and influential in the country, and he was able to protect the Biophysics Institute without ever compromising with the military regime.

At the beginning of the dictatorship, the bad guys were the communists, but they had a peculiar view of communism. Later on, in the 1960s, the bad guys were no longer the communists, but those who did not approve of the military regime were referred to as subversive. I never cared for politics, least of all for communism: I did not approve of the lack of freedom and, above all, if one of my students was arrested and tortured by the regime. My colleagues from the institute and I used to go from prison to prison looking for missing students. If no one searched for them, the regime thought they were of no importance or in fact subversives whom nobody wanted to be involved with and simply made them disappear. The regime did not like the interference of those who went looking for prisoners.

One day, the president of the Brazilian Academy of Science told me he had heard rumors that my family and I should take a long sabbatical leave. He assured me that he would let me know when things got better and I could come back safely. My wife was a well known geomorphologist. We both wrote letters seeking positions in different places, mostly in the United States. We received several favorable answers, but none in the same place except for Germany, where my wife was accepted at the

University of Heidelberg and Professor Hasselbach (Fig. 6) offered me a nice visiting professor position at the Max Planck Institute. This would keep the family together, and I had a chance to work in the laboratory of the discoverer of the sarcoplasmic reticulum Ca^{2+}-transport ATPase (17).

The work with acetyl phosphate (9, 10) played an important role in my sojourn in Heidelberg during the military regime's reign in Brazil. In 1970, Hasselbach and his close collaborator, Makinose, were in the process of discovering that the Ca^{2+}-ATPase could use the energy derived from a Ca^{2+} gradient to synthesize ATP from ADP and P_i (19–21). In the course of their experiments, they needed vesicles loaded with Ca^{2+} in a suspension completely free of ATP and ADP. This was not easy to obtain using ATP to load the vesicles but could be achieved using acetyl phosphate. Years later, I learned from Makinose that my letter asking for a position reached Hasselbach's office shortly after they had read my work on acetyl phosphate, and this apparently contributed to the generous offer I received from Germany.

When I arrived at the Max Planck Institute, Hasselbach asked me what I would like to work on, and I suggested that I continue exploring the kinetic differences between acetyl phosphate and ATP hydrolysis. Hasselbach said fine, carry on, thus giving me freedom to plan my own experiments. When I had a sufficient amount of data, he would come around two or three days later and discuss the data with me, together with Makinose. I enjoyed my work but noticed that, every two days, Makinose and Hasselbach closed themselves in Makinose's office and had long *sotto voce* discussions. This was intriguing, but it was none of my business, so I got used to it and did not pay much attention. What I did not know was that the two of them were immersed in experiments that would completely change the prevailing view of energy interconversion.

They showed that a steep Ca^{2+} gradient was formed when vesicles loaded with acetyl phosphate were transferred to a medium without Ca^{2+} and with EGTA, a Ca^{2+}-chelating substance. In these conditions, the Ca^{2+} retained by the vesicles leaked at a slow rate to the medium. The rate of Ca^{2+} leaking did not vary if either P_i or a small amount of ADP was added to the medium. However, if both ADP and P_i were added, then Ca^{2+} was released at a fast rate, and coupled with the increment in Ca^{2+} release, there was net synthesis of ATP. In 1971, Makinose and Hasselbach published three short papers in *FEBS Letters* (19–21) demonstrating that the Ca^{2+}-ATPase, similar to mitochondrial ATPase, could use the

energy derived from an ionic gradient (Ca^{2+}) to synthesize ATP from ADP and P_i. In 1972, Makinose demonstrated that the synthesis of ATP was initiated by phosphorylation of the enzyme by P_i, forming an acyl phosphate residue at the catalytic site (22). In accordance with what was described in the Ca^{2+} uptake, it was concluded that during reversal of the pump, the energy derived from the gradient was captured by the enzyme to form the acyl phosphate residue from P_i (Reactions I and II).

$$E + ATP \underset{\text{ADP}}{\overset{\text{HOH}}{\longleftrightarrow}} E \sim P \xrightarrow[\text{Energy conversion}]{} E + P_i$$
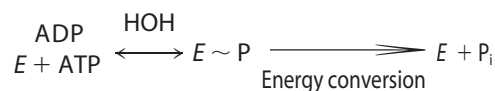
Reaction I. Pumping Ca^{2+}: converting chemical into osmotic energy.

$$E + P_i \xrightarrow[\text{Energy conversion}]{\text{HOH}} E \sim P \underset{\text{ADP}}{\overset{}{\longleftrightarrow}} E + ATP$$
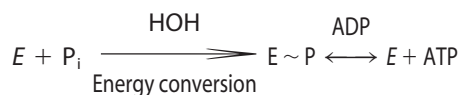
Reaction II. Reversal of the pump: osmotic into chemical energy.

These observations had an important impact on the bioenergetics field because they showed that in addition to mitochondria, other proteins could convert osmotic into chemical energy and that the interconversion could be catalyzed by a simple protein, a monomer with a molecular mass of 110 kDa. Because the mitochondrial F_1F_0 complex was a large polymer with many subunits of different molecular mass, it was thought that interconversion of osmotic into chemical energy required complex protein structures. At present, it is clear that the ability to interconvert these forms of energy is not related to the complexity of the enzyme. However, at the time of Mitchell, Hasselbach, and Makinose, these were the subjects of clamorous discussions in bioenergetics meetings.

I was fascinated by these new findings when Makinose told me about them a few days before publication. I could hardly believe that such important experiments were being discussed when Hasselbach went to Makinose's office.

We had a great time in Heidelberg, a small, lovely city crossed by the peaceful Neckar River. It contained quaint features: a castle on a hill; a romantic old bridge; and a happy section called the "Old City" that contained all sorts of small restaurants, stores selling exotic items, and lots of tourists. The institute directed by Hasselbach had a small administrative staff and practically no bureaucracy. It used to close on the weekends and was practically empty during week days after 6 p.m. However, the rest of the time, the work was intensive, disciplined, and productive.

The high point of my stay was the intimate relationships I formed with both Makinose and Hasselbach, in part due to music. Makinose played the violin and had two nieces who studied music at the university. I used to play the recorder reasonably well. Once a week, we met at Makinose's house after dinner and played for one hour, after which Makinose's wife would bring us snacks and a delicious tea. On the other hand, Hasselbach disliked music, at least our kind of music, but his wife enjoyed playing the recorder. She invited me to play and have a glass of wine once every two weeks at their home, which I was happy to do. The music sessions lasted less than half an hour, at which time Hasselbach would come in and state firmly that we had had enough music. He would then take me to the garden, where he would serve a very tasty Rhine wine, and we would become engrossed in marvelous conversations, in which I learned a lot about both science and life. I praised Frau Hasselbach's music skills, but I confess that what I really enjoyed was the wine and the highly inspiring conversation with Hasselbach. When my contract with the Max Planck Institute expired, Hasselbach offered me a permanent position at the institute with a higher salary. It was tempting, but I had just received letters from the president of the Brazilian Academy of Science and various colleagues at the university informing me that the situation in Brazil had improved greatly and that there was money available for research. Thus, I declined Hasselbach's excellent offer and returned home, where I was warmly received back at the Biophysics Institute. Before leaving Heidelberg, I asked Hasselbach and Makinose if they would mind if I followed the exciting new path they had discovered. Both of them warmly agreed, and for several years, most of my work focused on the reversal of the Ca^{2+} pump. As far as I know, unlike the ability to remove Ca^{2+} from the cytosol, there is no clear-cut evidence that the reversal of the pump has an important role in cell physiology. However, the pump reversal proved to be an excellent tool to study energy transduction, and through the reversal, it was possible to reveal the various steps of the enzyme catalytic cycle working both forward and backward. In addition, it provided information about the concept of high- and low-energy phosphate compounds.

How a Small Technical Detail Can Lead to Different Interpretations

In 1973, it was known that sarcoplasmic reticulum vesicles contain a small amount of endogenous Ca^{2+}. It was then hypothesized that if empty vesicles were suspended in a medium with EGTA, a small gradient should be formed across the sarcoplasmic reticulum vesicles due to
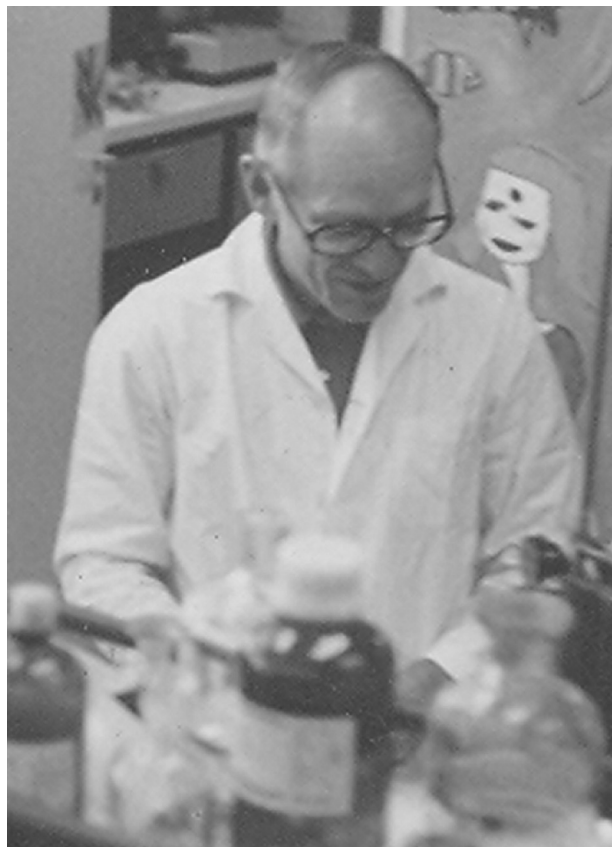

FIGURE 7. **Paul Boyer during his visit to Rio de Janeiro.** Paul and I together repeated the experiments with leaky vesicles.

the small Ca^{2+} inside the vesicles, and this should lead to a small enzyme phosphorylation by P_i. This possibility was simultaneously investigated in the laboratory of Paul Boyer and in my own laboratory. In 1973, Kanazawa and Boyer found that when empty vesicles were solubilized with the detergent Triton X-100 and incubated in a medium containing EGTA, P_i, and Mg^{2+}, a temporary, small amount of phosphoenzyme was formed (23). This was orders of magnitude smaller than that measured by Makinose with Ca^{2+}-loaded vesicles. The low P_i phosphorylation was abolished rapidly (in seconds) when the SERCA vesicles were disrupted with the detergent Triton X-100. These data led the authors to conclude that the small gradient formed with the endogenous Ca^{2+} could indeed lead to a small phosphorylation, which was abolished with the dissipation of the microgradient after a short incubation time, meaning that the presence of a gradient was an absolute requirement for the phosphorylation of the enzyme by P_i.

In Rio de Janeiro, we performed experiments similar to those of Kanazawa and Boyer (Figs. 7 and 8), but instead of using Triton X-100, we permeabilized the vesicles with a small amount of ethyl ether, which was known to disrupt

the vesicles' membrane (24, 25). With the leaky vesicles and in the absence of Ca^{2+} (excess EGTA), we measured phosphorylation of the Ca^{2+}-ATPase to a level that was smaller than that measured by Makinose but was an order of magnitude higher than that measured by Kanazawa and Boyer. An important feature was that the phosphoenzyme was stable and did not decrease even after a period of 30 min in the presence of excess EGTA. This indicated that, in contrast to the results of Kanazawa and Boyer, the Ca^{2+}-ATPase could be phosphorylated by P_i in the absence of a gradient, *i.e.* in the absence of an apparent source of energy. We then decided to vary the experimental conditions in an attempt to increase the level of stable phosphoenzyme measured in the absence of a gradient, and the best results were attained when the pH of the medium was decreased from 7.0 to a range of 5.5–6.0 and the P_i concentration in the medium was raised from 1–2 mM to a range of 5–8 mM. Under these conditions, we could obtain the same level of phosphoenzyme as that measured with the gradient by Makinose (22). Different tests confirmed that the acyl phosphate formed in the absence of the gradient was the very same aspartyl phosphate residue as that formed with the intact vesicles and gradient. It was known that the Ca^{2+}-ATPase could be solubilized with Triton X-100 and remained stable for hours provided that Ca^{2+} (0.1 mM) was included in the medium. Later, Kanazawa (Fig. 8) showed that the Ca^{2+}-ATPase was quickly denatured if solubilized with Triton X-100 in the absence of Ca^{2+} (26). This explained the earlier findings obtained with Boyer, which gave the incorrect impression that there was no phosphorylation by P_i in the absence of a gradient. We were lucky to permeate the vesicles with ethyl ether, a treatment that does not damage the Ca^{2+}-ATPase. Otherwise, we also would have missed the phosphorylation by P_i in the absence of a Ca^{2+} gradient.

Eventually, Paul Boyer came to Rio, and we repeated the experiment with leaky vesicles (Fig. 7). Paul was convinced that in fact phosphorylation could occur without a gradient. I learned a lot from Paul's sojourn and started to admire him not only because of his excellence in science but also for his kind and unassuming personality. During his visit, Paul explained to us his recent $P_i \leftrightarrow HO^{18}H$ exchange experiments with mitochondria indicating that ATP could be synthesized spontaneously at the catalytic site of the mitochondrial F_1-ATPase without the need for energy. The amount of ATP synthesized was very small and tightly bound and could not dissociate from the enzyme. Thus, its formation could only be inferred from $P_i \leftrightarrow HO^{18}H$ exchange measurements. According to Paul's
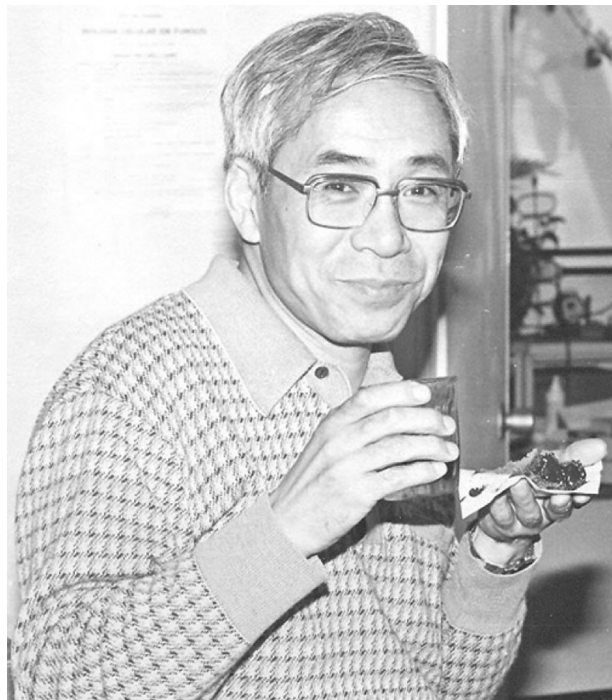

FIGURE 8. **Kanazawa in Rio de Janeiro.** He showed us the deleterious effects of the detergent Triton X-100 on sarcoplasmic vesicles when Ca^{2+} was removed from the medium with an excess of the Ca^{2+}-chelating agent EGTA.

findings, first published in 1973, energy was needed not for the synthesis of ATP but for the dissociation of the tightly bound ATP from the enzyme (27). After Paul's return to the United States, we started to collaborate from a distance, measuring $P_i \leftrightarrow HO^{18}H$ exchange catalyzed by the Ca^{2+}-ATPase under different conditions. The experiments were performed in Rio and the analysis of O^{18} in Los Angeles. In 1997, Paul was awarded the Nobel Prize for the discovery of the mechanism through which the mitochondrial ATP synthase works.

The ATP $\leftrightarrow$ P_i Exchange Reaction and the Low-affinity Ca^{2+}-binding Site

Phosphorylation of the Ca^{2+}-ATPase by P_i is very sensitive to the presence of Ca^{2+} in the medium regardless of whether the vesicles are leaky or loaded with Ca^{2+}. The Ca^{2+} concentration needed in the assay medium for half-maximal inhibition is 1 μM (24). Unlike P_i, phosphorylation of the Ca^{2+}-ATPase by ATP requires the presence of Ca^{2+} in the medium (28, 29). Coincidentally, the Ca^{2+} concentration needed for half-maximal phosphorylation by ATP was found to be the same as that needed to inhibit phosphorylation by P_i, indicating that the binding of Ca^{2+} to a single binding site located in the outer surface of the vesicles determines whether the enzyme will be phosphorylated by ATP or by P_i (25, 30, 31). Despite the fact

that the enzyme can be phosphorylated by P_i in the presence and absence of a gradient, there is a major difference between the two forms: the ability to synthesize ATP. Although the vesicles loaded with Ca^{2+} were able to transfer their phosphate to ADP, forming ATP, the phosphoenzyme formed in the absence of a gradient was not able to synthesize ATP. The details of the transference of phosphate to ADP will be discussed in further detail below, but this observation led to the following two conclusions.

(i) The same chemical species, an acyl phosphate, can be of high or low energy. When the concept of phosphate compounds of high and low energy was formulated by Lipmann in 1941 (12), it was thought that the energy level of a phosphate residue would always be the same, and on the basis of the data available at that time, the possibility that the energy of hydrolysis might change in the surface of an enzyme was not taken into consideration. (ii) At least for the Ca^{2+}-ATPase, the energy derived from the gradient (osmotic) is not used for the formation of the acyl phosphate bond at the catalytic site but is needed to convert it from low to high energy.

The ATP $\leftrightarrow$ P_i exchange reaction was first described in mitochondria when a proton gradient is formed across the membrane. During the exchange, mitochondria are able to hydrolyze and synthesize ATP simultaneously, *i.e.* although some units of the ATP synthase cleave ATP, other units synthesize ATP from ADP and P_i using the energy derived from the H^+ gradient. Inspired by the mitochondrial ATP $\leftrightarrow$ P_i exchange, in 1971, Makinose observed that the Ca^{2+}-ATPase also can catalyze the simultaneous hydrolysis and synthesis of ATP when the vesicles are filled with Ca^{2+} and a steady state between Ca^{2+} influx and Ca^{2+} efflux is reached (20). Because of the need for an ionic gradient, the ATP $\leftrightarrow$ P_i exchange reaction was one of the parameters used to characterize the interconversion between osmotic and chemical energy. In fact, the disruption of the muscle vesicle integrity with either phospholipase A or diethyl ether abolished the synthesis of ATP, and the Ca^{2+}-ATPase was only able to cleave the ATP available in the medium. This led to the conclusion that at steady state, the energy derived from the hydrolysis of ATP was used to maintain the Ca^{2+} gradient, and at the same time, the energy derived from the gradient was used to synthesize ATP from ADP and P_i.

The general view of Ca^{2+} transport was that the ion bound in a part of the transport ATPase facing the outer surface of the vesicles, and the energy derived from the cleavage of ATP was then used to promote a conformational change in the protein that would permit the release of the ion into the vesicle lumen. Thus, in physiological conditions, when a gradient is formed, the Ca^{2+} concentration in the solution facing the outer surface of the vesicle is very low, whereas inside the vesicle, it reaches a range of 2–10 mM. We then raised the possibility that the conversion of the phosphoenzyme from low to high energy could be related to the binding of Ca^{2+} to a low-affinity site of the ATPase facing the vesicle lumen. Thus, the conversion from low to high energy was related to Ca^{2+} binding (binding energy) and not to the gradient itself (osmotic energy). To test this hypothesis, we prepared leaky vesicles and incubated them in medium containing various Ca^{2+} concentrations from the range similar to that found on the outer surface of the vesicles during transport (submicromolar) up to the range found in the vesicle lumen during Ca^{2+} accumulation (millimolar). The result of this experiment was very rewarding. In the presence of low Ca^{2+} concentrations, the ATPase was only able to catalyze the hydrolysis of ATP, but as the Ca^{2+} concentration was raised to the millimolar range, there was a simultaneous inhibition of the hydrolysis of ATP and the activation of ATP synthesis. The Ca^{2+} concentration needed for both half-maximal inhibition of ATP cleavage and half-maximal activation of ATP synthesis was in the range of 1–2 mM (30–32). These experiments supported the hypothesis that the synthesis of ATP was promoted by the asymmetrical binding of Ca^{2+} on the two sides of the membrane and *not* by the osmotic energy derived from the gradient.

The Reaction Sequence

The data accumulated from the studies of the pump working forward (ATP hydrolysis) and backward (ATP synthesis) led us to the proposal of a basic catalytic cycle shown in Fig. 9*A*. During catalysis, the enzyme cycles through two distinct conformations, E_1 and E_2 (originally E and *E) (30, 31, 33–35). The enzyme form E_1 binds calcium with high affinity ($K_s \sim 1$ μM) on the outer surface of the vesicles and can be phosphorylated by ATP (Fig. 9, *reactions 1* and *2*) but not by P_i. This reaction has a K_{eq} value of ~ 1 and is therefore fully reversible (38). The ATPase undergoes a conformational change from $E_1 \sim P$ to E_2-P (*reaction 3*). During this transition, Ca^{2+} remains bound to the enzyme but now faces the luminal side of the vesicle. The Ca^{2+} affinity of the enzyme form E_2 is ~ 3 orders of magnitude smaller than that of E_1 ($\sim 1–2$ mM). During Ca^{2+} dissociation (*reaction 4*), the energy of hydrolysis of the phosphoenzyme decreases to practically zero ($K_{eq} = 1$) and becomes fully reversible, thus explaining why the ATPase can be phosphorylated by P_i in the absence of a gradient (*reaction 5*, reverse direction). Two
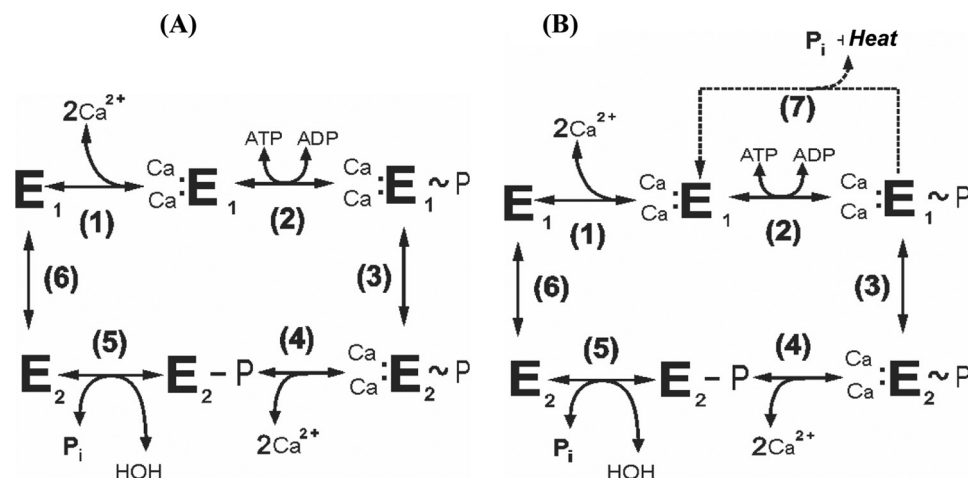
(A)

(B)

FIGURE 9. **Catalytic cycle of the Ca^{2+}-ATPase.** *A*, the sequence includes two distinct enzyme conformations, E_1 and E_2. The Ca^{2+}-binding sites in the E_1 form face the external surface of the vesicle and have a high affinity for Ca^{2+} ($K_a = 10^{-6}$ M at pH 7). In the E_2 form, the Ca^{2+}-binding sites face the vesicle lumen and have a low affinity for Ca^{2+} ($K_a = 10^{-3}$ M at pH 7). The enzyme form E_1 is phosphorylated by ATP but not by P_i, forming the high-energy phosphoenzyme $2Ca{:}E_1{\sim}P$ (K_{eq} hydrolysis of $\sim 10^6$ and $\Delta G^0 \sim -8.4$ kcal/mol). The enzyme form E_2 is phosphorylated by P_i but not by ATP, forming the low-energy phosphoenzyme $Ca{:}E_2$-P (*reaction 5*; K_{eq} hydrolysis of ~ 1 and $\Delta G^0 \sim 0$ kcal/mol). When the Ca^{2+} concentration on the two sites of the membrane is <50 μM (leaky vesicles), *reaction 4* is irreversible, and this forces the sequence to flow forward from *reactions 1* to *6*. When the Ca^{2+} concentration in the vesicle lumen rises above 1 mM, the catalytic cycle is reversed from *reactions 5* to *2*, and the Ca^{2+} efflux is coupled with ATP synthesis. These steps were described by our laboratory (30). *B*, *reaction 7* was described by the Inesi laboratory (68, 69) as was referred to as Ca^{2+} slippage. Later, we showed that this step regulates the amount of work and heat released during ATP hydrolysis (42).

Ca^{2+} ions are bound to each enzyme unit, and the energy required for the decrease in the Ca^{2+} K_s from 10^{-6} to 10^{-3} M is about -18 kcal/mol. This is in the range of the energy derived from ATP hydrolysis. This value was confirmed later in calorimetric experiments (see "Thermogenesis: Heat Production by the Ca^{2+}-ATPase" below). The finding of phosphoenzyme of low and high energy indicates that the energy derived from ATP is not released at the time of phosphoenzyme cleavage, but it is captured by the enzyme before the cleavage is completed. In this case, binding energy and conformational energy are involved. It is as if the enzyme sucks the energy from the phosphoenzyme and converts it into work before being cleaved (*reactions 3* and *4*).

This cycle has been confirmed in different laboratories (34–36), and the equilibrium constants (K_{eq}) of the intermediate steps have been determined by transient kinetics in our laboratory as well as several others. Recently, the structures of the two monomeric states (E_1 and E_2) have been resolved by x-ray refraction by Toyoshima and Mizutani (36). The structures obtained indicate that the region of the catalytic site forms a concavity that undergoes a dramatic change in the transition of E_1 into E_2.

Net Synthesis of ATP

Under the conditions used to measure ATP $\leftrightarrow$ P_i exchange, there is no net synthesis of ATP; the rate of ATP hydrolysis is always faster than the rate of ATP synthesis.

A year after our reports on the ATP $\leftrightarrow$ P_i exchange in the absence of a gradient, Knowles and Racker (37) confirmed the formation of the low-energy phosphoenzyme in leaky vesicles at pH 5.5– 6.0, and using the concept of asymmetrical Ca^{2+} binding (32), they were able to promote the net synthesis of ATP after a single catalytic cycle of the enzyme flowing back in the direction of the reversal of the Ca^{2+} pump. This was achieved using leaky vesicles and a two-step procedure in which, initially, the enzyme was phosphorylated by P_i in the absence of Ca^{2+}, and then ADP and 3–4 mM $CaCl_2$ were added rapidly to the medium, a procedure referred to as a "jump." After the Ca^{2+} jump, half of the phosphoenzyme phosphate was transferred to ADP, forming ATP. Shortly after, we confirmed this experiment using the transient kinetics technique (38).

Solvation Energy and Phosphate Compounds of High and Low Energy

In 1970, George *et al.* (39) proposed a new theory for the concept of high-energy ($\sim P_i$) and low-energy (–P) phosphates. The working title of the article was "$\sim H_2O$," and in it, the authors proposed that the energy of hydrolysis of the phosphate compound (K_{eq}) was determined by the solvation energies of reactant and products. Solvation energy is the amount of energy needed to remove the solvent molecules that organize around a molecule in solution. Later, Hayes *et al.* (40) calculated the energy of hydrolysis of several phosphate compounds in the gas phase, *i.e.* in an

aqueous medium but in a theoretical condition in which the solvent would not bind to the reactants. The values found were quite different from those measured in water, thus supporting the proposal of George *et al.* (39). According to these calculations, the energy of hydrolysis of an acyl phosphate residue solvated in water would be in the range of -8 to -10 kcal/mol but increased to $+5$ to $+32.5$ kcal/mol in the gas phase. We then reasoned that the interaction of water with the reactant should decrease if we decreased the water activity of the solvent, thus mimicking the gas phase. We then raised the possibility that in the E_2 conformation, the catalytic site of the enzyme would be hydrophobic and that the solvation of both P_i and the aspartate residue would decrease to a point that phosphorylation would occur spontaneously (E_2-P). In this case, the major thermodynamic barrier for the formation of the acyl phosphate residue would be not the formation of the covalent bond (Fig. 9*A*, *reaction 5*) but the binding of P_i to the enzyme, *i.e.* the partitioning of a hydrophilic ion (P_i) from the aqueous assay medium into the hydrophobic environment of the catalytic site. Factors facilitating this partition should also facilitate the phosphorylation of the enzyme by P_i. The phosphoenzyme E_2-P formed from P_i would not be able to transfer its phosphate to ADP because of the large difference in the energies of hydrolysis of the acyl phosphate in a hydrophobic environment and ATP in an aqueous solution. The binding of Ca^{2+} to the low-affinity site of the enzyme would then promote a conformational change in the protein that would allow the entry of water into the catalytic site with the subsequent solvation of both the acyl phosphate residue and ADP. As a result, the energy values for the hydrolysis of the acyl phosphate and ATP would become equally high, and ATP synthesis would proceed spontaneously. Experimental conditions that reduce the entry of water into the catalytic site should also impede the synthesis of ATP. According to this hypothesis, the existence of high- and low-energy forms of the phosphoenzyme would be related solely to the water activity in the catalytic site. We tested this hypothesis by measuring the phosphorylation of the enzyme by P_i and the synthesis of ATP in the presence of water and various concentrations of nondenaturing organic solvents such as dimethyl sulfoxide, glycerol, and *N,N*-dimethylformamide (34, 41–43). In aqueous mixtures, these solvents markedly increase the partition coefficient of P_i from the aqueous medium into an organic phase containing isobutyl alcohol and benzene. In previous studies, we observed that in the absence of a gradient, the phosphorylation of E_2 by P_i demonstrated a saturation behavior (24, 25, 31, 35), indicating the occurrence of a

phosphate-enzyme complex prior to the phosphorylation reaction (Reaction III).

$$E_2 + P_i \underset{k_{-1}}{\overset{k_1}{\longleftrightarrow}} E_2 \cdot P_i \underset{k_{-2}}{\overset{k_2}{\longleftrightarrow}} E_2 - P$$
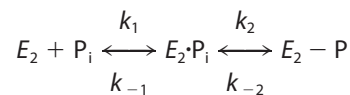
Reaction III. Reversal of the pump: osmotic into chemical energy.

If the catalytic site of the enzyme is hydrophobic, then the partitioning of P_i from the assay medium into the catalytic site (k_1/k_{-1}) should be facilitated when the difference in hydrophobicity between the two compartments is decreased by the addition of organic solvent to the medium, and this should promote a decrease in the apparent K_m for P_i. Accordingly, after replacing 40% of the water in the assay medium with dimethyl sulfoxide, we observed that the pronounced pH dependence was abolished and that the apparent K_m of E_2 for P_i decreased by up to a thousandfold (42). However, the phosphoenzyme formed in the presence of 40% dimethyl sulfoxide was not converted into a high-energy form after the addition of ADP and a Ca^{2+} concentration sufficient to bind to the low-affinity site of the enzyme, *i.e.* there was no synthesis of ATP after the addition of Ca^{2+} and ADP to the medium. The inhibition of ATP synthesis was related to the decrease in water activity caused by the organic solvent because the synthesis of ATP was restored if, after the addition of ADP and Ca^{2+}, the dimethyl sulfoxide concentration was suddenly decreased from 40 to 2%. The hypothesis proposed states that high- and low-energy forms of the phosphoenzyme are correlated with the availability of water at the catalytic site of the enzyme. The experimental results were consistent with this hypothesis. As predicted, phosphorylation of the enzyme by P_i was facilitated when the hydrophobicity of the medium was increased by the organic solvents, and after the addition of Ca^{2+}, the phosphoenzyme was only able to transfer its phosphate to ADP if the water activity of the medium was increased by dilution of the organic solvent with water. These data were reproduced in different laboratories and extended to other enzymes such as the mitochondrial F_1-ATPase (34, 44, 45) and inorganic pyrophosphatase (46). In these two systems, the addition of 40% dimethyl sulfoxide promoted both a large decrease in the apparent K_m for P_i and a substantial increase in the equilibrium level of the tightly bound ATP (soluble mitochondrial F_1-ATPase) and the level of the PP_i synthesized by the pyrophosphatase. Finally, the recently reported crystal structure of the Ca^{2+}-ATPase indicates that the conversion of the E_1 form into the E_2 form involves structural changes in

the region of the catalytic site that are compatible with the hydrophobic-hydrophilic transition proposed for the conversion of the phosphoenzyme from low to high energy (36).

Can SERCA1 Interconvert Other Forms of Energy Such as pH Gradients and Temperature?

The Ca^{2+} affinity of the two enzyme forms E_1 and E_2 varies with the pH of the medium. Both Ca^{2+}-binding sites exhibited an increased affinity for Ca^{2+} at high pH and a reduced affinity at lower pH values, with the relative differences in affinities of the two sites remaining unchanged at all pH values. Based on this finding, the following rationale was developed. No ATP synthesis will be observed when the same Ca^{2+} concentration exists on each side of the membrane and at a level sufficient to saturate the high-affinity site but not sufficient to allow significant binding of Ca^{2+} to the low-affinity site (Fig. 9*A*, reversal of *reaction 4*). However, this system is potentially capable of synthesizing ATP if the Ca^{2+} affinity of the two binding sites is modified by varying the pH on the two sides of the membrane: alkaline inside the vesicles and acidic in the assay medium. In such a situation, the phosphorylation of the enzyme by P_i will no longer be inhibited, and the Ca^{2+} concentration could become sufficient to allow the transfer of phosphate to ADP. At pH 5, the level of E_2-P is smaller than that measured at pH 6.0. However, at pH 5.0, the Ca^{2+} concentration needed in the medium for 50% inhibition of E_2-P formation is in the range of 1–2 mM Ca^{2+}. The transfer of phosphate from E_2-P to ADP is dependent upon the saturation of a low-affinity Ca^{2+}-binding site. After phosphorylation by P_i, it is possible to transfer the phosphate to ADP at progressively lower Ca^{2+} concentrations as the pH is raised from 6.0 to 8.1. Maximal ATP synthesis at pH 8.1 is attained at a Ca^{2+} concentration range of 0.5–1 mM. When E_2 is phosphorylated at pH 5.0 in the presence of 0.6 mM Ca^{2+}, net synthesis of ATP is observed if, at the time of ADP addition, the pH is rapidly increased to 8.0. These measurements indicate that it is possible to promote the synthesis of ATP after a single catalytic cycle without changing the Ca^{2+} concentration but by modifying the Ca^{2+} affinity of the enzyme forms E_1 and E_2. The measurements also sustain the hypothesis that the sarcoplasmic reticulum vesicles can drive the synthesis of ATP when a 3-pH unit gradient is formed across the vesicle membrane provided that Ca^{2+} is present in the medium in equal concentrations on the two sides of the membrane (no Ca^{2+} gradient). In absence of Ca^{2+}, the pH transition does not promote ATP synthesis. A similar procedure can be used by varying rapidly the temperature

of the assay medium. The equilibrium of the enzyme forms E_1 and E_2 and their respective Ca^{2+} affinities vary significantly with the assay medium temperature. These findings raised the possibility that the Ca^{2+}-ATPase can use the energy derived from either a pH or thermal gradient (33, 41, 43, 47).

Pyrophosphate of High and Low Energy

A direct way to study the role of the solvent in the energy of hydrolysis of a phosphate compound was to measure the K_{eq} and ΔG^0 *in vitro* using media with different water activities. These measurements can substantiate both the theoretical calculation for the gas phase and the assumption for the energy change at the catalytic site of the Ca^{2+}-ATPase. The water molecules that organize around a protein in solution have properties that are different from those of the bulk water medium, *e.g.* a lower vapor pressure, a lower mobility, and a greatly reduced freezing point. Similar changes in the properties of water are observed in mixtures of solvents and water. The simplest known high-energy phosphate compound is PP_i. Thus, we temporarily changed our focus from the Ca^{2+}-ATPase to PP_i, aiming to understand better the role of solvation energy. In a totally aqueous medium, the ΔG^0 of PP_i hydrolysis varies between -3.5 and -4.0 kcal/mol. We found that the energy of hydrolysis of PP_i decreased when different organic solvents were included in the medium, reaching ΔG^0 values of $+2.0$ kcal/mol, *i.e.* it is possible to convert PP_i from high energy ($\Delta G^0 = -4.0$) to low energy ($\Delta G^0 = +2.0$), and this can be achieved by simply modifying the water activity of the medium (48–51). In Boyer's laboratory, it was also shown that the energy of hydrolysis of PP_i varies greatly depending on whether it is in solution or bound to the yeast inorganic pyrophosphatase. In the presence of organic solvent, it was possible to decrease the energy of hydrolysis of PP_i to values even lower than those measured on the surface of inorganic pyrophosphatase (52). After these findings, I felt much more confident about our proposal that the change in energy of hydrolysis that occurs during the process of energy transduction was indeed related to a change in solvent structure around reactants and products at the catalytic site of enzymes. Thus, I decided to study other enzymes besides the Ca^{2+}-ATPase. The aim was to see if the correlation between water activity and changing energies of hydrolysis at the catalytic site could be extended to different enzymes involved in energy transduction.

FIGURE 10. **Armando (*left*) and Marietta (*right*) Gómez Puyou, distinguished scientists from the Universidad Nacional Autónoma de México (UNAM).**

Role of Water Activity in the Process of Energy Transduction by Different Enzymes

The chromatophores of the photosynthetic bacteria *Rhodospirillum rubrum* retain a membrane-bound inorganic pyrophosphatase that is able to catalyze both the synthesis and hydrolysis of PP_i. When illuminated, the chromatophores use the energy derived from light to form a proton gradient across the membrane, and the membrane-bound pyrophosphatase uses the energy derived from the gradient to catalyze the synthesis of pyrophosphate from inorganic phosphate. With Armando and Marietta Gómez Puyou (Fig. 10), we were able to show that (i) like the Ca^{2+}-ATPase, the *R. rubrum* inorganic pyrophosphatase is able simultaneously to catalyze the synthesis and hydrolysis of PP_i in the absence of a proton gradient; (ii) organic solvent promotes a decrease in the apparent K_m of the pyrophosphatase for P_i; and (iii) with purified soluble pyrophosphatase, it was possible to mimic the PP_i synthesis of the chromatophores in the light solely by changing the water activity of the medium (46).

After our studies on the effect of organic solvents on the Ca^{2+}-ATPase, Sakamoto (44) reported that the affinity of the soluble mitochondrial F_1-ATPase for P_i increased by several orders of magnitude when part of the water of the medium was replaced with dimethyl sulfoxide. As a result, there was an increase of several orders of magnitude in the formation of the tightly bound ATP, and this could now be easily measured using ^{32}P. In the absence of organic solvent, the presence of the ATP attached to the enzyme could only be inferred from $HOH \leftrightarrow O^{18}$ measurements. Sakamoto's findings were reproduced in different laboratories, including that of Gómez Puyou (53), with whom I had the privilege of collaborating. These findings indicated

FIGURE 11. **André Goffeau from the Université Catholique de Louvain in Belgium, well known for his important findings on the H^+-ATPase and DNA sequencing.**

that, as proposed by us for the Ca^{2+}-ATPase, during oxidative phosphorylation, the catalytic site of mitochondrial ATP synthase undergoes a hydrophobic-hydrophilic transition. In his 1997 Nobel Prize lecture, Boyer quoted our proposal as "the probable mechanism that promotes the spontaneous synthesis of the tightly bound ATP" (54).

Embedded in the plasma membrane of yeast is an H^+-ATPase that uses the energy derived from ATP hydrolysis to pump H^+ across the membrane. The catalytic cycle of this enzyme is similar to that of the Ca^{2+}-ATPase. Andre Goffeau, an expert on the yeast H^+ pump, is one of my best friends (Fig. 11), and by my good luck, he visited my laboratory several times. Together, we found that like the Ca^{2+}-ATPase, the yeast H^+-ATPase is able to catalyze the synthesis of ATP (ATP $\leftrightarrow P_i$ exchange) in the absence of an H^+ gradient and that a decrease in the water

activity of the medium leads to both a decrease in the apparent K_m for P_i and a severalfold increase in the rate of ATP synthesis (55, 56).

Like the Ca^{2+}-ATPase, the $(Na^+ + K^+)$-ATPase could be phosphorylated by P_i, and after the addition of excess Na^+, the phosphoenzyme could transfer the phosphate to ATP, synthesizing ATP after a single catalytic cycle in the absence of a transmembrane gradient. A few years later, it was found that in the absence of a gradient, a 200-fold decrease in the P_i concentration needed for half-maximal phosphorylation was obtained when 40–60% of the water of the assay medium was replaced with dimethyl sulfoxide. At this solvation concentration, the phosphoenzyme was not able to transfer its phosphate to ADP after the addition of excess Na^+. However, synthesis took place if the dimethyl sulfoxide concentration was decreased suddenly, as described for the muscle Ca^{2+}-ATPase. These findings indicated that, as with Ca^{2+}-ATPase, the conversion of the phosphoenzyme from a low- to high-energy form of the $(Na^+ + K^+)$-ATPase is related to a hydrophobic-hydrophilic transition at the catalytic site (57–59).

Thermogenesis: Heat Production by the Ca^{2+}-ATPase

Heat generation plays a key role in the regulation of the energy balance of the cell, and alterations of thermogenesis are noted in several diseases such as adiposity and thyroid hormone alterations (60–64). Skeletal muscle is by far the most abundant tissue of the human body and accounts for >50% of the total oxygen consumption in a resting human and can rise up to 90% during very active muscular work. Different studies have indicated that the hydrolysis of ATP by the muscle Ca^{2+}-ATPase is a major heat source contributing to animal thermogenesis (61, 63). During catalysis, a significant part of the energy derived from ATP hydrolysis is dissipated into the surrounding medium as heat. Until recently, it was assumed that the portion converted into heat was always the same, as if the energy released during ATP cleavage was divided into two non-interchangeable parts, one used for work (Ca^{2+} transport) and the other converted into heat. In more recent reports (65–67), we found that depending on the conditions used, the amount of heat released during the hydrolysis of ATP by the Ca^{2+}-ATPase can vary between 7 and 32 kcal/mol. This finding indicates that this enzyme is able to handle the energy derived from ATP hydrolysis in such a way as to determine the amount used for Ca^{2+} transport and the amount that is dissipated as heat. In this view, the total amount of energy released during ATP hydrolysis is always the same, but the fraction of the total

FIGURE 12. **Giuseppe Inesi, an expert on Ca^{2+}-ATPase and a dear friend from the University of Maryland School of Medicine in Baltimore.**

energy that is converted into either work or heat seems to be modulated by the Ca^{2+}-ATPase.

In 1995, Yu and Inesi (Fig. 12) (68) observed that the progressive rise in the Ca^{2+} concentration in the vesicle lumen promotes the hydrolysis of the phosphoenzyme form $2Ca:E_1\sim P$ (Fig. 9B, *reaction 7*) before being converted into E_2-P, and as a result, the cleavage of ATP is processed without a concomitant Ca^{2+} translocation through the membrane. This finding explained our results of heat production. The rate of reaction 7 determines how much of the energy derived from ATP cleavage is converted into heat and how much into work (Ca^{2+} translocation). These data were confirmed in different laboratories (65-73), and reaction 7 was referred to as uncoupled ATPase activity. During transport, a substantial amount of ATP is cleaved through the uncoupled route, and depending on the conditions used, the rate of the uncoupled ATPase activity can be 2–8-fold faster than that the ATPase activity coupled to Ca^{2+} transport. When the uncoupled ATPase activity is inhibited, there is both an increase in the Ca^{2+}/ATP ratio and a decrease in the yield of heat produced during cleavage of each ATP molecule (ΔH^{cal}), thus abolishing the difference of ΔH^{cal}. Both the coupled and uncoupled ATPase activities have the same Ca^{2+} dependence. The uncoupled ATPase activity can be specifically inhibited by fluoride and by curcumin, an inhibitor of carcinogenesis. Inhibition of the uncoupled ATPase activity is also observed in the presence of a low ATP and a high ADP concentration and when the assay medium temperature is decreased from 35 °C in homeo-

therms such as rabbits or below 25 °C in poikilotherms such as fish (65–73).

Final Comments

As I mentioned above, I like to spend some of my working time doing research in the social sciences and in science education. In these studies (1, 73), after a significant number of interviews, I found out that both senior and junior scientists are able to identify the competence of the speaker in a lecture by the way he or she presents data. If he or she mentions only the main experiments in simple language that can be easily understood with one set of data clearly related to the next and forming a cycle where beginning and end are obviously interconnected, then he or she is considered to be a top scientist. If a speaker presents various data that are not clearly related and do not form a circular story, then he or she is assumed to have a smaller degree of competence. Finally, if the speaker presents a lot of data, none of it necessarily related to the next, giving the impression that he or she does not clearly know the goal of the lecture, then he or she gets a very low score. Frequently, this type of speaker keeps mentioning that he or she has much more data (and slides) and, should the audience desire it, he or she can go on speaking. Although I am far being sure, I hope that my report was circular, but I know that it takes time to form a circular history. If one has the opportunity to repeat one's tale in different laboratories and in different systems, then one gets more confident, and the feeling is very rewarding. It is not the same temporary, frenetic joy that comes with a new finding. It is a sensation of plentitude that brings reassurance. This, however, does not prevent one from continuing the frantic search for the new. For instance, now I am studying brown adipose tissue in rats. This strange thermogenic tissue, found in both rats and humans, has a peculiar interconnection between mitochondria and reticulum rich in SERCA1, the same pump isoform found in white muscle. Unlike all other mitochondria, we found that SERCA1 is found not only in the reticulum but also inside mitochondria, and mitochondrial thermogenesis is activated by physiological Ca^{2+} concentrations. The amazing thing is that other mitochondria (liver, for instance) not only produce very little heat but are simply knocked down by the same Ca^{2+} concentrations that activate brown adipose tissue mitochondria (Ref. 74 and references within).

I had a strategy that proved useful for my students and me. When I went abroad and visited a colleague hoping to improve my way of thinking, I was able to talk with most of them for only a short period of time due to the many duties of a laboratory leader. Thus, I changed strategies. After several years working together, there is a tendency among the members of a team to think in a similar way. Thus, to expose the whole team to different forms of thinking, we invited scientists to our laboratory. That proved helpful in breaking the vicious thinking circle not only for me but for all the staff members as well. When outside his or her laboratory, the scientist is free of administrative obligations and can spend more time discussing science with others.

I like to work on two different types of research projects. The first type involves working on my own with my technician without involving students. In this type of work, I like to chase the improbable. These projects frequently result in no publication, a lot of reagent wasted, and intense frustration not shared by collaborators, but if something comes out, then I come a little closer to the ways of nature, and the emotion is also stronger and unshared.

In the second type of project, I work in strict collaboration with my students, including doing experiments together at the bench. The reasoning is that students cannot dedicate a limitless amount of time to the project. The university imposes strict time periods to graduate. In addition, students need publications to prove what they have done. As I have described above, to be a student is not an easy task. Finally, I must confess that when working with students, it never has been clear to me who learns more: me or the students. Frequently, students tell me that they are insecure and not sure if science is really their vocation. I have learned that a good test is how you feel on Friday and Sunday. If you feel happy on Friday because the next two mornings you do not need go to work and sad on Sunday because tomorrow it will start all over again, then you are in trouble because work is just work, a tedious task that one has to do to survive. Perhaps this is the root of the so-called Friday happy hour. However, if there is no difference in humor between Friday and Sunday, then you do not work; you simply follow your vocation, and you are doing what you really like to do. It is painful for me not be able to go to the laboratory when other matters prevent me from doing so.

⌘ *Author's Choice*—Final version full access.
Address correspondence to: leodemeis@terra.com.br.

REFERENCES

1. de Meis, L., Longo, P. H., and Falcáo, E. B. M. (1989) The learning process in science: a study among Brazilian biochemists. *Biochem. Educ.* **17,** 127–132

2. de Meis, L. (2003) in *Comprehensive Biochemistry: VII. Selected Topics in the History of Biochemistry* (Semeza, G., and Turner, A. J., eds) Volume 42, pp. 591–642, Elsevier B.V., Amsterdam

3. de Meis, L., and Leta, J. (1997) Modern science and the explosion of new knowledge. *Biophys. Chem.* **68,** 243–253

4. Tabor, H., Tabor, C. W., and de Meis, L. (1971) Chemical synthesis of *N*-acetyl-1,4-diaminobutane, N^1-acetylspermidine, and N^8-acetylspermidine. *Methods Enzymol.* **17B,** 829–833

5. de Meis, L., Rubin-Altschul, M., and Machado, R. D. (1970) Comparative data of Ca^{2+} transport in brain and skeletal muscle microsomes. *J. Biol. Chem.* **245,** 1883–1889

6. Wuytack, F., Papp, B., Verboomen, H., Raeymaekers, L., Dode, L., Bobe, R., Enouf, J., Bokkala, S., Authi, K. S., and Casteels, R. (1994) A sarco/endoplasmic reticulum Ca^{2+}-ATPase 3-type Ca^{2+} pump is expressed in platelets, in lymphoid cells, and in mast cells. *J. Biol. Chem.* **269,** 1410–1416

7. Lytton, J., Westlin, M., Burk, S. E., Shull, G. E., and MacLennan, D. H. (1992) Functional comparisons between isoforms of the sarcoplasmic or endoplasmic reticulum family of calcium pumps. *J. Biol. Chem.* **267,** 14483–14489

8. Cardoso, C. M., Rumjanek, V. M., and de Meis, L. (1997) Uncoupling of Ca^{2+} transport ATPase in muscle and blood platelets by diacylglycerol analogues and cyclosporin A antagonism. *Biochem. J.* **327,** 795–801

9. de Meis, L. (1969) Activation Ca^{2+} uptake by acetyl phosphate in muscle microsomes. *Biochim. Biophys. Acta* **172,** 343–344

10. de Meis, L. (1969) Ca^{2+} uptake and acetyl phosphatase of skeletal muscle microsomes. Inhibition by Na^+, K^+, Li^+, and adenosine triphosphate. *J. Biol. Chem.* **244,** 3733–3739

11. de Meis, L., and Hasselbach, W. (1971) Acetyl phosphate as substrate for Ca^{2+} uptake in skeletal muscle microsomes. Inhibition by alkali ions. *J. Biol. Chem.* **246,** 4759–4763

12. Lipmann, F. (1941) Metabolic generation and utilization of phosphate bond energy. *Adv. Enzymol.* **1,** 99–162

13. Fiske, C. H., and SubbaRow, Y. (1925) The colorimetric determination of phosphorus. *J. Biol. Chem.* **66,** 375–400

14. Keilin, D. (1969) in *Development of Concepts* (Kalckar, H. M., ed) pp. 178–191, Prentice Hall, Englewood Cliffs, NJ

15. Mitchell, P. (1961) Coupling of phosphorylation to electron and hydrogen transfer by a chemiosmotic type of mechanism. *Nature* **191,** 144–148

16. Skou, J. C. (1957) The influence of some cations on an adenosine triphosphatase from peripheral nerves. *Biochim. Biophys. Acta* **23,** 394–401

17. Hasselbach, W., and Makinose, M. (1961) The calcium pump of the "relaxing granules" of muscle and its dependence on ATP splitting. *Biochem. Z.* **333,** 518–528

18. Ebashi, S., and Lipmann, F. (1962) Adenosine triphosphate-linked concentration of calcium ions in a particulate fraction of rabbit muscle. *J. Cell Biol.* **14,** 389–400

19. Barlogie, B., Hasselbach, W., and Makinose, M. (1971) Activation of calcium efflux by ADP and inorganic phosphate. *FEBS Lett.* **12,** 267–268

20. Makinose, M. (1971) Calcium efflux-dependent formation of ATP from ADP and orthophosphate by the membranes of the sarcoplasmic vesicles. *FEBS Lett.* **12,** 269–270

21. Makinose, M., and Hasselbach, W. (1971) ATP synthesis by the reverse of the sarcoplasmic calcium pump. *FEBS Lett.* **12,** 271–272

22. Makinose, M. (1972) Phosphoprotein formation during osmo-chemical energy conversion in the membrane of the sarcoplasmic reticulum. *FEBS Lett.* **25,** 113–115

23. Kanazawa, T., and Boyer, P. D. (1973) Occurrence and characteristics of a rapid exchange of phosphate oxygens catalyzed by sarcoplasmic reticulum vesicles. *J. Biol. Chem.* **248,** 3136–3172

24. Masuda, H., and de Meis, L. (1973) Phosphorylation of the sarcoplasmic reticulum membrane by orthophosphate. Inhibition by calcium ions. *Biochemistry* **12,** 4581–4585

25. de Meis, L., and Masuda, H. (1974) Phosphorylation of the sarcoplasmic reticulum membrane by orthophosphate through two different reactions. *Biochemistry* **13,** 2057–2062

26. Kanazawa, T. (1975) Phosphorylation of solubilized sarcoplasmic reticulum by orthophosphate and its thermodynamic characteristics. The dominant role of entropy in the phosphorylation. *J. Biol. Chem.* **250,** 113–119

27. Boyer, P. D., Cross, R. L., and Momsen, W. (1973) A new concept for energy coupling in oxidative phosphorylation based on a molecular explanation of the oxygen exchange reactions. *Proc. Natl. Acad. Sci. U.S.A.* **70,** 2837–2839

28. Makinose, M. (1966) *Abstracts of the Second International Congress of Biophysics, Vienna*, No. 276

29. Makinose, M. (1969) The phosphorylation of the membranal protein of the sarcoplasmic vesicles during active calcium transport. *Eur. J. Biochem.* **10,** 74–82

30. de Meis, L., and Vianna, A. L. (1979) Energy interconversion by the Ca^{2+}-dependent ATPase of the sarcoplasmic reticulum. *Annu. Rev. Biochem.* **48,** 275–292

31. de Meis, L. (1981) in *The Sarcoplasmic Reticulum: Transport and Energy Transduction* (Bittar, E., ed) Volume 2, John Wiley & Sons, Inc., New York

32. de Meis, L., and Costa Carvalho Mda, G. (1974) Role of the Ca^{2+} concentration gradient in the adenosine 5′-triphosphate-inorganic phosphate exchange catalyzed by sarcoplasmic reticulum. *Biochemistry* **13,** 5032–5038

33. Carvalho, M. G., de Souza, D. G., and de Meis, L. (1976) On a possible mechanism of energy conservation in sarcoplasmic reticulum membrane. *J. Biol. Chem.* **251,** 3629–3636

34. de Meis, L. (1989) Role of water in the energy of hydrolysis of phosphate compounds: energy transduction in biological membranes. *Biochim. Biophys. Acta* **973,** 333–349

35. Tanford, C. (1984) Twenty questions concerning the reaction cycle of the sarcoplasmic reticulum calcium pump. *CRC Crit. Rev. Biochem.* **17,** 123–151

36. Toyoshima, C., and Mizutani, T. (2004) Crystal structure of the calcium pump with a bound ATP analogue. *Nature* **430,** 529–535

37. Knowles, A. F., and Racker, E. (1975) Formation of adenosine triphosphate from P_i and adenosine diphosphate by purified Ca^{2+}-adenosine triphosphatase. *J. Biol. Chem.* **250,** 1949–1951

38. de Meis, L., and Tume, R. K. (1977) A new mechanism by which an H^+ concentration gradient drives the synthesis of adenosine triphosphate, pH jump, and adenosine triphosphate synthesis by the Ca^{2+}-dependnet adenosine triphosphatase of sarcoplasmic reticulum. *Biochemistry* **16,** 4455–4463

39. George, P., Witonsky, R. J., Trachtman, M., Wu, C., Dorwart, W., Richman, L., Richman, W., Shurayh, F., and Lentz, B. (1970) "∼H_2O." An enquiry into the importance of solvation effects in phosphate ester and anhydride reactions. *Biochim. Biophys. Acta* **223,** 1–15

40. Hayes, D. M., Kenyon, G. L., and Kolman, P. A. (1978) Theoretical calculations of the hydrolysis energies of some "high-energy" molecules. 2. A survey of some biologically important hydrolytic reaction. *J. Am. Chem. Soc.* **100,** 4331–4340

41. de Meis, L., Martins, O. B., and Alves, E. W. (1980) Role of water, hydrogen ion, and temperature on the synthesis of adenosine triphosphate by the sarcoplasmic reticulum adenosine triphosphatase in the absence of a calcium ion gradient. *Biochemistry* **19,** 4252–4261

42. de Meis, L. (2002) Ca^{2+}-ATPases (SERCA): energy transduction and heat production in transport ATPases. *J. Membr. Biol.* **188,** 1–9

43. de Meis, L., and Inesi, G. (1982) ATP synthesis by sarcoplasmic reticulum ATPase following Ca^{2+}, pH, temperature, and water activity jumps. *J. Biol. Chem.* **257,** 1289–1294

44. Sakamoto, J. (1984) Effect of dimethylsulfoxide on ATP synthesis by mitochondrial soluble F_1-ATPase. *J. Biochem.* **96,** 483–487

45. Kandpal, R. P., Stempel, K. E., and Boyer, P. D. (1987) Characteristics of the formation of enzyme-bound ATP from medium inorganic phosphate by mitochondrial F_1-adenosine triphosphatase in the presence of dimethyl sulfoxide. *Biochemistry* **26,** 1512–1517

46. de Meis, L., Behrens, M. I., Celis, H., Romero, I., Gómez Puyou, M. T., and Gómez Puyou, A. (1986) Orthophosphate-pyrophosphate exchange catalyzed by soluble and membrane-bound inorganic pyrophosphatases. Role of H^+ gradient. *Eur. J. Biochem.* **158,** 149–157

47. Meltzer, S., and Berman, M. C. (1984) Effects of pH, temperature, and calcium concentration on the stoichiometry of the calcium pump of sarcoplasmic reticulum. *J. Biol. Chem.* **259,** 4244–4253

48. de Meis, L. (1984) Pyrophosphate of high and low energy. Contributions of pH, Ca^{2+}, Mg^{2+}, and water to free energy of hydrolysis. *J. Biol. Chem.* **259,** 6090–6097

49. de Meis, L., Behrens, M. I., Petretski, J. H., and Politi, M. J. (1985) Contribution of water to free energy of hydrolysis of pyrophosphate. *Biochemistry* **24,** 7783–7789

50. Behrens, M. I., and de Meis, L. (1985) Synthesis of pyrophosphate by chromatophores of *Rhodospirillum rubrum* in the light and by soluble yeast inorganic pyrophosphatase in water-organic solvent mixtures. *Eur. J. Biochem.* **152,** 221–227

51. Romero, P. J., and de Meis, L. (1989) Role of water in the energy of hydrolysis of phosphoanhydride and phosphoester bonds. *J. Biol. Chem.* **264,** 7869–7873

52. Janson, C. A., Degani, C., and Boyer, P. D. (1979) The formation of enzyme-

bound and medium pyrophosphate and the molecular basis of the oxygen exchange reaction of yeast inorganic pyrophosphatase. *J. Biol. Chem.* **254,** 3743–3749

53. Gómez Puyou, A., Tuena de Gómez Puyou, M., and de Meis, L. (1986) Synthesis of ATP by soluble mitochondrial F_1-ATPase and F_1-inhibitor-protein complex in the presence of organic solvents. *Eur. J. Biochem.* **159,** 133–140

54. Boyer, P. D. (1998) Energy, life, and ATP. *Biosci. Rep.* **18,** 97–117

55. de Meis, L., Blanpain J. P., and Goffeau, A. (1987) P_i in equilibrium ATP exchange in the absence of proton gradient by the H^+-ATPase from yeast plasma membranes. *FEBS Lett.* **212,** 323–327

56. Goffeau, A., and de Meis, L. (1990) Effects of phosphate and hydrophobic molecules on two mutations in the β-strand sector of the H^+-ATPase from the yeast plasma membrane. *J. Biol. Chem.* **265,** 15503–15505

57. Post, R. L., Toda, G., and Rogers, F. N. (1975) Phosphorylation by inorganic phosphate of sodium plus potassium ion transport adenosine triphosphatase. Four reactive states. *J. Biol. Chem.* **250,** 691–701

58. Jørgensen, P. L. (1982) Mechanism of the Na^+,K^+ pump. Protein structure and conformations of the pure $(Na^+ + K^+)$-ATPase. *Biochim. Biophys. Acta* **694,** 27–68

59. de Moraes, V. L., and de Meis, L. (1987) ATP synthesis by the $(Na^+ + K^+)$-ATPase in the absence of an ionic gradient. Effects of organic solvent. *FEBS Lett.* **222,** 163–166

60. Nunes, M. T., Bianco, A. C., Migala, A., Agostini, B., and Hasselbach, W. (1985) Thyroxine induced transformation in sarcoplasmic reticulum of rabbit soleus and psoas muscles. *Z. Naturforsch. C* **40,** 726–734

61. Clausen, T., Van Hardeveld, C., and Everts, M. E. (1991) Significance of cation transport in control of energy metabolism and thermogenesis. *Physiol. Rev.* **71,** 733–774

62. Levine, J. A., Eberhardt, N. L., and Jensen, M. D. (1999) Role of nonexercise activity thermogenesis in resistance to fat gain in humans. *Science* **283,** 212–214

63. Janský, L. (1995) Humoral thermogenesis and its role in maintaining energy balance. *Physiol. Rev.* **75,** 237–259

64. Lowell, B. B., and Spiegelman, B. M. (2000) Towards a molecular understanding of adaptive thermogenesis. *Nature* **404,** 652–660

65. de Meis, L. (1998) Control of heat production by the Ca^{2+}-ATPase of rabbit and trout sarcoplasmic reticulum. *Am. J. Physiol.* **274,** C1738–C1744

66. de Meis, L. (2001) Role of the sarcoplasmic reticulum Ca^{2+}-ATPase on heat production and thermogenesis. *Biosci. Rep.* **21,** 113–137

67. de Meis, L. (2001) Uncoupled ATPase activity and heat production by the sarcoplasmic reticulum Ca^{2+}-ATPase. Regulation by ADP. *J. Biol. Chem.* **276,** 25078–25087

68. Yu, X., and Inesi, G. (1995) Variable stoichiometric efficiency of Ca^{2+} and Sr^{2+} transport by the sarcoplasmic reticulum ATPase. *J. Biol. Chem.* **270,** 4361–4367

69. Fortea, M. I., Soler, F., and Fernandez-Belda, F. (2000) Insight into the uncoupling mechanism of sarcoplasmic reticulum ATPase using the phosphorylating substrate UTP. *J. Biol. Chem.* **275,** 12521–12529

70. Logan-Smith, M. J., Lockyer, P. J., East, J. M., and Lee, A. G. (2001) Curcumin, a molecule that inhibits the Ca^{2+}-ATPase of sarcoplasmic reticulum but increases the rate of accumulation of Ca^{2+}. *J. Biol. Chem.* **276,** 46905–46911

71. Reis, M., Farage, M., de Souza, A. C., and de Meis, L. (2001) Correlation between uncoupled ATP hydrolysis and heat production by the sarcoplasmic reticulum Ca^{2+}-ATPase: coupling effect of fluoride. *J. Biol. Chem.* **276,** 42793–42800

72. de Meis, L., Arruda, A. P., and Carvalho, D. P. (2005) Role of sarco/endoplasmic reticulum Ca^{2+}-ATPase in thermogenesis. *Biosci. Rep.* **25,** 181–190

73. Grynszpan, D., and de Meis, L. (1990) The concept of creativity among biochemists. *Biochem. Educ.* **18,** 167–170

74. de Meis, L., Ketzer, L. A., da Costa, R. M., de Andrade, I. R., and Benchimol, M. (2010) Fusion of the endoplasmic reticulum and mitochondrial outer membrane in rats brown adipose tissue: activation of thermogenesis by Ca^{2+}. *PLoS ONE* **5,** e9439