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A Fortunate Journey on Uneven Grounds

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Abstract

I was surprised to be invited to write a prefatory chapter for the *Annual Review of Microbiology*. Indeed, I did not feel that I belonged to that class of eminent scientists who had written such chapters. Perhaps it is because I am a kind of mutant: In spite of having experienced war, both German and Soviet occupations, repeated bombardments, dictatorships, and a revolution, I managed nonetheless to engage in scientific research, thus realizing a childhood dream. After having obtained my Doctor Rerum Naturalium degree in Budapest, Hungary, I was fortunate to meet Jacques Monod at the Pasteur Institute, and this became a turning point in my scientific career. In his laboratory, I contributed to the definition of the lactose operon promoter, uncovered intracistronic complementation in β-galactosidase, and investigated the role of cAMP in *Escherichia coli*. In my own laboratory, together with many gifted students and collaborators, I studied the role of adenylate cyclase in bacterial virulence. This allowed the engineering of recombinant adenylate cyclase toxin from *Bordetella pertussis* for the development of protective and therapeutic vaccines.

Contents

BEGINNINGS

I was born in 1927 in Transylvania, a region in the Carpathian Basin, which now belongs to Romania. To many people, this region is associated with the bloodthirsty vampire Count Dracula. Transylvania was always a multiethnic and multilingual region; it belonged intermittently to Hungarians, Romanians, and Germans and was even part of the Ottoman Empire. It was considered one of the cultural centers in Central Europe. Most cities in Transylvania carried three names in three different languages, the last one used depending on the last occupant.

As a child, I was trilingual, speaking Hungarian, Romanian, and German. According to distinguished linguists, when a child begins talking by speaking three languages, she ends up

speaking none of them correctly. Alas, in my case, they were right. I attended public schools and, from age eleven, I was more interested in sports than in what was taught in school. I was doing athletics, rowing and playing tennis and ping-pong (I even earned medals!). Sports helped me in many ways. I was twelve when World War II began. I spent much of my time on sport fields (except during bombardments), which kept me away from the daily war problems. I had an excellent athletic trainer who taught me how to practice correctly. Once, having lost a 100-m sprint during a competition, I had asked him whose example I should follow to be successful. He answered that I didn't have to compare myself to anybody; all I had to do was to carry a stopwatch while running and try to do better than the day before. Maybe that lesson helped me later in science.

After finishing college, I decided to study science at the University of Cluj in Romania. My father, who had always hoped that I would become a doctor like my grandfather and my aunts, was not entirely surprised by my choice. Apparently, as a child, I constantly pestered him with odd questions such as "How come cows who eat green grass give white milk?"

My first real interest in science began when I was 14, after my father had given me for my birthday a Hungarian translation of Paul de Kruif's *Microbe Hunters*, a beautifully bound book with many pictures of microbes and scientists such as Antoni van Leeuwenhoek, Louis Pasteur, Robert Koch, and Paul Ehrlich. I had dreamed of becoming a microbe hunter and had chosen Pasteur as my hero because of his incredible accomplishments in so many fields. Seventeen years later, my dream was fulfilled: I began working at the Pasteur Institute in Paris. But many things happened in the years in between.

I earned my high school diploma (baccalauréat) in 1945 from a Romanian public school in Arad, Transylvania (now Romania), just as the war ended. I then attended the Faculty of Science at the University of Cluj. During World War II, Cluj, a charming city in the hills of Transylvania, belonged to Hungary and still bore its Hungarian name of Kolozsvár (Klausenburg in German) when I arrived there. Transylvania returned to Romania after the Paris Peace Treaties in 1947. During my first two years at the University, Cluj was a kind of no-man's-land. The civil population had fled Transylvania, not knowing to whom it might belong next. The Opera House was running. Attendance was almost free for students; furthermore, it was heated during our ice-cold winters. Artists and poets came from many Western countries: Jacques Thibaud and Yehudi Menuhin played violin sonatas for the students, and Tristan Tzara lectured on the Dada movement and surrealism, which I had never even heard of. Furthermore, even though the postwar period was not easy and we were often hungry and cold, we were totally immersed in a true climate of culture. By contrast, science instruction at the university was so inadequate that, after a couple of years, I decided to transfer to the University of Budapest in Hungary. But then, of course, I had to switch from Romanian to Hungarian.

In 1949, I obtained my chemistry diploma from the University of Science in Budapest. Predictably, in addition to the scientific subjects, I had to pass an exam in Marxism. Then, I started to work in the Department of Medical Chemistry, directed by Bruno F. Straub, a former student of the Nobel Prize winner Albert Szent-Györgyi. Everyday life was difficult not only for political reasons but also because salaries were very low. The Soviet Army had occupied Hungary since the end of the war, and a real Communist dictatorship was established in 1947. Later, Hungary became the Hungarian People's Republic. Political purges and show trials began; around 100,000 persons were arrested or deported to concentration camps and several thousands were executed. For several years, Hungary experienced the harshest dictatorship in Europe. I was also arrested but miraculously released after 48 hours. My schoolmate, who was arrested at the same time as me, was sentenced for no reason and, as I later learned, executed.

FIRST STEPS IN RESEARCH

Actin

Since the discoveries of Szent-Györgyi on muscle contraction, research at the Department of Medical Chemistry in the late 1940s had been dedicated to muscle proteins. Just before I arrived in 1949, Straub had discovered that actin contained bound ATP and that, during its polymerization into filamentous F-actin, ATP was hydrolyzed to ADP, yielding ADP-F-actin. I was charged with studying the mechanism of this polymerization as a function of ATP dephosphorylation, the hypothesis being that the energy of polymerization was provided by the hydrolysis of ATP. By studying the kinetics of polymerization as a function of temperature, I found that at 0°C F-actin still contained bound ATP instead of ADP. To explain this result, I suggested that actin itself might exhibit some ATPase activity. This idea was rejected offhand as an unorthodox hypothesis and the project was brought to an end. Nevertheless, many years later, it was shown by others that actin, indeed, had intrinsic ATPase activity.

It may be interesting to recall how we measured ATP concentrations when no spectrophotometer was available. We used a method set up by Szent-Györgyi himself based on the observation that a highly polymerized actomyosin gel dissociates and depolymerizes in the presence of ATP. So, we measured the kinetics of depolymerization as a function of known concentrations of ATP in a viscosimeter kept in ice. Once the reaction was calibrated, it was easy to determine the experimental ATP values.

Erythrocyte ATPase

A small group in the laboratory was studying the potassium permeability of erythrocytes. They found that the intracellular concentration of K^+ was dependent on the level of ATP. This was in the 1950s, when assays for measuring the concentration of K^+ were extremely time-consuming. We thought that an ATPase might be involved in potassium permeability; therefore, I decided to purify this hypothetical ATPase because the inorganic phosphate generated could easily be determined colorimetrically. At that time, one could not purchase ATP. We had to prepare the material ourselves from rabbit skeletal muscle, which was time-consuming. When I accumulated enough ATP, I started to purify the enzyme from different blood sources, but to my surprise, I could never purify it more than approximately 50-fold, nor could I obtain it in soluble form. Fortunately, an electron microscope was available in the building, and to my amazement, all the purified preparations were revealed as beautiful erythrocyte ghosts. Clearly, the enzyme was membrane bound and I could show that treatments that damaged the membrane integrity led to a loss of enzymatic activity. Due to my inexperience, I was not able to demonstrate any correlation between potassium transport and ATPase activity; I also missed the Na^+ and K^+ activation of the enzyme. Nevertheless, to my knowledge, this was the first observation of a membrane-bound ATPase. The results were published in German in 1952 in a practically unknown journal, *Acta Physiologica Hungarica*, with the obligatory abstract in Russian.

In Vitro Protein Synthesis

In the early 1950s, I became interested in protein biosynthesis. One had to choose a model system compatible with our limited working possibilities. We found a publication from Fritz Lipmann's laboratory describing the in vitro synthesis of cytochrome *c*. Even though I strictly followed their experimental conditions, I could not for months duplicate their results and finally gave up. Straub

thought that I was totally incompetent if I couldn't even repeat an experiment coming from Lipmann's lab, but because I carried heavy teaching duties, he needed me around and accepted that I choose another project.

The cytochrome *c* story came to an end when many years later I met Fritz Lipmann and asked him what I could have done wrong for not having been able to reproduce his experiments. He said, "Thank God you could not reproduce them." It turned out that one of his collaborators had falsified the data and the paper had to be retracted. Lipmann was genuinely sorry for me and, from then on, each time I was in New York, arranged for me to stay in the guesthouse of Rockefeller University. Several years later, I told that story to Albert Szent-Györgyi, and he remarked that cheating was unfair toward Fritz because "Fritz was a person who only perceived the music of nature without listening to the background noise."

After my failure with cytochrome *c*, a small group of us in Straub's laboratory decided to study the formation of α-amylase under a variety of in vitro conditions with the hope of obtaining some information about the mechanism of its biosynthesis. L.E. Hokin had described in 1951 an increase of amylase activity in pancreas slices in vitro. These data were perfectly reproducible and the amylase system kept me busy for about four years. I set up different cell-free systems and studied the requirements to obtain an increase in amylase activity. In retrospect, much work was invested but the cell-free systems we had set up were ill-defined and some of the conclusions reached were not straightforward. When we finally obtained radioactive glycine, thanks to a microscale one-step isolation procedure we had devised, we were able to demonstrate a specific incorporation of glycine into amylase. The microscale method consisted of adsorbing amylase-containing extracts at 0°C on insoluble starch we had prepared, washing it exhaustively in the cold, and finally dissociating the starch-amylase complex in buffer at 37°C. The method yielded practically pure amylase. When we incubated pancreas slices in the presence of radioactive glycine and measured the incorporation of radioactivity in purified amylase, we observed a considerable time lag in the labeling of the enzyme compared with that of total proteins. This finding suggested the presence of some intermediary steps (a precursor?) in the synthesis of amylase. In cell-free systems, we could observe an increase in amylase activity but no specific labeling of the enzyme. Because ribonuclease inhibited this reaction, we concluded that RNA was somehow implicated in the process. The precursor hypothesis was never substantiated, and at that time, we were not able to define the intermediary steps in enzyme formation. This work led to eight publications, seven of them in *Acta Physiologica Hungarica*, and formed the substance of my doctoral thesis.

INTERMISSION

As mentioned above, when I arrived in Budapest in 1947, the Communist dictatorship was already installed and the Soviet Army occupied Hungary. From 1949 onward, political trials followed by death sentences were daily events. Those who were not arrested or purged lived in constant fear.

University studies were totally corrupted and the teaching of genetics was forbidden. Only the theories of the Russian agronomist Trofim Lysenko were taught. He rejected the science of genetics, believed in the heritability of acquired characteristics, and claimed that their inheritance played a major role in evolution. He considered Mendel's principles incompatible with dialectic materialism, which pleased Stalin. He not only succeeded in ruining Soviet agriculture but also caused the elimination of the best Soviet geneticists. It was during the Lysenko affair, in 1948, that I first heard of Jacques Monod. Western newspapers were banned in Hungary but a friend of mine succeeded in smuggling a newspaper called *Combat*, directed by Albert Camus and dated September 19, 1948. In it was an article by a certain Dr. Monod entitled "The Victory of Lyssenko

Has No Scientific Basis." To me the article came as a revelation and I decided on the spot that I would meet this Dr. Monod one day. Indeed I did 10 years later.

In 1949, when I started research and teaching, the political climate was not at all conducive to quiet scientific pursuits (though this was certainly not my only excuse for having been unsuccessful in research). Teaching was not easy: One was compelled to tell the students that all the main discoveries in science were made by Russian scientists. Once, at the end of a general chemistry lecture, a student quietly asked me who was the Western scientist who had really discovered the Mendeleev Periodic Table. He couldn't believe me when I told him that it really was Mendeleev. Everything associated with the Russians was summarily dismissed.

After Stalin's death in 1953, Hungary's Communist regime started to crumble. The voice of the opposition was no longer silenced; many of the persons who had been deported or arrested and who were still alive were rehabilitated. Public debates occurred everywhere (I participated in many of them) and justice was demanded for those guilty of murders. But we did not anticipate the 1956 Revolution. It actually started on October 23 during the course of a huge demonstration. We were about 50,000 marching peacefully to pay homage to the heroes of the 1848 Hungarian Revolution. Toward its end, when the crowd began to disperse and a few of the participants went to the radio station to deliver an appeal for freedom, the men of the secret police began to shoot. This happened next to the university while we were holding a meeting to discuss the follow-up of our afternoon protest. That evening, one of the symbols of the dictatorship, a giant bronze statue of Stalin, was toppled and smashed to pieces. The next day, groups of young people with newly acquired weapons roamed Budapest, and in the following days, the uprising spread throughout the country. On October 27, the Revolutionary Committee of Intellectuals was founded and established itself in one of the university buildings. I joined them there and I helped them coordinate their daily activities. It was an unbelievably exciting period of time because, for once, we felt free. We stayed there day and night because nobody owned cars and public transportation had stopped. Young people requisitioned Soviet tanks (unbelievably, in those days the Russians let them do that). Once, they even picked me up and drove me home. That was the noisiest ride I ever experienced.

We were full of enthusiasm and totally naïve. We really believed the Russian promises that they would withdraw from Hungary. But at 4 AM on November 4, 15 Russian armored divisions equipped with 6,000 tanks launched their attack on the unsuspecting country, putting an end to the revolution. Fighting, however, went on for many days and chaos continued to reign for many months. Hundreds of people were arrested and more than 1% of the population fled the country. By the end of the year, I was back at the bench and resumed teaching, but times were hard because, once again, we were living in constant fear.

MEETING JACQUES MONOD

In 1957, I received an invitation to attend a Symposium of the Society for Experimental Biology in London. Obtaining a passport was like a lottery. I was 30 years old and I had never left the country. Unexpectedly, I received my passport but I still needed an English entry visa plus transit visas to travel through Austria, Switzerland, and France. For unknown reasons, the British refused to grant me a visa. Fortunately, I knew the French consul in Budapest and he provided me with a French visa. So, I left for Paris (with only the five dollars I was allowed to carry with me), with the hope that I would meet the famous Dr. Monod who had been fighting against Lysenko's theories.

After arriving in Paris in January 1958, I immediately asked for an appointment with Professor Monod, which I rapidly obtained. I was nervously waiting in his secretary's office at the Pasteur Institute, fully expecting to meet a gray-bearded, distinguished old gentleman. To my surprise, a young fellow came whistling down the corridor and introduced himself as Jacques Monod. He

was friendly but looked very busy. In fact, that was when the famous Pardee–Jacob–Monod (the PaJaMa) experiment, which led to the operon model, was being carried out. The fastest way to get rid of me was to invite me to present a seminar the next day. Following that, Monod politely asked me what I was doing in Paris and how long I intended to stay. Gathering all my courage, I told him that if he allowed me to work in his lab, I could extend my stay to six weeks; otherwise, I would have to return to Budapest very shortly. The next morning, I started working with François Gros, who at that time was interested in the mechanism by which chloramphenicol inhibited protein synthesis. I learned, for example, how to prepare bacterial extracts and how to measure aminoacyl-tRNA complexes, but I did not obtain any conclusive results during my stay (the inhibitory effect of chloramphenicol on peptidyl-transferase was discovered many years later).

One Saturday morning, the Spinco centrifuge in the corridor refused to start. A group of people was standing around it, rather perplexed, trying to figure out what was going on. I approached them asking if I could be of help. They looked at me incredulously, wondering how somebody coming from an Eastern country could have the technical know-how to repair a Spinco. I solved the problem in two minutes: The needle that makes the electric contact with the rotor had fallen off. That was for me an enormous success. They didn't know that, in Hungary, many such instruments were embargoed and could only be delivered in separate parts; we had to put everything together ourselves. Before coming to Paris, we had just reconstructed a Spinco but at the end of putting it together we found a needle in a small box. After working our way through the instructions for another hour, we finally discovered that it was an essential item that had to be installed last.

My collaboration with François Gros developed into a close friendship to the point that I felt I could speak to him openheartedly. One day, I confided to him that I wanted to leave Hungary for good, come to France, and go on working forever in the lab. Somewhat puzzled, he advised me to discuss this problem with Monod, but since I did not dare do it, he did it for me. The next day Jacques Monod invited me for dinner at his home. After a few hours during which I recounted what had happened during the revolution, the unending menace of persecution under which we were living, constantly fearing for our security, Jacques told me that he was willing to do everything in his power to help me. When I asked him why, he simply answered: "It is a matter of human dignity."

BACK IN BUDAPEST

After almost two months in total bliss, I had to leave Paris, which I did with a heavy heart. I had to go back to Budapest; otherwise I would have endangered my husband, my colleagues, and my friends. But I knew Jacques Monod would try to help. Indeed, a few weeks later, I received a letter from him indicating that I had to return to Pasteur for a few months to finish some crucial experiments. Of course, it was only a pretext for me to renew my passport.

Meanwhile, I wanted to take advantage of the techniques I had learned with François Gros and decided to study the mechanism of action of streptomycin. My husband, Tom Erdös, a former student of Szent-Györgyi who had been jailed after the revolution for a few months for political reasons, was working in a large tuberculosis institute where nonpathogenic strains of mycobacteria, including some streptomycin-sensitive, resistant and dependent strains of *Mycobacterium friburgiensis*, were available. The strains were certainly not isogenic but behaved as expected in bacterial cultures. We prepared cell extracts and looked for the incorporation of radioactive tyrosine (the only labeled amino acid we had available at that time) in the presence or absence of streptomycin. We observed no effect of streptomycin on the formation of the aminoacyl-tRNA complex but, to our great satisfaction, found that streptomycin did inhibit the incorporation of labeled tyrosine into a subcellular fraction. It was the first time that streptomycin had been

shown to inhibit protein synthesis. We were even more satisfied when we could show that in streptomycin-resistant strains, streptomycin did not inhibit amino acid incorporation whereas in streptomycin-dependent strains, the antibiotic was required for incorporation. Although we could not characterize our "subcellular fraction" (later identified as ribosomes), we sent two short letters to *Nature* that were rapidly published (8, 9).

Around February 1959 I obtained for the second time a passport that would allow me to return to Paris. In no time, I secured the needed French and transit visas and left Budapest with the hope of never coming back. No return meant that something would have to be organized for my husband, Tom, to leave Hungary illegally. That was Jacques Monod's original idea: He figured that it would be easier to smuggle one person out rather than two, and thought that we would have plenty of time to plan for his escape during the six months I would be in Paris. With Tom, we agreed on a simple telephone code by which I would let him know how to proceed if his escape could be organized. This was essential because, since his arrest, he was under constant police surveillance. When my train departed Budapest, I was hoping that everything would work out as we had planned.

THE ESCAPE

François Gros was waiting for me at the Paris railway station and drove me directly to Pasteur. At that time, François was trying to identify messenger RNA, whose existence had been suggested by the PaJaMa experiment (37). He proposed that I share this task with him. For many weeks, we labeled *Escherichia coli* cultures with several millicuries of ^{32}P. After centrifugation, we ground the bacteria with alumina powder in a cooled mortar, suspended the mixture in buffer, and loaded the centrifuged suspension on a sucrose gradient. After eight hours of centrifugation, we counted the drops. The ribosomes were well separated but the radioactivity was never where it was expected to be found. When I asked how long I had to grind the bacteria, someone told me until I heard "the bacteria crying." All these procedures would seem quite folkloric today. A year later, when François spent some time in Jim Watson's laboratory, he finally succeeded in identifying the messenger (16).

Meanwhile, with the help of a friend (a Hungarian refugee who was living in Vienna, close enough to Hungary), Jacques Monod was trying to arrange for Tom's escape. Several strategies were considered, such as constructing a special car with a hiding place or stowing away aboard one of the Danube tankers. That latter plan nearly went through but for the first time in 50 years, the Danube was so shallow that all river traffic was interrupted. Jacques had collected money from many friends and colleagues but all our successive plans collapsed. We finally ran out of time and I had to return home. But I never doubted Jacques's total commitment to get us both out of Hungary, one way or another.

Before leaving, I had an idea of how we should correspond with Jacques and his secretary, Madeleine Brunerie, to avoid the surveillance by the secret police. For the first time, my work with amylase paid off. Knowing that one can detect starch with iodine, I proposed to write my messages with a starch solution that Jacques or Madeleine would reveal with an iodine solution. I demonstrated how this would work and Jacques liked the idea because it reminded him of his time in the Resistance. François Jacob and his wife, Lise, accompanied me to the railway station. Lise bought me books by Albert Camus, Jean-Paul Sartre, and Simone de Beauvoir, among others, most of which were prohibited in Hungary. So I spent the 20 hours of travel time reading those books, throwing away all those that were banned before reaching the border. I was particularly sorry to get rid of the books by George Orwell. But in Budapest, a colleague working in the zoology department succeeded in ordering a single copy of Orwell's *Animal*

Farm, along with other scientific books, and it was then circulated among several hundreds of us. Another friend in the history department never dared to order Orwell's *1984*: It was simply too dangerous.

Between September 1959 and April 1960, nothing happened in Budapest, except some exchanges of messages written with starch on the cover sheets of records by Béla Bartók or, more often, Mstislav Rostropovich (Jacques Monod was a cello player). Most of the strategies for getting us out of Hungary had failed. All Monod's correspondence concerning these plans, their organization, and attempts to raise the needed money can now be found in several folders in the *Fonds Monod* at the Archives of the Pasteur Institute. Once in Paris, it was a breathtaking experience for me to go over them.

In May 1960, Jacques arrived in Budapest with another carefully prepared scheme for our escape. Officially, he had been invited by the Hungarian Academy of Sciences to present some lectures. His first lecture on regulation was a tremendous success: It was the first time since 1948 that the word "gene" had been pronounced. His entire visit was a memorable event for the Hungarian scientific community. The new escape plan he presented seemed feasible to us: We were to meet at a given place with an Austrian "tourist" owning a trailer modified to contain an appropriate hiding place. Once inside it, he would drive us to Austria. At that time, Hungarian borders were tightly sealed and crossing could be nightmarish. Jacques asked us what would happen if the police caught us? We answered "prison for twenty years." When we assured Jacques that we were definitely willing to take that risk, we drove along the Danube to find an adequately isolated place where the rigged trailer could pick us up.

Our escape was scheduled for a Saturday in June 1960. The trailer arrived as planned and we crammed ourselves under a bathtub. At the border, two customs officers searched every corner of the vehicle with a flashlight for more than one hour. It was an absolute miracle that they did not catch us. The officers finally left the trailer and, after a few more controls, we were allowed to proceed. Shortly afterward, I smelled gasoline. Because there were no gas stations on the Hungarian side of the border, I knew we were in Austria.

We became political refugees in Vienna, and our only possessions were the clothes on our backs. Monod and other friends made arrangements to send some money and thanks to Philippe Monod, Jacques's brother, a diplomat who would later be named ambassador to Australia, we rapidly obtained all the official documents that would permit us to travel to France.

AT THE PASTEUR INSTITUTE FOR GOOD

Having participated emotionally in this rescue operation for two years, Madeleine Brunerie was waiting for us at the Paris train station and offered us a cup of coffee and a "liberty croissant." Philippe Monod kindly lent us his apartment. Jacques secured for each of us a Rockefeller Fellowship. I stayed at the Pasteur Institute while Tom worked at a research institute outside Paris.

Lactose Repressor

I wanted to begin working immediately. Jacques Monod told me that a few months earlier François Gros and Georges Cohen had attempted to isolate the Lac repressor but had abandoned that project. He suggested that I take it over. At that time, the Lac repressor was thought to be RNA that would bind inducer. I started to induce wild-type, constitutive, and repressor-negative bacteria with labeled ^{14}C-methyl-thiogalactoside (TMG) and succeeded in isolating labeled RNA. However, we still obtained stable TMG-RNA complexes in strains that were deleted for the lactose region and furthermore inducers other than TMG failed to bind to RNA. Therefore, we had to

relinquish the idea that the repressor could form a stable complex with inducer. Later, when I analyzed certain TMG-labeled RNA fractions, I found to my great surprise that it was the methyl group, not TMG, that was bound to RNA: TMG served as a methyl-donor to yield methyl-adenine. In short, I had discovered RNA methylation before Ernest Borek! But because our goal was to isolate the repressor, I never investigated this phenomenon further.

At the same time, a number of other pieces of evidence came to light suggesting that the repressor might be a protein and that its inactivation would not necessarily involve a covalent interaction. Because the repressor was supposed to exhibit a strong affinity for both DNA and the inducer, I thought of making use of this property to purify it. I set up DNA-affinity columns (the first: it was 1960) and tried to elute specific fractions with inducer. The results were consistently negative. We then turned to a genetic approach, i.e., measured β-galactosidase basal levels in diploid strains harboring varying numbers of operator and regulatory genes. The data indicated that the number of repressor molecules was no higher than 10 per cell. This was so discouraging that I asked François Jacob if it were possible to obtain a strain that would make ten or a hundred times more repressor? He said that it was hopeless. Well, a few years later, Wally Gilbert & Benno Müller-Hill (13) did obtain those mutants, which allowed them to purify the Lac repressor and show that it was indeed a protein.

Lactose Promoter

By the end of 1963, most of the major concepts regarding the structure of the Lac operon had been delineated by Jacob and Monod. One question that remained to be solved was the site at which the transcription of the structural genes would commence. Genetic evidence suggested that transcription would not start at the operator. If an additional site existed, then joining structural genes of one operon to the control system of another would result in the subordination of those structural genes to the regulatory mechanism of the other operon. To check this prediction, without running the risk of deleting essential genes, François Jacob isolated a series of deletions on *Flac* episomes. The deletions extended on one side over various lengths of the *lacZ* gene (they were selected for the presence of a functional permease); on the other side, we expected them to extend into the alkaline phosphatase region. I participated in this project and was hoping that François would isolate a *pho-lac* fusion in which the Lac permease would be repressed by inorganic phosphate. But after having analyzed several thousand clones without success, François obtained deletions extending into *purE*. We proceeded like workers on an assembly line: François analyzed the petri dishes, his technician cultured the clones, and I analyzed the cultures to determine whether the permease would be regulated by purine (not an easy assay, by the way). I usually arrived at the lab late but also left late. Every morning, François would be pacing the corridor, growing increasingly impatient, and greet me with such remarks as "It is quite late to start." In the evening, he left early to have dinner with his children, but before leaving, he passed by my lab to ask whether there was anything new. "Not yet," I would always reply. Each time he mumbled something like, "Had you started earlier . . . call me if there is anything new."

One evening around 9 PM I found a clone in which purine repressed both permease and transacetylase synthesis. The *purE-lacZY* fusion had just created a new operon. Instead of calling François right away, I went to a movie. I called him when I came back, around midnight. Obviously I woke him up. All he said, curtly, was "Merci." The next day I arrived even later than usual. François did not make any remarks; he only asked to see the results. He then told me that next time he would prefer that I call him before 10 PM. It was the last time I ever heard him comment on my working hours.

These experiments represented the first example of in vivo genetic engineering (21). Gene fusions were to become a major tool to study genetic analysis and regulation. I sent a telegram to Monod, who was at the Salk Institute in La Jolla, California, to tell him of the result and took off to go skiing for 10 days.

β-GALACTOSIDASE COMPLEMENTATION

Early studies by Melvin Cohn in Jacques Monod's laboratory in the 1950s demonstrated that the *E. coli* β-galactosidase was a tetramer composed of four identical polypeptides (36). Later, David Perrin's work (38) on complementation between various *lacZ* point mutants favored the idea that the *lacZ* gene consisted of a single cistron.

Looking for gene fusions while working on the *lac* promoter, François Jacob obtained a large collection of deletions. Some of those covering the *lac* regulatory region and extending to various sites of the *Z* gene did complement certain promoter-distal point mutants. This raised the possibility that the *Z* gene might consist of several cistrons. François refined the in vivo complementation tests while I set up an in vitro system with crude extracts of different deletion mutants. Fortunately, the two approaches converged with the conclusion that all deletions not extending beyond a certain point in the gene (which we called the ω-barrier) would complement all promoter-distal point mutants or deletion mutants of the *Z* gene. We called this phenomenon ω-complementation. I purified the ω-peptide from the largest deletion still displaying complementation and showed that the ω-region represented about one-third of the total genetic length of *lacZ* (53). In 1994, Matthews and colleagues (22) determined the three-dimensional structure of β-galactosidase and showed that the polypeptide chain folded into five sequential domains. They concluded that the C-terminal third of β-galactosidase could fold independently from the rest and probably corresponded to the ω-fragment. It was most rewarding to see a crystallographic confirmation of our results 30 years after they had been obtained by genetic and biochemical approaches.

In the main corridor of the Institute, called *avenue de l'opéron*, discussions whether the *lacZ* represented one or several cistrons were reawakened when Gary Craven in Chris Anfinsen's laboratory presented some evidence that the β-galactosidase monomer might consist of several peptides. Chris invited me to spend a few months in his laboratory at the National Institutes of Health to settle this point, but by the time I arrived in 1965, the *lacZ* had once again reverted to a single cistron. That was my first visit to the United States: I learned some protein chemistry, met many new colleagues, and gave a few seminars at different institutions. However, my trips were restricted and I could only travel with the permission of the US Attorney General's office (they granted most of my requests). Indeed, after an investigation that lasted a few months, the visa office of the US Embassy in Paris had concluded that I was an Eastern European spy, so I was issued only a "restrictive" visa that did not allow me to move freely throughout the United States.

In fact, I remained subject to these idiotic restrictions for the next 20 years. One of my later trips took a turn to the absurd. Around 1970, I had gone to the US Embassy for a visa to attend a scientific Battelle Conference in Seattle, Washington, to which I had been invited. The civil servant involved insisted in examining my travel reservations. When I told him that I was flying from London to Seattle, he asked how I would travel from Paris to London. Exasperated, I told him I would swim. He became furious and refused to issue the visa until I showed him my Paris-London ticket, but that was not the end of it. The London flight was delayed such that we arrived in Seattle a few minutes past midnight, and I was refused entry because my restrictive entry visa had expired at midnight. I tried to explain what had happened to no avail. Fortunately, I had met Manfred Eigen at Heathrow Airport; he was also flying to Seattle to attend the same meeting.

Manfred had been awarded the Nobel Prize in Chemistry three years earlier and he told them that if they did not allow me in, he wouldn't enter either. The greatly embarrassed customs officer finally relented and we were both allowed to proceed in the company of Eddy Fischer, who had come to pick us up.

Once back at the Pasteur Institute, I tried to understand the mechanism of ω-complementation. I purified both in vivo– and in vitro–complemented enzymes, studied their properties, and filled up several notebooks. The conclusion was straightforward: ω-Complementation involved noncovalent association between peptides corresponding to different fragments of the wild-type chain. This finding was confirmed by immunological studies carried out with Franco Celada. Having shown that antibodies directed against the ω-peptide reacted with the wild-type enzyme, we concluded that ω was able to fold itself into the correct wild-type structure, indicating that it had to contain an independent nucleation center (4). This appeared evident after examining Matthews's X-ray structure (22).

Once ω-complementation was settled, I wondered whether other combinations of deletion mutants would generate an active enzyme. When I mixed extracts containing partial deletions of the promoter proximal segment of *lacZ* with extracts of β-galactosidase-negative mutants whose promoter-proximal segments were intact, I recovered enzymatic activity: α-Complementation was born! Jacques liked it, but François was somehow reluctant to accept those results because he had not observed those effects previously on eosin-methylene blue (EMB)-lactose plates. After a while, by choosing the appropriate heterozygous strains, he eventually confirmed the in vitro results but considered that in vivo α-complementation was very inefficient (49). Today, when α-complementation has become the basis for the commonly used blue/white screen to identify recombinant DNA (insertion of foreign DNA into plasmid-borne *lacZα* results in the abolition of α-complementation), one would smile at those barely positive in vivo results. The reasons for the EMB data became clear later: *Flac* episomes used by François were not multicopy vectors and EMB is much less sensitive than X-gal.

I showed that the α-peptide could be obtained in a practically pure state by boiling a crude extract in 6M guanidine in a pressure cooker. While studying the mechanism of α-complementation, several pieces of convergent data suggested that the role of α-peptide was conformational rather than structural (50). This was confirmed later by the crystal structure of β-galactosidase. It showed that the polypeptide chain contains an extended segment at the N terminus (the α-peptide), which participates in subunit interaction and stabilizes it (22).

Encouraged by the discovery of α-complementation, I went on to look for a β- or γ-complementation by mixing three different extracts of deletion strains but never succeeded in recovering enzymatic activity. One day, at lunchtime, I was complaining to André Lwoff about my failure to detect complementation with three partners. "Nothing surprising about that," he said. "You are not yet French enough to be ready for a ménage à trois." So I gave up the project.

I enjoyed the fact that α-complementation had an unexpectedly long career, even though no reference was ever made to our work in subsequent papers reporting new cloning vectors based on α-complementation. An amusing story happened in the early 1980s at a Cold Spring Harbor meeting on bacteria and phage. A nice young man presented a new cloning vector based on a screening method involving the appearance of Lac^+ clones. In short, he explained what α-complementation was. Not wanting to intervene publicly, I approached him during the coffee break and told him that the phenomenon he just described had been discovered more than 15 years ago. "By whom?" he asked. "By me, it so happens." He turned pale and, panic-stricken, asked, "Did you patent it?" "No," I said. "I only published it." He didn't even ask if it was in a high-impact-factor journal.

THE TALE OF GLYCOGEN PHOSPHORYLASE

Late one evening in 1961, Jacques walked into my lab. His tie was loose and he looked tired and worried. He stood silently at my bench and after a few minutes he said, "I think I have discovered the second secret of life." I looked at him rather alarmed and I suggested that he sit down and have a drink. After downing his second or third glass of scotch, he started to explain his discovery of a phenomenon he had already named "allostery." He then pointed out that the regulatory role of allosteric proteins was absolutely fundamental, arguing that the "invention" of indirect allosteric interactions in the course of evolution opened the way to an infinite number of possible regulations. During the following month, one spoke only of allostery in the lab. In fact, it couldn't be helped because whenever we discussed whatever topic, we came back to allostery within minutes. During this period, Jacques desperately searched for a suitable experimental model with the idea of doing some bench work himself. He asked me one day whether it was difficult to prepare muscle phosphorylase and whether it was easy to assay. I answered that it was a matter of just a few days; I had done it myself in Budapest.

Act I

Around that time, P. Roy Vagelos arrived to work on bacterial genetics for a postdoctoral year. (He had isolated a deletion of the whole lactose region, called RV X74, which biotech companies sell as X74 even today.) He was, however, willing to help me perform sucrose gradient experiments to determine whether 5′-AMP, the activator of dimeric phosphorylase b, acted as an allosteric effector or whether it converted the inactive dimer to the active tetrameric form as proposed earlier by Carl and Gerty Cori. Our data clearly showed that the S values of phosphorylase b were identical whether the centrifugation was carried out in the presence or absence of 5′-AMP. This strongly suggested that the activation of phosphorylase b by 5′-AMP was not due to its association into the tetrameric a form (55). Jacques was pleased with the phosphorylase b system and set up a dye-binding procedure inspired by the observations of Eraldo Antonini that the effect of bromthymol blue (BTB) on hemoglobin was due to an allosteric heme-heme interaction (1). It therefore seemed of particular interest to test whether phosphorylase would exhibit a similar behavior, which it did. The dye associated with phosphorylase b was significantly increased in the presence of 5′-AMP. Interestingly, phosphorylase a bound the same amount of BTB as did AMP/phosphorylase b, but the nucleotide had no additional effect. We concluded that in the presence of its allosteric effector, 5′-AMP, phosphorylase b underwent a reversible conformational change that increased its capacity to bind certain ligands (55).

Act II

For various reasons, we had to determine the molecular weight of the subunits (protomers) of oligomeric proteins. I thought that it would be easy, in 1966, to do so in 6M guanidine using an analytical ultracentrifuge. By carrying out the centrifugation with proteins whose molecular structures were well known, we could devise a calibration curve. Michel Goldberg, whose knowledge in physical chemistry far exceeded mine, warned me that the experiment could be done, but a number of specific parameters would have to be taken into consideration for a correct evaluation of the results. Because the ultracentrifuge used schlieren optics, photos had to be taken every 6 hours. Michel took the first picture at 6 AM and I took the last at midnight. We ran six different proteins whose subunit molecular weight varied from 14,000 to 135,000 Da and found the expected values for all proteins except phosphorylase b, which repeatedly gave a value 92,500 Da

instead of 125,000 Da, published by the Coris 12 years earlier. I was terribly disappointed because after several months of hard work I thought that the method had to be abandoned. When we showed those results to Jacques, he was not discouraged at all and simply said, "Believe in your experiment; for once, Cori might have been wrong." I called Eddy Fischer in Seattle for his advice. He told me that they had just submitted a manuscript in which they had revised the molecular weight of phosphorylase b and found a value of 185,000 Da for the dimeric enzyme (43). That corresponded almost exactly to what we had found, so we rapidly sent in a paper to the same journal (48).

Act III

Henri Buc, who was working on the allosteric properties of phosphorylase b, came in one day worried, saying that if our new molecular weight value was correct, the number of bound 5′-AMP would no longer be 1 per monomer, as had been calculated for a molecular weight of 250,000. With Marie-Hélène Buc and Michel Goldberg, we decided to reexamine several parameters to solve this discrepancy. We measured molecular weights in nondenaturing conditions by analytical ultracentrifugation and light scattering. From the different measurements we obtained values between 190,000 and 198,000 Da. We then measured the molar extinction coefficient at 280 nm by five different methods and, to our satisfaction, found a value of 13.2 rather than 11.8, as published earlier. With these new values in hand, we established that there are precisely 2.0 stereospecific 5′-AMP sites per phosphorylase b dimer (3).

Act IV

When Maxime Schwartz joined the lab in 1963, Jacques suggested that he start working with me to learn how to handle rabbit phosphorylase b, with the idea that he would later study its allosteric conformational changes. But Maxime rapidly switched to a bacterial phosphorylase with the hope—at least Jacques's hope—that the bacterial enzyme would also be allosteric and that it would be amenable to genetic analysis. Maxime did indeed find a polysaccharide phosphorylase in *E. coli*. He showed that the enzyme was inducible by maltose, but to Jacques's disappointment, displayed no allosteric properties. Nevertheless, Maxime later discovered the positive regulation of the maltose regulon.

BACK TO THE LACTOSE OPERON

Since the early 1960s, several reports had appeared showing that clinical isolates of enterobacteria resistant to antibiotics carried plasmids that endowed them with the ability to utilize lactose. Normally, those bacteria are unable to ferment this sugar. With Nicole Guiso, we were interested in the origin of the Lac^+ character of these antibiotic-resistant plasmids. After having transferred the plasmids to the *E. coli* RV X74 Lac deletion strain, we compared their characteristics to those of the *E. coli* Lac operon. The proteins essential for lactose utilization, i.e., β-galactosidase and lactose permease, were biochemically and functionally similar to those of the *E. coli* Lac operon. The first surprise was that none of the plasmids carried enzymatically detectable thiogalactoside transacetylase. (Guy Cornelis later showed by hybridization techniques that the gene was also missing.) If the plasmids had acquired the lactose genes from an *E. coli* chromosome, then selection must have gotten rid of the transacetylase, an enzyme with no known physiological function in bacteria. The next and most unexpected surprise was that all repressors carried by the plasmids exhibited affinities for the inducer that were 10 to 30 times higher than that for the

E. coli Lac repressor. This might have given a selective advantage to the bacteria carrying these genes when growing in low lactose media. I might have been more successful in isolating the Lac repressor if I had been aware of these plasmids earlier (18).

β-Galactosidase Fusions as a Tool

The observation that the N-terminal 23 residues of β-galactosidase could be replaced with other amino acids without affecting enzymatic activity suggested that hybrid genes coding for hybrid proteins could be constructed. At that time, the purification of the hybrid proteins required several steps and the yields were poor. To avoid such low yields and protein degradation, I devised in 1984 a one-step procedure for the isolation of hybrid proteins exhibiting β-galactosidase activity (46). The procedure was based on affinity chromatography using a β-galactosidase inhibitor of high affinity. Starting with crude bacterial extracts, several milligrams of near-homogeneous proteins could be obtained within a few hours with an overall yield of around 90%. Some of these hybrid proteins contained up to 500 residues of the foreign sequence; they were nevertheless able to fold into a conformation leading to a tetrameric protein endowed with β-galactosidase activity. The method was a great success; for several years, colleagues from different laboratories arrived in the morning with their bacterial extracts and left in the afternoon with the pure protein. Since the discovery of the His-tag purification method, the β-galactosidase affinity chromatography procedure has been abandoned. *Sic transit gloria mundi*! (Thus passes the glory of the world!)

CYCLIC AMP EFFECTS IN BACTERIA

When Jacques Monod was appointed professor at the Collège de France in 1967, he devoted his first lectures to allosteric interactions. At the time, he was so convinced that only noncovalent interactions were important that, at a Gordon Conference on Proteins organized by Eddy Fischer and Dan Koshland, he proclaimed dismissively that, after all, peptide bonds were irrelevant, to which Fred Richards immediately countered by saying, "But Jacques, if you were right, proteins should be gases." Someone else in the back remarked that "the only person I know of who wasn't held together by covalent bonds was Lot's wife, after she had been converted into pillar of salt."

When Jacques asked me to give a seminar as part of a course on allosteric interactions at the Collège de France, I decided to play devil's advocate and talk about the cascade of covalent regulations of glycogen phosphorylase discovered earlier by Fischer et al. (12). The process involved a hormonal regulation, amplified by the second-messenger cAMP, which activates a cAMP-dependent protein kinase. This kinase then phosphorylates a second kinase, which in turn phosphorylates glycogen phosphorylase b, transforming it into active phosphorylase a (26). Reviewing the literature on this second messenger, I discovered a recent paper by Makman & Sutherland (33) describing the presence of cAMP in bacteria. I therefore decided to look for covalent modifications in *E. coli* triggered by cAMP.

François Jacob discouraged me from undertaking this project because, as he said, "There are no hormones in *E. coli*." Jacques also thought that it wasn't a good idea.

In those days, cAMP was not commercially available. With the help of Mic Michelson at the Institut de Biologie Physico-Chimique (IBPC), rue Pierre Curie (it had not yet been renamed rue Pierre et Marie Curie), on a Friday evening we synthesized a few grams of cAMP. The next day I grew *E. coli* in the presence of different carbon sources and found that addition of cAMP relieved bacteria from catabolite repression (CR) (52). On Monday morning Mic asked me to come to the IBPC. He took me into the dark room and showed me a petri dish that looked like a sky full of stars: He had discovered that bacterial luciferase synthesis was dramatically enhanced by cAMP. When it turned out that cAMP also relieved diauxic growth, which Jacques had discovered

25 years earlier (35), he started to become really interested. One day, Gerard Buttin entered the lab and, after looking at the diauxic curves, spontaneously remarked, "How nice, Jacques. Now you can at last complete your thesis."

It became obvious that covalent modifications were involved neither in CR nor in the relief of diauxic growth. On the contrary, evidence from us and others indicated that cAMP was an allosteric effector. By binding to CAP (the catabolite gene activator protein, its receptor protein), cAMP exerts a positive regulation on the otherwise negatively regulated *lac* operon (25). For 18 years, I worked on the role of cAMP in regulatory mechanisms in bacteria (47), and with my collaborators, we published approximately 50 papers on the subject.

Transcription Regulation

With François Gros's group, we were the first to show a role of cAMP in transcription initiation. Later on, with Antoine Danchin, Evelyne Joseph, and Chantal Guidi-Rontani, we studied its role in transcription termination. Our first observation concerned natural polarity, that is, the decreased expression of promoter distal genes as opposed to proximal genes. We showed that cAMP relieved the degree of natural polarity and interpreted this result by assuming that it was interfering with the transcription termination protein Rho. Indeed, direct measurements of mRNA synthesis corresponding to the promoter proximal and distal regions of the *lac* and *gal* operons showed that the relative synthesis of the promoter distal mRNAs was significantly increased in the presence of cAMP (17).

One of our manuscripts on the functional relationship between the cAMP-CAP complex and Rho was communicated by Roger Stanier to the *Proceedings of the National Academy of Sciences* (51). One referee requested minor modifications while the second just wrote, "I don't believe it." Because I did not want to argue with this referee about the role belief played in science, I simply quoted André Lwoff, who said "Once, the author of a discovery was subjected to three successive attacks. First, he was told that his findings were wrong; then, that they were already known. Lastly, when the poor fellow finally succeeded in demonstrating that his findings were right and original, he was told: 'That was obvious.'" The referee wrote to Roger: "Nice quotation; accept the manuscript."

Glucose Effects

After my first experiments on the relief of CR by cAMP, Jacques Monod's comment was that it was bad to get involved with a phenomenon that was far too complicated. Within a few years, I realized that he was right, but I was already too much involved in this unending story.

The terms catabolite repression and inducer exclusion were coined by Boris Magasanik (32) to describe a phenomenon called the glucose effect discovered in 1900 by Dienert (7). This phenomenon accounts for the "diauxic growth" observed by Monod in 1942 (35). Studying glucose inhibition of different catabolic enzymes, I succeeded in dissociating the relative contributions of CR and inducer exclusion. Surprisingly, I found that for some systems (*lac*, *tna*), glucose brought about mainly CR, whereas in other systems (*mal*, *gal*) glucose brought about inducer exclusion.

cAMP and Catabolite Repression

In the 1970s, the accepted model postulated that the extent of CR depended exclusively on the intracellular concentration of cAMP. This model was challenged when subsequent results indicated that it could be modulated independently of cAMP. They were based mainly on the observations (of Alain Dessein and Chantal Guidi-Rontani) that mutants deficient in adenylate cyclase and/or CAP were still subjected to CR (6). Searching for inhibitors that specifically affected

catabolite-sensitive operons without interfering with overall cellular mechanisms, we found with Brigitte Sanzey that low concentrations of urea that did not inhibit bacterial growth specifically repressed the expression of catabolite-sensitive operons, a repression that was not relieved by cAMP (40). These and other results led us to propose that CR could be accounted for by a negative regulation afforded by a mediator: a specific metabolite or a class of metabolites that would accumulate under growth conditions that led to CR, and was degraded when CR was relieved (47). Searching for such modulators with Françoise Tillier, we purified from boiled extracts of *E. coli* a low-molecular-weight fraction that exerted a strong repressive effect on catabolite-sensitive operons, without showing any effect on catabolite-insensitive systems. The chemical nature of the active compound has not been elucidated. The compound, designated catabolite modulator factor, was actively metabolized by the bacteria, and cAMP only partially reversed its repression effect. In all respects, the repression obtained by catabolite modulator factor was similar to physiological CR (54). The paper describing these results was the last one signed by Jacques Monod; when it appeared in the *Proceedings of the National Academy of Sciences* (communicated by Boris Magasanik) Jacques had already passed away.

Escherichia coli Adenylate Cyclase

In the early 1980s, very little was known about the expression, structure, and activity of the *E. coli* adenylate cyclase. Some reports had suggested that the enzyme was loosely bound to the membrane and extremely labile. Antoine Danchin succeeded in cloning the *cya* gene and demonstrated that the bacterial adenylate cyclase consisted of two functional domains: an amino-terminal domain exhibiting cyclase catalytic activity and a carboxy-terminal domain having regulatory functions. Antoine then constructed a *cya-lacZ* fusion that coded for both adenylate cyclase and β-galactosidase activities (39).

Using the one-step purification procedure mentioned above, we obtained homogeneous Cya-LacZ fusion protein with which we could confirm that the adenylate cyclase activity was indeed associated with the amino-terminal domain of the wild-type protein. From the specific activity of the purified protein we calculated that wild-type *E. coli* contains about 1,000 molecules of adenylate cyclase per cell (39).

FROM *ESCHERICHIA COLI* TO PATHOGENS

Having spent many years on the role of cAMP in *E. coli*, I wanted to switch to a different area, i.e., study the role of this nucleotide in pathogenesis. It was already known that two bacterial toxins (secreted by *Vibrio cholerae* and by *Bordetella pertussis*) acted by increasing cAMP levels of the infected organisms. This increase was due to an ADP ribosylation (from NAD) of one of the regulatory subunits of the eukaryotic adenylate cyclase. But at the time, this field seemed very crowded and competitive so I decided to turn to something else.

Upon reviewing the relevant literature, I discovered reports on the existence of adenylate cyclase activity in *B. pertussis*, the etiologic agent of whooping cough (20). It took a few more years for Wolff et al. (57) to realize that it was a secreted protein that exhibited the unusual property of being activated by calmodulin, a eukaryotic protein par excellence. Confer & Eaton (5) were the first to demonstrate in 1982 that *B. pertussis* adenylate cyclase is a toxin. After entering animal cells, it elicits an unregulated increase in cAMP, thereby disrupting normal cellular functions. More importantly, Alison Weiss (of Stanley Falkow's laboratory) had opened the way to *B. pertussis* genetic analysis by showing that mutants deficient in adenylate cyclase were avirulent (56). Around the same time, Leppla (31) had reported that *Bacillus anthracis* toxin (the edema factor) was also a calmodulin-activated adenylate cyclase.

At the beginning, I was tempted to choose the anthrax system, because when I was a student, for an end-of-the-year diploma, my organic chemistry professor, V. Bruckner, asked me to purify the capsule of *B. anthracis* (poly-D-γ-glutamic acid), the structure of which he was trying to establish. To start the purification, I had to centrifuge large amounts of bacteria. Because we had only glass centrifuge tubes without cover, I asked whether the bacteria were dangerous. I was told that they could be lethal and that I had better be careful. I thought it was the first step one had to take to become a microbe hunter. I purified large amounts of capsule and survived; I obtained my diploma.

For a while, I worked with Michèle Mock on the *B. anthracis* system but finally decided to work on the adenylate cyclase of *B. pertussis*. Michèle and I could not understand how two taxonomically distant organisms with such different genomic G+C content (30% for *B. anthracis* versus 66% for *B. pertussis*) could produce adenylate cyclase toxins that required the same eukaryotic activator for their enzymatic activity, namely calmodulin (34). We never did solve that evolutionary puzzle, but both our groups uncovered many aspects of the structure-function relationships of the two systems.

THE SAGA OF *BORDETELLA PERTUSSIS* ADENYLATE CYCLASE

When we decided in 1984 to work on the *B. pertussis* adenylate cyclase (with Octavian Bârzu, Nicole Guiso, Isabelle Crenon, and Daniel Ladant), very little was known about the enzyme itself. No homogeneous preparation had been obtained. Various groups had described both low- and high-molecular-weight forms that were activated to various extents by calmodulin. Therefore, our first priority was to purify the enzyme in order to study its structure-function relationship.

From supernatants of a virulent *B. pertussis* strain, Daniel Ladant succeeded in purifying a 45-kDa form of adenylate cyclase with high affinity for calmodulin (0.2 nM). Limited proteolysis with trypsin yielded two fragments of 25 and 18 kDa (T25 and T18) that could be separated by gel-filtration chromatography and easily detected by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). At that point, Daniel performed an experiment that only an uninhibited and imaginative young scientist would do: He excised the two fragments from the gel, eluted them in the presence of calmodulin, and recovered enzymatic activity. This showed that T25 and T18 represented separate domains of the adenylate cyclase that could complement one another in the presence of calmodulin, leading to a structure resembling the native enzyme (27). Fifteen years later, Daniel had the idea of setting up a bacterial two-hybrid screening system based on the T25 and T18 complementation (24).

We obtained antibodies against the 45-kDa form of the adenylate cyclase, which brought us two surprises. The first surprise came when Daniel isolated the enzyme from urea-treated bacterial extracts: He obtained mainly the ~200-kDa form that was fully active. Moreover, antibodies directed against the low-molecular-weight form recognized perfectly well the high-molecular-weight enzyme. The obvious conclusion was that the 45-kDa form resulted from a proteolytic cleavage of the high-molecular-weight form. Clearly, the 45-kDa species could represent the catalytic moiety of the native protein. The nature of the rest of the molecule was identified later thanks to antibodies raised against *E. coli* hemolysin (a gift from Werner Goebel): It recognized the same high-molecular-weight protein that our antibodies did. This result validated and extended Weiss & Falkow's (56) findings that several *Tn*5-induced nonhemolytic mutants were also adenylate cyclase deficient. The cloning and sequencing of the *cya* gene coding for adenylate cyclase (by Philippe Glaser and Antoine Danchin) confirmed the biochemical results and showed that the 5′-terminal end of the gene represented the catalytic moiety and the 3′-terminal end exhibited strong homology to the *E. coli* hemolysin (14).

The second surprise arose from a collaboration with Michèle Mock's group. We found that the antibodies directed against *B. pertussis* adenylate cyclase cross-reacted with the *B. anthracis* enzyme. Later, sequence analysis indicated that only the calmodulin-activated catalytic domain

shared those structural similarities (34). In 2005, Tang et al. (19) established the three-dimensional structures of both adenylate cyclases and concluded that their catalytic regions shared similar structures and mechanisms of activation. By contrast, the interactions with calmodulin were completely different, providing molecular details of how two structurally homologous bacterial toxins could have undergone divergent evolution to bind calmodulin (19).

In the late 1980s, I attended a Gordon Conference on Microbial Toxins and Pathogenicity also attended by several colleagues involved in pertussis research. I reported our results on adenylate cyclase and that afternoon, while we were discussing our projects with Rino Rappuoli, John Coote, and Roy Gross, I told them that I had learned from Jacques Monod that it was "better to collaborate than to compete." Shouldn't we, for instance, join forces and initiate a collaborative project and apply for a common international grant? They agreed, provided that I would be the coordinator. After several months of hard work, I sent in a grant request to the Human Frontier Science Program (HFSP) entitled "Molecular and Cellular Mechanisms Underlying Regulation of Virulence Factors of *Bordetella pertussis*." Even though I knew that our chance of success was less than 1%, we were funded, which was a bonanza for our research. This grant radically changed our everyday lives: For once, I could support postdocs, which was not possible with French grants. The HFSP provided us with many facilities and, more importantly, sealed a very close and fruitful collaboration among our groups.

Structure-Function Relationships

The genetic organization of the *cya* locus revealed, in addition to the structural gene (*cyaA*) encoding for adenylate cyclase, the existence of three additional genes (*cyaB*, *cyaD*, and *cyaE*) required for the secretion of CyaA. Analysis of the transcriptional organization of the *cya* locus showed that *cyaA*, *cyaB*, *cyaD*, and *cyaE* were organized in a single operon transcribed from the *cyaA* promoter. Adjacent to the *cyaA* gene, *cyaC* was transcribed in the opposite direction. Its product catalyzed the palmitoylation of CyaA, a prerequisite for the conversion of the protein to the active toxin.

Transcriptional regulation of *B. pertussis* virulence factors, achieved by a two-component system (BvgA/BvgS), was studied extensively by the Falkow and Rappuoli groups (2). My own group was interested mainly in some molecular mechanisms underlying specific promoter activations. We elucidated the transcriptional organization of the *cya* locus (with Brid Laoide) (30), characterized the specificity of BvgA binding to virulence promoter regions (with Gouzel Karimova) (23), carried out functional analysis of the *cya* promoter (with Sophie Goyard) (15), and studied the in vitro transcriptional activation of *bvg*-regulated promoters as well as the specific role of *B. pertussis* RNA polymerase subunits (with Pierre Steffen and Sophie Goyard) (45).

For several years our research was focused on the structure-function relationship of CyaA. The 1,706-residue CyaA is a bifunctional protein: It exhibits both adenylate cyclase and hemolytic activities and is constructed in a modular fashion. The calmodulin-activated catalytic domain is located within the 400 amino-proximal residues, whereas the 1,306 carboxy-terminal residues are responsible for the hemolytic activity, for the binding of the toxin to eukaryotic cells, and for its internalization. In addition, it contains the secretion signals (29).

Antigen Delivery

To study the mechanism of CyaA secretion and its entry into eukaryotic cells, we had to reconstruct in *E. coli* a high level of expression of the *cya* genes, which was achieved mainly by Peter Sebo (42). Using the reconstituted system, he and Daniel Ladant, Jacques Bellalou, and Hiroshi Sakamoto elucidated several aspects of the secretion mechanism. Daniel Ladant carried

out the next important experiment for our future understanding of the multiple capabilities of the CyaA toxin. He set up an insertional mutagenesis procedure for the introduction of as many as 16 codons into the *cyaA* gene and identified within the primary structure of the catalytic domain several permissive sites where insertions altered neither the activity nor the stability of the protein (28). This finding suggested that if we could insert foreign antigenic determinants into those permissive sites, we might be able to use the resulting recombinant CyaA toxin as a Trojan horse to deliver specific epitopes into antigen-presenting cells. Because I was not an immunologist, I approached Claude Leclerc at the Pasteur Institute. To my satisfaction, she agreed to test the idea, and it worked! Daniel Ladant and Peter Sebo constructed genetically detoxified (i.e., lacking adenylate cyclase catalytic activity) recombinant CyaA toxins carrying different $CD8^+$ epitopes. Then Leclerc's group, using different mouse models, showed that a recombinant CyaA toxin carrying a viral $CD8^+$ epitope could elicit epitope-specific cytotoxic T-cell immune responses and, more importantly, protect mice against infections triggered by high doses of the virus (11, 41). Also, recombinant CyaA toxin carrying specific tumor epitopes could protect mice against grafts of tumor cells (10). Further work fully confirmed that recombinant CyaA toxin represented an attractive nonreplicative vector for antigen delivery and could be used for the development of protective and therapeutic vaccines. After my mandatory retirement in 1995 (French law), the Pasteur Institute generously offered me an office so I could closely follow the development of a project entitled "Recombinant CyaA Toxins for Vaccinal Purposes" funded by the European Union. The results obtained so far have fully validated the vaccine potential of CyaA: Two of these recombinant molecules are currently under Phase I clinical trials.

Reporter for Protein Targeting

The structural flexibility of the CyaA catalytic domain proved to be very useful not only for antigen delivery but also for protein targeting and the characterization of protein-protein interactions. Guy Cornelis, who studied the *Yersinia* type III secretion system, which allows direct delivery of bacterial virulence proteins into the host cell cytosol, once mentioned that it was difficult to detect the proteins delivered because of their very low concentration. I suggested he try to construct a fusion between the C-terminal section of his protein and the catalytic domain of CyaA. The hybrid protein would exhibit no adenylate cyclase activity in *Yersinia* because bacteria do not produce calmodulin. By contrast, once internalized in eukaryotic cells, the hybrid enzyme would become activated by calmodulin and generate cAMP. As the CyaA catalytic domain alone is unable to penetrate into eukaryotic cells, appearance of cAMP in the target cell would be a direct measure of virulence protein internalization. After constructing the appropriate fusion proteins from the *cya*-containing plasmid we provided, Sory & Cornelis (44) were able to define the mechanism of internalization of the *Yersinia* virulence factors. The successful utilization of cAMP production as a selective reporter of eukaryotic environment inspired groups working on *Shigella* or *Salmonella* type III secretion systems to adopt this approach.

Bacterial Two-Hybrid System

Taking advantage of the modular structure of the catalytic domain of CyaA, Daniel Ladant designed a genetic system that would allow easy screening and selection of functional interactions between two proteins in vivo. The system is based on complementation between the T25 and T18 fragments of the catalytic domain that can be easily tested in an *E. coli cya* strain. If cAMP is produced, catabolic operons are activated (as I had shown 30 years earlier), thus giving rise to a selectable phenotype. T25 and T18 expressed in *E. coli cya* as separate entities are unable to associate

and reconstitute a functional enzyme. On the other hand, when T25 and T18 are fused to peptides or proteins that interact with one another, heterodimerization of those chimeric polypeptides results in a functional complementation of the two fragments and cAMP synthesis. The bacteria are thus able to ferment carbohydrates; therefore, it is easy to detect positive clones on petri dishes containing lactose or maltose (24). The system functions as a versatile reporter of protein associations and has been used successfully to reveal interactions between bacterial, viral, and eukaryotic proteins. It proved to be an attractive complementary approach to the yeast two-hybrid system.

Today, when scores of scientists work on different aspects of *B. pertussis* adenylate cyclase, it is gratifying to remember that it started from a shot-in-the-dark weekend experiment with homemade cAMP. At that time, I could never have dreamed that many years later it would lead to versatile reporters for protein targeting and protein association and, more importantly, to a useful and innovative vaccine.

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